Blumenthal, C.Z

A037

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Regulatory Toxicology and Pharmacology 39 (2004) 214–228
Regulatory Toxicology and Pharmacology
www.elsevier.com/locate/yrtph

Production of toxic metabolites in *Aspergillus niger*, *Aspergillus oryzae*, and *Trichoderma reesei*: justification of mycotoxin testing in food grade enzyme preparations derived from the three fungi

Cynthia Z. Blumenthal*

Genencor International, 925 Page Mill Road, Palo Alto, CA 94304, USA

Received 3 June 2003

Abstract

Aspergillus niger, *Aspergillus oryzae*, and *Trichoderma reesei* are three important production organisms used in industrial fermentations. Several of the fungal secondary metabolites produced by selected strains of these three fungi are capable of eliciting toxicity in animals. Among those toxic substances are the well-known mycotoxins 3-nitropropionic acid and ochratoxin A. However, many others, such as kojic acid, may not be true mycotoxins. The production, extraction, chemical structure, and the toxicity (expressed as LD_{50}) of these substances are reviewed. Production of toxic secondary metabolites in *A. niger*, *A. oryzae*, and *T. reesei* is strain-specific and environment-dependent. Considering all of the safety measures taken in the industrial production process, these three fungal species are safe to use. The recently revised JECFA specification for mycotoxins in food enzyme preparations is also discussed. The extent of mycotoxin tests in food enzyme preparations should be judged on a case-by-case basis, through a careful evaluation based on knowledge of taxonomy, biochemistry, and genetics. In many cases, the testing scope at the level of genus should be sufficient. In other cases, the scope can even be further narrowed based on scientific knowledge and assessment.

Keywords: Mycotoxin; *Aspergillus niger*; *Aspergillus oryzae*; *Trichoderma reesei*; JECFA; Review

1. Introduction

Aspergillus niger, *Aspergillus oryzae*, and *Trichoderma reesei* are three important species used as production organisms in industrial fermentations for the production of various substances. These products have wide application in numerous industrial fields including the food industry. A few examples are *A. niger*-derived citric acid, fermented foods produced using *A. oryzae*, and a number of enzyme preparations produced by the three microorganisms using traditional or modern biological technologies. A comprehensive list of those enzyme preparations used in today's food industry can be found in Pariza and Johnson's recent paper (2001).

The safety of any food-grade product is carefully evaluated before its commercialization and is assured throughout the manufacturing, processing, transportation, storage, and use of the product. Safety assurance measures range from the selection of manufacturing raw materials to a series of toxicological tests. The primary consideration in the safety evaluation of a food-grade product derived from a microorganism is the safety of the production organism. The primary issue in the safety evaluation of such a microbial production strain, according to Pariza and Johnson (2001), is "its toxigenic potential, specifically the possible synthesis by the production strain of toxins that are active via the oral route."

Toxins of fungal origin are called mycotoxins. They are secondary metabolites. Bennett (1987) defined mycotoxins as "natural products produced by fungi that evoke a toxic response when introduced in low concentration to higher vertebrates and other animals by a natural route." Natural routes may include ingestion, skin contact, inhalation, or others, as opposed to

* Fax: 1-650-845-6505.
E-mail address: cblumenthal@genencor.com.

0273-2300/$ - see front matter © 2003 Published by Elsevier Inc.
doi:10.1016/j.yrtph.2003.09.002

"unnatural" routes such as injection. To ensure the safety of food-grade enzymes, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) required, until last year, that food enzyme preparations derived from fungal sources should not contain detectable amounts of aflatoxin B1, ochratoxin A, sterigmatocystin, T-2 toxin (a major trichothecene toxin) or zearalenone (JECFA, 1989) (The new JECFA requirements will be discussed later in this paper). It should be pointed out that in the *A. niger*, *A. oryzae*, and *T. reesei* group, the only confirmed production of the above mycotoxins is that of ochratoxin A by a few *A. niger* isolates.

The safety of *A. niger*, *A. oryzae*, and *T. reesei* as production organisms for food-grade products has long been recognized. For example, the general use in food of *A. niger*-derived citric acid has been affirmed as GRAS by the FDA (21 CFR §184.1033) in 1994. A number of traditional fermented foods are produced by *A. oryzae* (Beuchat, 2001) and are still being consumed especially in Asia. As a production organism *T. reesei* emerged later than the aspergilli. Nevertheless, the use of cellulase enzyme preparations derived from *T. reesei* in food processing has been affirmed as GRAS by the FDA (21 CFR §184.1250). None of the three organisms is considered pathogenic. Mycotoxin production in some strains from the three species, however, has been reported. This article collects information on reported toxic metabolite production in *A. niger*, *A. oryzae*, and *T. reesei*, and assesses the safety of using those microbes as production strains for food-grade products.

2. Taxonomy of *A. niger*, *A. oryzae*, and *T. reesei*

The genus*Aspergillus* (*Eurotiales*; *Trichocomaceae*) includes approximately 150 recognized species. The genus was divided into 7 subgenera by Samson (1994). *A. niger* belongs to subgenus *Circumdati*, section *Nigri* (= *A. niger* group). The teleomorph of *A. niger* is unknown. Two RFLP (restriction fragment length polymorpism) patterns, N pattern and T pattern, are seen in the current *A. niger* strains, indicating that *A. niger* can be possibly divided into two genetically distinguishable species based on the RFLP pattern: *A. niger* (N pattern) and *A. tubigensis* (T pattern) (Accensi et al., 2001). Results from the sequencing of the mitochondrial cytochrome *b* genes of section *Nigri* also show the possibility of reevaluating the current taxonomy of the section (Yokoyama et al., 2001). The taxonomy of black aspergilli, a developing subject, is summarized recently by Schuster et al. (2002). *A. oryzae* belongs to subgenus *Circumdati*, section *Flavi* (= *A. flavus* group). The teleomorph of this section is *Petromyces* (Frisvad and Samson, 2000). Section *Flavi* contains powerful mycotoxin producers as well as production strains used in the food industry. Both *A. flavus* and *A. parasiticus* belong to that group. *A. oryzae* and *A. flavus* are so closely related that it was once proposed to redefine *A. oryzae* as a variant of *A. flavus* that likely had changed its morphology and physiology and lost certain biosynthetic pathways (Wicklow, 1984a,b). More recently, phylogenetic and genetic data suggested that *A. oryzae* may be derived from a non-aflatoxin-producing *A. flavus* progenitor and supported the individual status of *A. oryzae* (van den Broek et al., 2001; Geiser et al., 2000). *A. oryzae* is probably a domesticated fungus that emerged from the environment of fermentation activities such as the make of soy sauce.

Trichoderma reesei was first isolated from a cotton duck shelter in Bougainville Island in 1944. The original isolate, QM6a, and its subsequent derivatives have been the subject of intense research due to their usefulness in the production of cellulases. In the 1980s, it was suggested that *T. reesei* be placed into synonymy with *T. longibrachiatum* (Bissett, 1984). Later however, there subsequently appeared evidence that the two species were not identical (Meyer et al., 1992). The proposal by Kuhls et al. (1996) that *T. reesei* was a clonal derivative of *Hypocrea jecorina* is being accepted by more and more people, and the National Center for Biotechnology Information (NCBI) refers to *T. reesei* as the anamorph of *H. jecorina* (*Hypocreales*, *Hypocreaceae*) and no longer includes it in the genus *Trichoderma*.

3. Production of toxic secondary metabolites in *A. niger*, *A. oryzae*, and *T. reesei*

Fungi produce a number of secondary metabolites of which some have been found to be toxic to humans and animals. Secondary metabolites that were reported to be produced by *A. niger*, *A. oryzae*, and *T. reesei*, and whose toxicological data as LD_{50} values are available are presented in Table 1 (LD_{50} values) and Fig. 1 (chemical structures), respectively. Chromatography is the most commonly used method to isolate these compounds from culture or supernatant extracts (Smedsgaard, 1997), while immunological methods are more recent advances. Reference materials can be used to improve the quality assurance of mycotoxin analysis which often shows wide variability in results (van Egmond, 2000).

3.1. Aspergillomarasmine

Aspergillomarasmines were isolated from *A. oryzae* as phytotoxins (Haenni et al., 1965). Later they were shown to have inhibitory effects on angiotensin-converting enzymes (Mikami and Suzuki, 1983). The methods of isolation and purification were described by Haenni et al. (1965).

Table 1
Secondary metabolites produced by *A. niger*, *A. oryzae*, and *T. reesei* and their LD_{50} values

Metabolite	Organism	Reference	LD_{50} (mg/kg)	Route
Aspergillomarasmine	*A. oryzae*	Haenni et al. (1965)	160, mouse	IV
Cyclopiazonic acid	*A. oryzae*	Orth (1977)	2, rat	IP
			13, mouse	IP
			64, mouse	O
			12, chicken	O
Kojic acid	*A. oryzae*	EPA (1997a,b)	250, mouse	IP
Malformins	*A. niger*	Takahashi and Curtis (1961); Anderegg et al. (1976); Varoglu and Crews (2000)		
Malformin A			3.1, mouse	IP
Malformin C			0.9, rat	IP
Maltoryzine	*A. oryzae*	Iizuka and Iida (1962)	3, mouse	IP
Naphtho-r-pyrones	*A. niger*	Ehrlich et al. (1984)		
Aurasperone D			47, mouse	IP
Nigerazine B	*A. niger*	Iwamoto et al. (1983)	75, mouse	IP
Nigragillin	*A. niger*	Caesar et al. (1969)	150, cockerels*	O
3-Nitropropionic acid	*A. oryzae*	Nakamura and Shimoda (1954); Orth (1977)	67, rat	IP
			140, mouse	IP
			50, mouse	IP
			68.1, mouse	O
			25.1, chicken	O
			22, rat	SC
Ochratoxin A	*A. niger*	Abarca et al. (1994)	12.6, rat	IP
			22, mouse	IP
			12.8, rat	IV
			25.7, mouse	IV
			1, sheep	IV
			20, rat	O
			46, mouse	O
			3.3, chicken	O
			0.5, duck	O
			16.5, quail	O
			5.9, turkey	O
Oxalic acid	*A. niger*	Pier and Richard (1992)	270, mouse	IP
			7500, rat	O
			475, rat, male	O
			375, rat, female	O
Trichodermin	*T. reesei*	Watts et al. (1988)	500, mouse	IP, SC
Violacetin	*A. oryzae*	Kobayashi (1966)	45, mouse	IP
			37, mouse	IV
			375, mouse	O
			75, mouse	SC

IP, intraperitoneal; IV, intravenous; O, oral; SC, subcutaneous.
LD_{50} values are obtained from Registry of Toxic Effects of Chemical Substances (RTECS) unless indicated.
*Datum from Cole and Cox (1981).

3.2. Cyclopiazonic acid (CPA)

Cyclopiazonic acid is an indole-tetramic acid and a natural food/feed contaminant (EPA, 1997b). Production of cyclopiazonic acid by *A. oryzae* was first reported by Orth (1977). The compound was extracted from whole culture or culture filtrate of *A. oryzae* grown on both solid and liquid media. Cyclopiazonic acid abolishes the function of sarcoplasmic reticulum (SR) by inhibiting Ca^{2+}-ATPase (Daniel et al., 1995; Paul, 1998). Mycotoxicoses caused by this compound have been observed in several animals (Hill et al., 1986; Lomax et al., 1984; Morrissey et al., 1985; Nuehring et al., 1985; Pier et al., 1989). Cyclopiazonic acid is also related to debilitating illnesses in cattle and man in India (EPA, 1997b). This compound was reviewed by Burdock and Flamm recently (2000). The authors estimated that an appropriate acceptable daily intake (ADI) for CPA in humans is 700 μg/day.

CPA can be detected from extract of fungal cultures by thin-layer chromatography (TLC) (Munimbazi and Bullerman, 1996; Orth, 1977). Recent advances in analytical methodology were reviewed by Dorner (2002).

3.3. Kojic acid

Kojic acid is produced by many strains from the genera *Aspergillus* and *Penicillium*. In Section *Flavi*, the

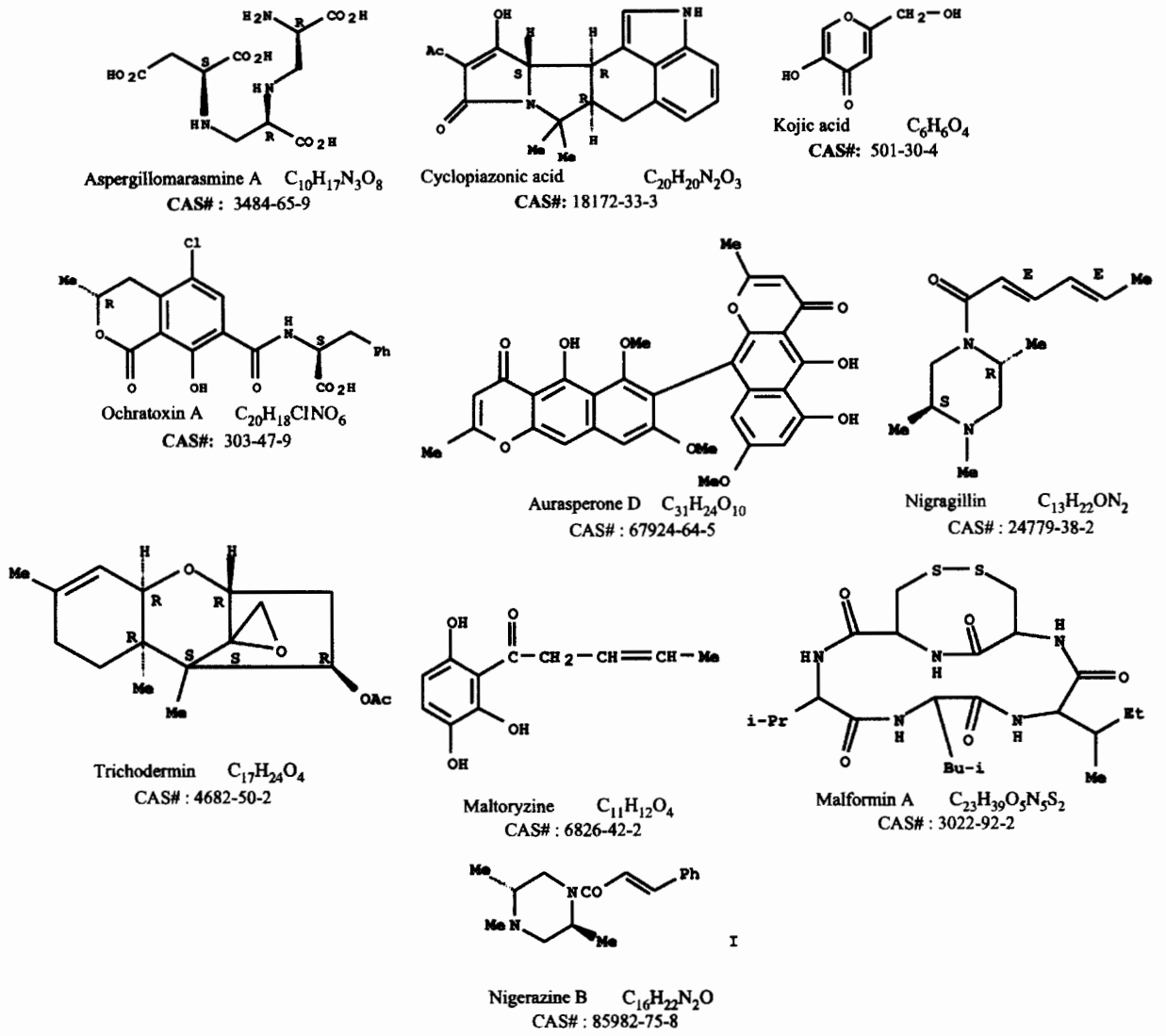


Fig. 1. Chemical structures of several toxic secondary metabolites produced by *A. niger*, *A. oryzae*, and *T. reesei*.

favored production media contain fairly high concentrations of glucose and low concentrations of phosphate (Roehr et al., 1992). The compound is excreted into the medium. A comprehensive review of kojic acid by Burdock et al. (2001) discussed the sources, chemical and biological characteristics, toxicological studies, and commercial applications of kojic acid. The authors evaluated the safety of kojic acid in foods and concluded that "consumption of kojic acid at levels normally found in food does not present concern for safety" based on the compound's long history of human exposure and industrial applications. The authors also mentioned that kojic acid is an approved additive for food and skin care products in Japan. Recently, however, the Japanese Ministry of Health, Labor, and Welfare (MHLW) reevaluated the use of kojic acid in the food and personal care industries due to concerns of its possible carcinogenicity. While the Japanese government "believes that there is no need to implement immediate measures for kojic acid" in food products (Evaluation and Review Panel for Food Additive Safety, 2002), it announced the suspension of the manufacture and importation of non-medicinal and other products containing kojic acid before more is known about the carcinogenicity and genetoxicity of this compound (MHLW, 2003).

The no-adverse-effect level of kojic acid was established in chickens at 146 mg/kg in a 21-day feeding study and the NOAEL (no observed adverse effect level) for thyroid tumor promoting effects of kojic acid, at 15.5 mg/kg/day in mice and rats (Burdock et al., 2001).Analytical methods of kojic acid can be found in Ogawa et al. (1995) and Burdock et al. (2001).

3.4. Malformins

Malformins are cyclic pentapeptides. The compounds acquired the name because they caused malformation in plants (Curtis, 1961). They were also found to be toxic to a variety of bacteria (Kobbe et al., 1977; Suda and Curtis, 1966) and to prevent IL-1 induced procoagulant changes in human endothelial cells (Herbert et al., 1994). Malformins are present in culture filtrate or extract of *A. niger* grown on solid or liquid medium (Anderegg et al., 1976; Kobbe et al., 1977; Takahashi and Curtis, 1961). In one study, Yukioka and Winnick (1966) demonstrated the effect of growth conditions on malformin biosynthesis and revealed that *A. niger* released this metabolite into growth medium. Malformins are also produced by other species in Section *Nigri* (Cole and Cox, 1981).

A complete procedure for the isolation of malformins was provided by Takahashi and Curtis (1961). Further analysis can be achieved by mass spectrometry, HPLC, and NMR (Anderegg et al., 1976; Kim et al., 1993).

3.5. Maltoryzine

This compound was first isolated from the culture broth of a strain of *A. oryzae* var. *microsporus* and was determined to be the cause of food poisoning among cows. The strain produced maltoryzine when grown in Czapek-Dox medium containing malt sprout extract and excreted the compound into the broth, as reported by Iizuka and Iida (1962). In the same article the authors also described the procedure for the isolation and purification of maltoryzine.

3.6. Naphthopyrones

Naphthopyrones are a group of aromatic compounds. They were shown to be able to cause central nervous system signs in albino mice and rats leading to death when dosed intaperitonially (Ghosal et al., 1979) and to reverse drug resistance in human KB cells (Ikeda et al., 1990). The production of naphthopryrones in *A. niger* was described by several investigators (Ehrlich et al., 1984; Fischer et al., 2000; Ghosal et al., 1979; Nielsen et al., 1999; Tanaka et al., 1966). Naphthopyrones have been isolated from the culture extract of *A. niger* grown on liquid and solid medium. In a separate study on fungal components from grain dust (Palmgren and Lee, 1986), aurasperone C was found to be mainly present in the spores. Naphthopyrones seem to accumulate within *A. niger* cells.

Isolation of naphthopyrones has been described by various authors (Ehrlich et al., 1984; Fischer et al., 2000; Nielsen et al., 1999).

3.7. Nigerazines

Nigerazines were first isolated by Iwamoto et al. (1983, 1985) from *A. niger* and found to inhibit root growth of lettuce seedlings. Nigerazines can be extracted from the mycelia of *A. niger* (Iwamoto et al., 1983).

3.8. Nigragillin

Nigragillin production by *A. niger* was first reported by Caesar et al. (1969). It can be extracted from culture filtrate and was demonstrated to be toxic to silkworm larvae (Isogai et al., 1975). The procedure for the isolation of nigragillin was described in the same article. The compound was also found to be produced by *A. phoenicis* which is a species in Section *Nigri* (Cole and Cox, 1981).

3.9. 3-Nitropropionic acid

Production of 3-nitropropionic acid (3-NPA) by *A. oryzae* was first reported by Nakamura and Shimoda (1954). This compound is considered a neurotoxin by irreversibly inhibiting succinate dehydrogenase (EPA, 1997b), leading to the dysfunction of mitochondria (Albin, 2000). The pathology and mechanism of action of 3-NPA were reviewed by Alexi et al. (1998) and Albin (2000). The possible association of 3-NPA with human health was reviewed by a Croatian research group (Peraica et al., 1999; Peraica and Dominjan, 2001). 3-NPA can be extracted from the culture filtrate (Orth, 1977) as well as from the culture (Penel and Kosikowsi, 1990) of *A. oryzae*. The spectrophotometric determination of 3-NPA was described by Masumoto et al. (1961).

Based on animal studies, Soni et al. (unpublished) recently proposed an ADI of 25 μg/kg/day for human intake of 3-NPA. The authors also estimated that the 3-NPA consumption from fermented foods is appropriate 5.5 mg/day/individual in the Japanese population.

3.10. Ochratoxin A

Ochratoxin A, a nephrotoxic and carcinogenic compound, is considered the most toxic among ochratoxins and its impact on human and animal health has been reviewed elsewhere (Abarca et al., 2001; Benford et al., 2001; Bondy and Pestka, 2000) and assessed by different jurisdictions as reviewed by Walker (2002). The toxin is receiving attention because it is a common food contaminant. Ochratoxins are derived primarily from *Aspergillus ochraceus* and *Penicillium verrucosum* (Larsen et al., 2001). Abarca et al. (1994) first reported the production of ochratoxin A in *A. niger* and isolated the toxin from the culture extract of *A. niger* grown on both liquid and solid medium. The toxin is also found to be

produced by other species in Section *Nigri* (Teren et al., 1996).

Screening and analytical methods for ochratoxin A are discussed by Benford et al. (2001) and Scott (2002). Until recently, JECFA required that food enzyme preparations should not contain detectable amounts of ochratoxin A, as determined by the specified method (JECFA, 1989).

3.11. Oxalic acid

Oxalic acid is produced by a variety of fungi including *A. niger*. The toxic effects of oxalic acid are due to complexing of oxalate with calcium, causing hypocalcemia and renal failure secondary to deposition of complexes in the renal tubules and vasculature (RTECS). Oxalic acid is produced and excreted to the environment by many fungi in abundance and can be found extensively in nature. Unlike secondary metabolites, the compound seems to be somehow beneficial to the producing organisms. The production of oxalic acid in fungi and its possible roles in fungal pathogenicity and ecology was reviewed by Dutton and Evans (1996). *A. niger* was found able to produce oxalic acid in liquid media and to secrete the acid into the medium as well as on solid natural substrates at both 26 and 35 °C. The acid could then be precipitated with calcium acetate at low pH (Wilson and Wilson, 1961).

3.12. Trichodermin

Trichodermin is a member of the trichothecene toxin family—a group of sesquiterpenes. While some toxins in this family, such as T-2 toxin, are potent food contaminants, trichodermin is much less toxic although it is similar to T-2 toxin in structure and biochemical activity. Trichodermin was reported to be produced by a strain of *T. reesei* (Watts et al., 1988) but this discovery may need further confirmation. One opinion is that the only producer of trichodermin in *Trichoderma* is a strain from *T. harzianum* (van Dijck, personal communication). The metabolite can be produced in synthetic medium and is excreted into the culture medium.

Trichothecenes can be detected using chromatographic methods (Eppley, 1975; Frisvad and Thrane, 1987; Nielsen and Thrane, 2001). Trichodermin can be detected with HPLC (Watts et al., 1988).

3.13. Violacetin

Violacetin was only once reported to be produced on malt extracts by a strain of *A. oryzae* as a candidate antibiotic and its pharmacology was reviewed by Kobayashi (1966). The compound is also produced by *Streptomyces* and exhibited a broad spectrum of antibiotic activity. Isolation of violacetin could be achieved by DE (diatomaceous earth) extraction (Aiso et al., 1955).

Some other secondary metabolites have been reported to be produced by *A. niger* or *A. oryzae*. Although considered as mycotoxins by some (Lawrence, 1995), they are not included in this article for the lack of toxicological data on humans and animals. Such compounds include flaviolin, a naphthoquinone produced by a strain of *A. niger* (Astill and Roberts, 1953; McGovern and Bentley, 1975) and oryzacidin produced by a strain of *A. oryzae* (Shimoda, 1951). Although some naphthoquinones are reported as cytotoxic (Dubin et al., 2001), no animal toxicity data can be found on flaviolin.

A few antibiotic substances derived from aspergilli were once named aspergillin by researchers, for example Stanley (1944). In the RTECS database, aspergillin and gliotoxin are considered as synonymous terms with the same CAS number 67-99-2. A proteolytic compound from *A. oryzae* is also named aspergillin (Stefanini et al., 1959) (CAS number 9000-99-1 in the RTECS database). The term aspergillin should, as proposed by Tobie (1946), be reserved for the black pigment from *A. niger*, characterized as a humic acid, that was discovered and named aspergillin by Linossier in 1891.

As a common fungus, *A. niger* is involved in a number of reports on toxicity of food or feed contaminated with molds. Those reports are not discussed in this article because it is difficult to determine the source of toxicity from various contaminating organisms or because the source of toxicity, although perhaps associated with *A. niger*, is not clearly identified. Similarly, toxic factors reported to be produced by *A. niger* isolated from other sources, but chemically unidentified (Fischer et al., 2000; Speth et al., 2000) are also not included in this article.

4. Discussion

4.1. Aflatoxin production in A. niger and A. oryzae

Although low-level production of aflatoxins in *A. niger* was reported (Glinsukon et al., 1979), according to a recent review (Schuster et al., 2002), this species "does not have the ability to produce aflatoxins" and previous reports may result from errors in detection methodology.

Closely related to *A. flavus*, *A. oryzae* has caused more controversy in its potential for aflatoxin production. While aflatoxin production in *A. oryzae* was reported by some authors (Adebajo, 1992; Atalla et al., 2003; Basappa et al., 1967; El-Kady et al., 1994), others were unable to detect aflatoxin production in this fungus (Wicklow, 1983). In one review, the authors claimed

that "aflatoxins are never formed" in *A. oryzae* (Barbesgaard et al., 1992). It is possible that the aflatoxin-producing strains were incorrectly identified as *A. oryzae* because of the close taxonomic relatedness between this fungus and other members of *A. flavus* group. For example, one early report (El-Hag and Morse, 1976) described aflatoxin production by *A. oryzae* strain NRRL 1988, but the strain was later re-identified as *A. parasiticus* (Fennell, 1976). Today, it is agreed by the majority of the scientific community that *A. oryzae* does not produce aflatoxins (Z. Kozakiewicz, personal communication).

Among all important mycotoxins, the genetics of aflatoxin synthesis is the most studied. With the completion of the sequencing of the *A. niger* genome, it is now possible, at least to persons having access to the sequence, to examine whether the genes involved in aflatoxin synthesis are present in *A. niger*. On the other hand, there is evidence that *A. oryzae* possesses some genes from the aflatoxin biosynthesis pathway but, among the examined strains, the genes appear to be inactive (Bhatnagar, 2002; Kusumoto et al., 1998a,b; Kusumoto et al., 2000; Liu and Chu, 1998; Watson et al., 1999; Wei and Jong, 1986).

4.2. Potency of toxic metabolites

As cited previously in this article, mycotoxins are defined as "natural products produced by fungi that evoke a toxic response when introduced in low concentration to higher vertebrates and other animals by a natural route." While *A. niger* and *A. oryzae* do produce some important mycotoxins such as ochratoxin A and perhaps 3-nitropropionic acid, it is questionable that many other secondary metabolites they produce should be classified as mycotoxins, especially when some researchers propose to define mycotoxins as being toxic to vertebrates only (van Dijck, personal communication).

To evaluate the toxic potency of a chemical, the dosage at which the chemical exhibits toxicity, the identity of the recipient, and the route by which the chemical is introduced to the recipient must all be specified. In their recent review, Bhatnagar et al. (2002) pointed out that the toxicity of the mycotoxins "varies considerably with the toxin, the animal species exposed to it, and the extent of exposure, age and nutritional status." In Table 1, the acute toxicity of the metabolites produced by *A. niger*, *A. oryzae*, and *T. reesei* is reported as LD_{50}. The degree of acute toxicity of a chemical substance as LD_{50} and the conditions under which the LD_{50} is determined are specified in 16 CFR §1500.3. Many LD_{50} values from Table 1 were obtained under conditions different from the CFR specifications,—therefore, these values may not truly describe the acute toxicity of those metabolites and cannot be used to determine whether the substances can be claimed as a mycotoxin under the current definition.

Easy to determine, LD_{50} used to be widely used as an indicator of toxicity. Nowadays, however, it is being replaced by NOAEL which is considered more useful to describe the toxicity of a substance. Future information of NOAEL values of the secondary metabolites of *A. niger*, *A. oryzae*, and *T. reesei* will better help determine the toxic potency of those chemicals and clarify their status among mycotoxins.

4.3. Conditional production of toxic metabolites

Mycotoxins are a large group of structurally diverse compounds. Despite the knowledge of the biosynthesis of several mycotoxins (Franck, 1984), the pathways and regulations for the biosynthesis of many mycotoxins are yet to be elucidated. The Environmental Protection Agency (EPA, 1997a) evaluated the safety of *A. niger* and concluded that "mycotoxin production appears to be controlled by the conditions of fermentation" and be strain specific. EPA (1997b) also evaluated the safety of *A. oryzae* and concluded that "under usual conditions of culture, well-established commercial strains of this species do not seem to produce significant levels of mycotoxins, although certain moderately potent toxins can be produced after extended culture." "Mycotoxin production can most likely be avoided by properly controlling the fermentation conditions." The production of mycotoxins in fungi, being strain-dependent and growth condition-dependent, is a combined consequence of the genetic feature of the strain and its environment. It is completely possible that non-toxic strains are selected as production organisms and, furthermore, that production conditions are designed and controlled to favor the selective production of the product of interest, in order to preclude the presence of mycotoxins in the product.

4.3.1. Strain-specificity

Production of mycotoxins is limited in certain strains, a fact which has been demonstrated in a number of studies. Aurasperone production on rice by *A. niger* was found to be strain-specific (Ehrlich et al., 1984). In separate studies, 50% of *A. oryzae* strains tested were found to produce CPA in YES broth (Munimbazi and Bullerman, 1996), 33.3% of *A. oryzae* strains tested were found to produce kojic acid on mixed feed (Kharchenko and Yatsyshin, 1984) and 11.5% of *A. niger* strains tested were found to produce ochratoxin A (Urbano et al., 2001). A number of toxicological investigations of natural fungal isolates also demonstrate that many *A. niger* isolates are non-toxic (Davis et al., 1975; El-Shanawany et al., 1999; Glinsukon et al., 1979; Shank et al., 1972; Youssef et al., 2000). It should be pointed out however, that the production and detection of toxicity

may be limited by growth conditions and detection methodology.

Knowledge of the genetics and metabolism of mycotoxins is still lacking despite attempts to research the production of mycotoxins in *A. niger* and *A. oryzae* at the level of genetics. Benkhemmar et al. (1985) reported the artificial mutation of CPA-producing *A. oryzae* (CPA+) strains into non-CPA-producing (CPA-) ones, and vice versa. They also examined CPA production among heterokaryons resulting from pairing of those mutants and concluded that "the risks of producing this toxin from 2 CPA- individuals are not high." Accensi et al. (2001) examined 92 *A. niger* strains for ochratoxin A production and found that all the 6 positive strains were N pattern ones. It is foreseeable that the decoding of the *A. niger* genome will help the studies of ochratoxin A production in *A. niger* and provide a powerful tool for the screening of non-toxic strains.

4.3.2. Time

Mycotoxins are secondary metabolites. Secondary metabolites are usually produced during the stationary phase of microbial culture growth, although exceptions do seem to occur (Kharchenko, 1999). Production of cyclopiazonic acid in *A. oryzae* was reported to start 50 h after inoculation and to reach a maximum level in 2 weeks (Goto et al., 1987). Kojic acid does not seem to be produced within the typical 2-day koji fermentation time (EPA, 1997b). Production of malformins and maltoryzine usually occurs in stationary phase (EPA, 1997b; Yukioka and Winnick, 1966). Calvo et al. (2002) associated mycotoxin production with sporulation in aspergilli.

4.3.3. Environment

Microbial metabolism is significantly influenced by the physical and chemical environment. For given strains of *A. niger*, aurasperone production was found to be substrate-dependent (Ehrlich et al., 1984). Production of kojic acid by *A. oryzae* is associated with the availability of oxygen and high dosage of glucose (Ogawa et al., 1995). EPA (1997a) concluded that production of malformins by *A. niger* is related to growth conditions, preferably on solid media and that of maltoryzine by *A. oryzae* is related to the composition of the growth media (EPA, 1997b). The environmental pH appeared to be a main factor governing oxalic acid production in *A. niger* (Roehr et al., 1992; Ruijter et al., 1999). In one study, *A. oryzae* was grown on various high protein and carbohydrate-rich solid foods and was found to produce 3-nitropropionic acid on some of the foods (Penel and Kosikowsi, 1990).

4.4. Relevance of mycotoxins to human health: JECFA's new specifications

Recently, JECFA revised the general specifications and considerations for enzyme preparations used in food processing including those for mycotoxins. Noting that the list of five mycotoxins contained in the 1989 General Specifications was not relevant to all food enzyme preparations from fungal sources, the Committee "agreed that enzyme preparations derived from fungal sources be evaluated for those mycotoxins that are known to be produced by strains of the species used in the production of the enzyme preparation or related species" (JECFA, 2001). Compared to the previous requirement which was specific but arbitrary, the new one, which mandates mycotoxin testing but de-specifies mycotoxins to be tested in fungal enzyme preparations, attempts to avoid both unnecessary and irrelevant testing for mycotoxins and to avoid missing a test for relevant mycotoxins other than the five previously listed ones for a specific species. Yet the details of the degree of relatedness, the species that are considered as related to *A. niger*, *A. oryzae*, and *T. reesei*, the mycotoxins that need to be tested, the testing methods, and the minimal allowance all need to be explored.

4.4.1. The scope of relatedness of fungal species—the relevancy among fungi

The new JECFA specifications require that fungal-derived food enzyme preparations be tested for mycotoxins produced not only by the production species but also by fungal species related to the production species. This consideration of testing relevancy based on the biological relatedness of fungi defines the scope of mycotoxin testing for each production organism. Such relatedness can be obvious. For example, aflatoxins should continue to be tested in *A. oryzae*-derived food enzyme preparations because of the close relatedness between *A. oryzae* and *A. flavus*. However, the specifications did not define, on a measurable scale, the degree of the relatedness at which testing for mycotoxins becomes necessary.

The determination of such a degree of relatedness, and thus the name of the related fungal species, will require a complete and concise examination of the genomic sequences of microorganisms. These genomic sequence data currently are not always available. Fortunately, the development of modern taxonomy has enabled scientists to group microorganisms using both genotypical and phenotypical measures including the pattern of secondary metabolite production. Often species in the same natural series produce the same secondary metabolites. If a significant mycotoxin is found to be produced by a closely related species, in the current taxonomic frame, of the production fungal species, this mycotoxin should be tested in the enzyme preparations derived from this production organism. The extent of this relatedness, however, should be judged on a case-by-case basis, through a careful evaluation based on knowledge of taxonomy, biochemistry, and genetics. In many cases, the testing scope at the level of genus should

be sufficient. In other cases, the scope can even be further narrowed.

4.4.2. What to test—the medical importance of mycotoxins

The removal by JECFA of the specification for testing of specific mycotoxins in food enzyme preparations leads to the question of which mycotoxins are "significant" and so should be tested in those preparations. Since an attempt to list all known mycotoxins of potential concern is "impractical and unwarranted" (JECFA, 2001), it can be expected that mycotoxin testing in food enzyme preparations will focus on mycotoxins that are medically important via ingestion and relevant to the production organism. Therefore, even though the scope of testing is extended from the mycotoxins produced by one species to those produced by a few species, the number of mycotoxins to be tested can be limited, at least in food enzyme preparations.

Mycotoxins that have been related to human intoxication include aflatoxins, cyclopiazonic acid, citreoviridin, ergot (not considered a threat today), fumonisins, 3-nitropropionic acid, ochratoxin A, certain trichothecenes, and zearalenone (Peraica and Dominjan, 2001). Aflatoxins perhaps are the best-known mycotoxin of medical importance. Others currently considered as important to human health may include ochratoxin A, fumonisins, certain trichothecenes, and zearalenone, judged by their potency and the degree of possibility of human exposure (Pitt, 2000). The impact of these mycotoxins, all active via the oral route, on human health and their mechanisms have been reviewed elsewhere (Bondy and Pestka, 2000; van Egmond, 2000; Peraica et al., 1999; Peraica and Dominjan, 2001; Pitt, 2000). These mycotoxins should be the primary testing candidates in food enzyme preparations of fungal origin. Table 2 demonstrates the currently known sources of those mycotoxins (JECFA, 1989; Peraica and Dominjan, 2001; Pitt, 2000).

Traditionally, mycotoxins that are important to human health and thus important to the food industry need to be active via the oral route. The development of biotechnology, however, has opened the possibility of using fungi to produce therapeutic biologics. This means that toxic fungal metabolites, if present in the biologics, can enter the human body via a route that has not been considered relevant to human health: injection. To cause a similar toxic reaction, the dosage of a toxic substance by injection is usually lower than that by ingestion. The impact of this new route on mycotoxin testing in those products can be significant not only because a wide spectrum of testing will be required but also because the standard of detection levels may need to be lowered. Certain mycotoxins that nowadays need not to be considered as important in the food industry, such as malformins by *A. niger*, may become relevant to human health in biologics.

4.4.3. What to test in food grade enzyme preparations derived from A. niger, A. oryzae, and T. reesei?

Among those mycotoxins in Table 2, aflatoxins, 3-NPA, sterigmatocystin, and ochratoxin A are known to be produced by *Aspergillus*. Trichothecene T-2 has been reported to be produced by *Trichoderma* although some experts seriously doubt that *Trichoderma* can produce this toxin (van Dijck, personal communication).

Table 2
Production of some mycotoxins known to be associated with human health

Mycotoxin	Producers*	Confirmed production in		
		A. niger	*A. oryzae*	*T. reesei*
Aflatoxin B1[a,b,c]	*Aspergillus*	No	No	No
Citreoviridin[c]	*Penicillium*	No	No	No
Fumonisins[b,c]	*Fusarium***	No	No	No
3-Nitropropionic acid[c]	*Aspergillus, Penicillium Arthrinuim****	No	Yes	No
Ochratoxin A[a,b,c]	*Aspergillus, Penicillium*	Yes	No	No
Sterigmatocystin[a]	*Aspergillus, Bipolaris Chaetomium*****	No	No	No
Trichothecenes				
T-2[a]	*Fusarium, Trichoderma*	No	No	No
Deoxynivalenol[b,c]	*Fusarium*	No	No	No
Nivalenol[c]	*Fusarium*	No	No	No
Diacetoxyscirpenol[c]	*Fusarium, Gibberella*	No	No	No
Zearalenone[a,b,c]	*Fusarium*	No	No	No

[a] Testing previously required by JECFA in all fungal-derived food enzyme preparations.
[b] Considered medically important by Pitt (2000).
[c] Postulated human mycotoxicoses by Peraica and Dominjan (2001).
* From Cole and Cox (1981) unless indicated.
** Pitt (2000); Peraica and Dominjan (2001).
*** Peraica et al. (1999).
**** Udagawa et al. (1979).

Aflatoxins are reported to be produced by several *Aspergillus* species (Ito et al., 2001; Klich et al., 2000; Peterson et al., 2001; Pitt, 2000). Most aflatoxin-producing aspergilli belong to Section *Flavi*. Because of the close relatedness of *A. oryzae* to the aflatoxin-producing fungi, aflatoxin testing should continue in enzyme preparations derived from *A. oryzae*. Although *A. niger* was never found to produce aflatoxins and some data may indicate the possible lacking of a complete gene family for aflatoxin biosynthesis in *A. niger* (Chen et al., 2002), given the medical significance of this toxin, aflatoxins should continue to be tested in *A. niger*-derived enzyme preparations unless future data ensure that genetically this fungus is incapable of aflatoxin production.

Sterigmatocystin is known to be produced by species from *Aspergillus* sections *Nidulantes*, *Versicolores*, and *Usti* (Cole and Cox, 1981). All sections belong to subgenus *Nidulantes*. Sterigmatocystin production has been reported also in sections *Circumdati* and *Nigri* (Begum and Samajpati, 2000; Klich et al., 2000) but these reports have not been confirmed by other researchers. Since sterigmatocystin is also an intermediate in aflatoxin synthesis by Section *Flavi*, the testing of this toxin in *A. oryzae* can be justified but that in *Nigri* may require stronger evidence of sterigmatocystin production by this section before testing is justified.

Ochratoxins are first known to be produced by species from Section *Circumdati* (*A. orchraceus* group) (Cole and Cox, 1981). Later, the production of this toxin was repeatedly reported in Section *Nigri* including *A. niger* (Abarca et al., 1994; Da et al., 2002; Teren et al., 1996). Ochratoxin should continue to be tested in *Aspergillus*-derived enzyme preparations.

Compound 3-NPA is known to be produced by species from *Aspergillus* sections *Flavi* and *Wentii* (Cole and Cox, 1981; Peterson et al., 2001). Both sections, as well as Section *Nigri*, belong to subgenus *Circumdati*. 3-NPA is a potent neurotoxin irreversibly dysfunctioning an important enzyme in TCA cycle. It is produced by *Aspergillus* including *A. oryzae*. Human intoxication by 3-NPA was reported (reviewed by Peraica et al., 1999). While it may be too early to determine the necessity of testing this compound in enzyme preparations, the development of the production of 3-NPA in aspergilli, the toxicology, and the clinical prevalence of this compound should be monitored by the enzyme industry. If the medical importance of this toxin is further confirmed, it may in the future become a candidate for testing in enzyme preparations derived from *A. niger* and *A. oryzae*.

Trichoderma produces trichothecenes including T-2 toxin (Cole and Cox, 1981). This toxin should continue to be tested in *Trichoderma*-derived enzyme preparations.

Hypocrea does not seem to produce any medically important mycotoxin. Other miscellaneous toxic substances were reported to be produced by Section *Nigri* (Akatsu, 1952; Alfatafta et al., 1996; Andersen et al., 1977; Cole and Cox, 1981; TePaske et al., 1989), Section *Flavi* (Brookes et al., 1963; Cole and Cox, 1981; Kimura et al., 1982), *Trichoderma* (Corley et al., 1994; Hou et al., 1972; Landreau et al., 2002; Leclerc et al., 2001; Rebuffat et al., 1991; Vicente et al., 2001), and *Hypocrea* (Brueckner et al., 1991; Nair and Carey, 1979; Yamamoto et al., 1969). None of them is recognized as medically important and as a known product of *A. niger*, *A. oryzae*, or *T. reesei*.

Our understanding of mycotoxins—its production, mechanism, and medical importance—is constantly developing and impacting our decisions regarding what to test in enzyme preparations. It must again be pointed out that it is not uncommon that in literature of mycotoxin production the identification of the production organisms is incorrect. This has caused much confusion. Also the production of mycotoxins is dependent upon the growth environment. Caution must be exercised when information is analyzed and conclusions are drawn. Furthermore, when discussing which mycotoxins to test in enzyme preparations derived from *A. niger*, *A. oryzae*, or *T. reesei*, the assessment made by this article is limited to the production organism itself and does not extend to possible contamination from raw materials used in manufacturing.

5. Conclusion: information regarding the safe use of *A. niger*, *A. oryzae*, and *T. reesei*

A. niger, *A. oryzae* and *T. reesei* are well-known production organisms used in the food and food/feed additives industry. To ensure the safety of these products, the industry carefully selects and maintains, and sometimes modifies, the production strains. Fermented foods produced by *A. niger* and *A. oryzae* were shown to be aflatoxin-free (Liu and Chen, 1966). Some soy sauce production strains of *A. oryzae* were tested and demonstrated that none of those strains produced detectable amount of cyclopiazonic acid (Goto et al., 1987). EPA (1997b) concluded that commercial strains of *A. oryzae* "apparently do not produce maltoryzine." In the field of enzyme production, the safety of the three species as production organisms including toxigenicity has been assessed (Barbesgaard et al., 1992; Coenen et al., 1995; van Dijck et al., 2003; Nevalainen et al., 1994; Schuster et al., 2002). Toxicity tests on several enzyme preparations derived from the three species indicate that they are safe for use in their intended applications (Bergman and Broadmeadow, 1997; Broadmeadow et al., 1994; Greenough et al., 1996; Lane et al., 1997; Ye and Fields, 1989). In the United States, a number of enzyme preparations derived from *A. niger*, A. *oryzae*, and *T. reesei* have been granted GRAS status in various

food-processing applications based on publicly available information and scientific studies (GRN89; GRN90; GRN113; 21 CFR §184.1250). Despite the wide use of *A. niger, A. oryzae*, and *T. reesei* in the fermentation industry, reports on the production of aflatoxin B, 3-NPA, ochratoxin A, sterigmatocystin, T-2 toxin, and zearalenone from production strains from the 3 species are extremely rare. One example is *A. niger* CBS 618.78 that has been extensively used for enzyme preparation and is found to be able to produce ochratoxin A under certain lab conditions (Schuster et al., 2002). Ochratoxin A production is inhibited, however, when growth conditions are modified.

When developing a new production strain from *A. niger*, *A. oryzae*, and *T. reesei*, it is of primary importance that the strain is tested for the production of any toxicologically significant amount of relevant mycotoxins. This is especially important if the microorganism and/or its products are to be used in the food industry. Care must be taken to ensure these production strains do not produce mycotoxins under commercial enzyme production conditions. The toxigenicity of *A. niger*, *A. oryzae*, and *T. reesei* is controllable, these organisms are safe to use as production organisms, and the products derived from these microorganisms are safe to use, given that (1) the production species is correctly identified, (2) the production strain is carefully selected, manipulated, and tested to ensure its lack of relevant mycotoxin production, (3) the research, development, and manufacturing processes including the establishment of a validated seed bank are carefully designed, operated, and monitored, and (4) the product is routinely tested for mycotoxin contamination although there has not been any report of spontaneous transformation of non-toxic production strains into toxic ones. It should be stressed that most industrial production strains of *A. niger*, *A. oryzae*, and *T. reesei* have successfully demonstrated a long history of safe use.

Acknowledgments

The author thanks Ms. A. Caddow, Dr. P. van Dijck, Mr. J. Mercer, Dr. M. Pariza, Dr. M. Ward, and members of the Enzyme Technology Association (ETA) for their review and comments.

References

Abarca, M.L., Bragulat, M.R., Castella, G., Cabanes, F.J., 1994. Ochratoxin A production by strains of *Aspergillus niger* var. *niger*. Appl. Environ. Microbiol. 60, 2650–2652.

Abarca, M.L., Accensi, F., Bragulat, M.R., Cabanes, F.J., 2001. Current importance of ochratoxin A-producing *Aspergillus* spp. J. Food Prot. 64, 903–906.

Accensi, F., Abarca, M.L., Cano, J., Figuera, L., Cabanes, F.J., 2001. Distribution of ochratoxin A producing strain in the *A. niger* aggregate. Antonie van Leeuwenhoek 79, 365–370.

Adebajo, L.O., 1992. Spoilage moulds and aflatoxins from poultry feeds. Nahrung 36, 523–529.

Aiso, K., Arai, T., Shidara, I., Kurihara, H., Morita, Y., 1955. A new broad spectrum antibiotic, violacetin. J. Antibiot. Japan 8 (A), 33–38.

Akatsu, K., 1952. The antibiotic substance produced by *Aspergillus japonicus*. II. Molecular formula, toxicity, and some practical tests. J. Agr. Chem. Soc. Japan 1951–1952 25, 517–519.

Albin, R.L., 2000. Basal ganglia neurotoxins. Neurol. Clin. 18, 665–680.

Alexi, T., Hughes, P.E., Faull, R.L., Williams, C.E., 1998. 3-Nitropropionic acid's lethal triplet: cooperative pathways of neurodegeneration. Neuroreport 9, R57–R64.

Alfatafta, A.A., Dowd, P.K., Gloer, J.B., Wicklow, D.T., 1996. Carbonarin insecticidal fungal metabolites. US Patent 5,519,052.

Anderegg, R.J., Biemann, K., Buchi, G., Cushman, M., 1976. Malformin C, a new metabolite of *Aspergillus niger*. J. Am. Chem. Soc. 98, 3365–3370.

Andersen, R., Buechi, G., Kobbe, B., Demain, A.L., 1977. Secalonic acids D and F are toxic metabolites of *Aspergillus aculeatus*. J. Org. Chem. 42, 352–353.

Astill, B.D., Roberts, J.C., 1953. Studies in mycological chemistry. Part I. Flaviolin, 2 (or 3):5:7-trihydroxy-1:4-naphthaquinone, a metabolic product of *Aspergillus citricus* (Wehmer) Mosseray. J. Chem. Soc., 3302–3307.

Atalla, M.M., Hassanein, N.M., El-Beih, A.A., Youssef, Y.A., 2003. Mycotoxin production in wheat grains by different aspergilli in relation to different relative humidities and storage periods. Nahrung 47, 6–10.

Barbesgaard, P., Heldt-Hansen, H., Diderichsen, B., 1992. On the safety of *Aspergillus oryzae*: a review. Appl. Microbiol. Biotechnol. 36, 569–572.

Basappa, S.C., Jayaraman, A., Sreenivasamurthy, V., Parpia, H.A.B., 1967. Effect of B-group vitamins and ethyl alcohol on aflatoxin production by *Aspergillus oryzae*. Indian J. Exp. Biol. 5, 262–263.

Begum, F., Samajpati, N., 2000. Mycotoxin production on rice, pulses and oilseeds. Naturwissenschaften 87, 275–277.

Benford, D., Boyle, C., Dekant, W., Fuchs, R., Gaylor, D.W., Hard, G., McGregor, D.B., Pitt, J.I., Plestina, R., Shephard, G., Solfrizzo, M., Verger, P.J.P., Walker, R., 2001. Ochratoxin A. JECFA 47. Available from http://www.inchem.org/documents/jecfa/jecmono/v47je04.html.

Benkhemmar, O., Gaudemer, F., Bouvier-Fourcade, I., 1985. Heterokaryosis between *Aspergillus oryzae* cyclopiazonic acid-defective strains: method for estimating the risk of inducing toxin production among cyclopiazonic acid-defective industrial strains. Appl. Environ. Microbiol. 50, 1087–1093.

Bennett, J.W., 1987. Mycotoxins, mycotoxicoses, mycotoxicology and *Mycopathologia*. Mycopathologia 100, 3–5.

Bergman, A., Broadmeadow, A., 1997. An overview of the safety evaluation of the *Thermomyces lanuginosus* xylanase enzyme (SP 628) and the *Aspergillus aculeatus* xylanase enzyme (SP 578). Food Addit. Contam. 14, 389–398.

Beuchat, L.R., 2001. Traditional fermented foods. In: Doyle, M.P., Beuchat, L.R., Montville, T.J. (Eds.), Food Microbiology Fundamentals and Frontiers, second ed. ASM Press, Washington, DC, pp. 701–720.

Bhatnagar, D., 2002. Aflatoxins: are they biological weapons? Symp. 261. 102nd ASM general meeting, Salt Lake City, UT.

Bhatnagar, D., Yu, J., Ehrlich, K.C., 2002. Toxins of filamentous fungi. Chem. Immunol. 81, 167–206.

Bissett, J., 1984. A revision of the genus *Trichoderma*.I. Section *Longibrachiatum* sect. Nov. Can. J. Bot. 62, 924–931.

Bondy, G.S., Pestka, J.J., 2000. Immunomodulation by fungal toxins. J. Toxicol. Envir. Health. B 3, 109–143.

Broadmeadow, A., Clare, C., de Boer, A.S., 1994. An overview of the safety evaluation of the *Rhizomucor miehei* lipase enzyme. Food Addit. Contam. 11, 105–119.

van den Broek, P., Pittet, A., Hajjaj, H., 2001. Aflatoxin genes and the aflatoxigenic potential of Koji moulds. Appl. Microbiol. Biotechnol. 57, 192–199.

Brookes, D., Tidd, B.K., Turner, W.B., 1963. Avenaciolide, and antifungal lactone from *Aspergillus avenaceus*. J. Chem. Soc., 5385–5391.

Brueckner, H., Maisch, J., Reinecke, C., Kimonyo, A., 1991. Use of α-aminoisobutyric acid and isovaline as marker amino acids for the detection of fungal polypeptide antibiotics. Screening of *Hypocrea*. Amino Acids 1, 251–257.

Burdock, G.A., Flamm, W.G., 2000. Review article: safety assessment of the mycotoxin cyclopiazonic acid. Int. J. Toxicol. 19, 195–218.

Burdock, G.A., Soni, M.G., Carabin, I.G., 2001. Evaluation of health aspects of kojic acid in food. Regul. Toxicol. Pharmacol. 33, 80–101, doi:10.1006/rtph.2000.1442.

Caesar, F., Jansson, K., Mutschler, E., 1969. Nigragillin, a new alkaloid from the *Aspergillus niger* group.1. Isolation and structure clarification of nigragillin and a dioxopiperazine. Pharm. Acta Helv. 44, 676–690.

Calvo, A.M., Wilson, R.A., Bok, J.W., Keller, N.P., 2002. Relationship between secondary metabolism and fungal development. Microbiol. Mol. Biol. Rev. 66, 447–459, doi:10.1128/MMBR.66.3.447-459.2002.

Chen, R.S., Tsau, J.G., Huang, Y.F., Chiou, R.Y., 2002. Polymerase chain reaction-mediated characterization of molds belonging to the *Aspergillus flavus* group and detection of *Aspergillus parasiticus* in peanut kernels by a multiplex polymerase chain reaction. J. Food Prot. 65, 840–844.

Coenen, T.M.M., Schoenmakers, A.C.M., Verhagen, H., 1995. Safety evaluation of beta-glucanase derived from *Trichoderma reesei*: summary of toxicological data. Food Chem. Toxicol. 33, 859–866.

Cole, R.J., Cox, R.H., 1981. Handbook of Toxic Fungal Metabolites. Academic Press, New York, NY.

Corley, D.G., Miller-Wideman, M., Durley, R.C., 1994. Isolation and structure of harzianum A: a new trichothecene from *Trichoderma harzianum*. J. Nat. Prod. 57, 422–425.

Curtis, R.W., 1961. Studies on the response of bean seedlings and corn roots to malformin. Plant Physiol. 36, 37–43.

Da, R.R., Palacios, V., Combina, M., Fraga, M.E., De, O.R., Magnoli, C.E., Dalcero, A.M., 2002. Potential ochratoxin A producers from wine grapes in Argentina and Brazil. Food Addit. Contam. 19, 408–414.

Daniel, E.E., van Breemen, C., Schilling, W.P., Kwan, C.Y., 1995. Regulation of vascular tone: cross-talk between sarcoplasmic reticulum and plasmalemma. Can. J. Physiol. Pharmacol. 73, 551–557.

Davis, N.D., Wagener, R.E., Dalby, D.K., Morgan-Jones, G., Diener, U.L., 1975. Toxigenic fungi in food. Appl. Microbiol. 30, 159–161.

van Dijck, P.W.M., Selton, G.C.M., Hempenius, R.A., 2003. On the safety of a new generation of DSM *Aspergillus niger* enzyme production strains. Regul. Toxicol. Pharmacol. 38, 27–35, doi:10.1016/S0273-2300(03)00049-7.

Dorner, J.W., 2002. Recent advances in analytical methodology for cyclopiazonic acid. Adv. Exp. Med. Biol. 504, 107–116.

Dubin, M., Fernandez, V.S.H., Stoppani, A.O., 2001. Cytotoxicity of beta-lapachone, a naphthoquinone with possible therapeutic use. Medicina (B Aires) 61, 343–350.

Dutton, M.V., Evans, C.S., 1996. Oxalate production by fungi: its role in pathogenicity and ecology in the soil environment. Can. J. Microbiol. 42, 881–895.

van Egmond, H.P., 2000. Mycotoxins: detection, reference materials and regulation. In: Samson, R.A., Hoekstra, E.S., Frisvad, J.C., Filtenborg, O. (Eds.), Introduction to Food- and Airborne Fungi, sixth ed. CBS, The Netherlands, pp. 332–338.

Ehrlich, K.C., deLucca II, A.J., Ciegler, A., 1984. Naphtho-γ pyrone production by *Aspergillus niger* isolated from stored cottonseed. Appl. Environ. Microbiol. 48, 1–4.

El-Hag, N., Morse, R.E., 1976. Aflatoxin production by a variant of *Aspergillus oryzae* (NRRL 1988) on cowpeas (*Vigna sinensis*). Science 192, 1345–1346.

El-Kady, I., El-Maraghy, S., Zohri, A., 1994. Mycotoxin producing potential of some isolates of *Aspergillus flavus* and *Eurotium* groups from meat products. Microbiol. Res. 149, 297–307.

El-Shanawany, A.A., Mahmoud, A.L.E., Baraket, A., 1999. Interacting effects of temperature and storage period on lipolytic activity and mycotoxin-production potential of fungi inhabiting Egyptian raw butter. Afr. J. Mycol. Biotechnol. 7, 25–38.

Environmental Protection Agency (EPA), 1997a. Final decision document: TSCA section 5(H)(4) exemption for *Aspergillus niger*. Attachment I. Item #: 3171.

Environmental Protection Agency (EPA), 1997b. Final decision document: TSCA section 5(H)(4) exemption for *Aspergillus oryzae*. Attachment I. Item #: 3173.

Eppley, R.M., 1975. Methods for the detection of trichothecenes. J. Assoc. Off. Anal. Chem. 58, 906–908.

Evaluation and Review Panel for Food Additive Safety, 2002. Food additive "kojic acid." Meeting summary. December, 2002.

Fennell, D.I., 1976. *Aspergillus oryzae* (NRRL strain 1988): a clarification. Science 194, 1188.

Fischer, G., Muller, T., Schwalbe, R., Ostrowski, R., Dott, W., 2000. Species-specific profiles of mycotoxins produced in cultures and associated with conidia of airborne fungi derived from biowaste. Int. J. Hyg. Environ. Health 203, 105–116.

Franck, B., 1984. Mycotoxins from mold fungi—weapons of uninvited fellow-boarders of man and animal: structures, biological activity, biosynthesis, and precautions. Angew. Chem. Int. Ed. Engl. 23, 493–505.

Frisvad, J.C., Thrane, U., 1987. Standardized high-performance liquid chromatography of 182 mycotoxins and other fungal metabolites based on alkylphenone retention indices and UV–VIS spectra (diode array detection). J. Chromatogr. 404, 195–214.

Frisvad, J.C., Samson, R.A., 2000. *Neopetromyces* gen. Nov. and an overview of teleomorphs of *Aspergillus* subgenus *Circumdati*. Stud. Mycol. (Baarn) 45, 201–207.

Geiser, D.M., Dorner, J.W., Horn, B.W., Taylor, J.W., 2000. The phytogenetics of mycotoxin and sclerotium production in *Aspergillus flavus* and *Aspergillus oryzae*. Fungal Genet. Biol. 31, 169–179.

Ghosal, S., Biswas, K., Chakrabarti, D.K., 1979. Toxic naphtha-gamma-pyrones from *Aspergillus niger*. J. Agric. Food Chem. 27, 1347–1351.

Glinsukon, T., Romruen, K., Visutasunthorn, C., 1979. Preliminary report on toxigenic fungi isolates of *Aspergillus niger* in market foods and foodstuffs. Experientia 35, 522–523.

Goto, T., Shinshi, E., Tanaka, K., Masaru, M., 1987. Production of cyclopiazonic acid by koji molds and possibility of cyclopiazonic acid contamination of Japanese fermented foods. Shokuhin Sogo Kenkyusho Kenkyu Hokoku 51, 23–28.

Greenough, R.J., Perry, C.J., Stavnsbjerg, M., 1996. Safety evaluation of a lipase expressed in *Aspergillus oryzae*. Food Chem. Toxicol. 34, 161–166.

Haenni, A.L., Robert, M., Vetter, W., Roux, L., Barbier, M., Lederer, E., 1965. The chemical structure of the aspergillomarasmines A and B. Helv. Chim. Acta 48, 729–750.

Herbert, J.M., Savi, P., Lale, A., Laplace, M.C., Baudry, N., Pereillo, J.M., Emonds-Alt, X., 1994. Malformin-A1 inhibits the binding of interleukin-1 beta (IL1 beta) and suppresses the expression of tissue factor in human endothelial cells and monocytes. Biochem. Pharmacol. 48, 1211–1217.

Hill, J.E., Lomax, L.G., Cole, R.J., Dorner, J.W., 1986. Toxicologic and immunologic effects of sublethal doses of cyclopiazonic acid in rats. Am. J. Vet. Res. 47, 1174–1177.

Hou, C.T., Ciegler, A., Hesseltine, C.W., 1972. New mycotoxin, trichotoxin A, from *Trichoderma viride* isolated from southern leaf blight-infected corn. Appl. Microbiol. 23, 183–185.

Iizuka, H., Iida, M., 1962. Maltoryzine, a new toxic metabolite produced by a strain of *Aspergillus oryzae* var. *microsporus* isolated from the poisonous malt sprout. Nature 196, 681–682.

Ikeda, S., Sugita, M., Yoshimura, A., Sumizawa, T., Douzono, H., Nagata, Y., Akiyama, S., 1990. *Aspergillus* species strain M39 produced two naphtha-gamma-pyrones that reverse drug resistance in human KB cells. Int. J. Cancer 45, 508–513.

Isogai, A., Horii, T., Suzuki, A., Murakoshi, S., Ikeda, K., Sato, S., Tamura, S., 1975. Isolation and identification of nigragillin as an insecticidal metabolite produced by an *Aspergillus niger*. Agr. Biol. Chem. 39, 739–740.

Ito, Y., Peterson, S.W., Wicklow, D.T., Goto, T., 2001. *Aspergillus pseudotamarii*, a new aflatoxin producing species in *Aspergillus* section *Flavi*. Mycol. Res. 105, 233–239.

Iwamoto, T., Shima, S., Hirota, A., Isogai, A., Sakai, H., 1983. Nigerazine B, a new metabolite from *Aspergillus niger*. Screening, isolation, and chemical and biological properties. Agr. Biol. Chem. 47, 739–743.

Iwamoto, T., Hirota, A., Shima, S., Sakai, H., Isohai, A., 1985. Nigerazine A, an isomer of nigerazine B, from *Aspergillus niger*. Agr. Biol. Chem. 49, 3323–3325.

Joint FAO/WHO Expert Committee on Food Additives (JECFA), 1989. Specifications for Identity and Purity of Certain Food Additives. JECFA 35th session. Rome.

Joint FAO/WHO Expert Committee on Food Additives (JECFA), 2001. Summary and Conclusions. JECFA 57th meeting. Rome.

Kharchenko, S.N., 1999. The biosynthesis of kojic acid by *Aspergillus flavus* Link strains isolated from feed. Mikrobiol. Zh. 61, 15–21.

Kharchenko, S.N., Yatsyshin, A.I., 1984. Species of *Aspergillus* Mich. producing kojic acid on mixed feed and its role in pathology of animals. Mikrobiol. Zh. (Klev) 46, 41–47.

Kim, K., Sugawara, F., Yoshida, S., Murofushi, N., Curtis, R.W., 1993. Structure of malformin B, a phytotoxic metabolite produced by Aspergillus niger. Biosci. Biotech. Biochem. 57, 787–791.

Kimura, Y., Hamasaki, T., Nakajima, H., Isogai, A., 1982. Structure of aszonalenin, a new metabolite of *Aspergillus zonatus*. Tetrahedron Lett. 23, 225–228.

Klich, M.A., Mullaney, E.J., Daly, C.B., Cary, J.W., 2000. Molecular and physiological aspects of aflatoxin and sterigmatocystin biosynthesis by *Aspergillus tamarii* and *A. ochraceoroseus*. Appl. Microbiol. Biotechnol. 53, 605–609.

Kobayashi, T., 1966. Food poisoning. Chiba Daigake Fuhai Kenkyusho Hokoku 19, 92–107.

Kobbe, B., Cushman, M., Wogan, G.N., Demain, A.L., 1977. Production and antibacterial activity of malformin C, a toxic metabolite of *Aspergillus niger*. Appl. Envir. Microbiol. 33, 996–997.

Kuhls, K., Liechfeldt, E., Samuals, G.J., Kovacs, W., Meyer, W., Petrini, O., Gams, W., Borner, T., Kubicek, C.P., 1996. Molecular evidence that the asexual industrial fungus *Trichoderma reesei* is a clonal derivative of the ascomycete *Hypocrea jenorina*. Proc. Natl. Acad. Sci. USA 93, 7755–7760.

Kusumoto, K.I., Yabe, K., Nogata, Y., Ohta, H., 1998a. *Aspergillus oryzae* with and without a homolog of aflatoxin biosynthetic gene ver-1. Appl. Microbiol. Biotechnol. 50, 98–104.

Kusumoto, K.I., Yabe, K., Nogata, Y., Ohta, H., 1998b. Transcript of a homolog of aflR, a regulatory gene for aflatoxin synthesis in *Aspergillus parasiticus*, was not detected in *Aspergillus oryzae* strains. FEMS Microbiol. Lett. 169, 303–307.

Kusumoto, K.I., Nogeta, Y., Ohta, H., 2000. Directed deletions in the aflatoxin biosynthesis gene homolog cluster of *Aspergillus oryzae*. Curr. Genet. 37, 104–111.

Landreau, A., Pouchus, Y.F., Sallenave-Namont, C., Biard, J.F., Boumard, M.C., Robiou du Pont, T., Mondeguer, F., Goulard, C., Verbist, J.F., 2002. Combined use of LC/MS and a biological test for rapid identification of marine mycotoxins produced by *Trichoderma koningii*. J. Microbiol. Methods 48, 181–194.

Lane, R.W., Yamakoshi, J., Kikuchi, M., Mizusawa, K., Henderson, L., Smith, M., 1997. Safety evaluation of tannase enzyme preparation derived from *Aspergillus oryzae*. Food Chem. Toxicol. 35, 207–212.

Larsen, T.O., Svendsen, A., Smedsgaard, J., 2001. Biochemical characterization of ochratoxin A-producing strains of the genus *Penicillium*. Appl. Environ. Microbiol. 67, 3630–3635.

Lawrence, Z., 1995. *Aspergillus* and *Penicillium*. In Identification of Industrially Important Fungi. Course material of International Mycological Institute (IMI). Egham, England.

Leclerc, G., Goulard, C., Prigent, Y., Bodo, B., Wroblewski, H., Rebuffat, S., 2001. Sequences and antimycoplasmic properties of longibrachins LGB II and LGB III, two novel 20-residue peptaibols from *Trichoderma longibrachiatum*. J. Nat. Prod. 64, 164–170.

Liu, B.H., Chu, F.S., 1998. Regulation of *aflR* and its product, AflR, associated with aflatoxin biosynthesis. Appl. Environ. Microbiol. 64, 3718–3723.

Liu, P., Chen, S., 1966. Studies on toxin production by fungi used in fermentation industry. Chung-Kuo Nung Yeh Hua Hsueh Hui Chih 4, 65–73.

Lomax, L.G., Cole, R.J., Dorner, J.W., 1984. The toxicity of cyclopiazonic acid in weaned pigs. Vet. Pathol. 21, 418–424.

Masumoto, H., Unrau, A.M., Hylins, J.W., Temple, B., 1961. Spectrophotometric determination of b-nitropropionic acid in biological extracts. Anal. Chem. 33, 1442–1444.

McGovern, E.P., Bentley, R., 1975. Biosynthesis of flaviolin and 5,8-dihydroxy-2,7-dimenthoxy-1,4-naphthoquinone. Biochemistry 14, 3138–3143.

Meyer, W., Morawetz, R., Borner, T., Kubicek, C.P., 1992. The use of DNA-fingerprint analysis in the classification of some species of the *Trichoderma* aggregate. Curr. Genet. 21, 27–30.

Mikami, Y., Suzuki, T., 1983. Novel microbial inhibitors of angiotensin-converting enzyme, aspergillomarasmines A and B. Agric. Biol. Chem. 47, 2693–2695.

Ministry of Health, Labor and Welfare (MHLW), 2003. Safety measures for nonmedicinal products containing kojic acid. March 7, 2003.

Morrissey, R.E., Noored, W.P., Cole, R.J., Dorner, J., 1985. Toxicity of the mycotoxin, cyclopiazonic acid, to Sprague–Dawley rats. Toxicol. Appl. Pharmacol. 77, 94–107.

Munimbazi, C., Bullerman, L.B., 1996. Molds and mycotoxins in foods from Burundi. J. Food Prot. 59, 869–875.

Nair, M.S.R., Carey, S.T., 1979. Metabolites of Pyrenomycetes: XII. Polyketides from the Hypocreales. Mycologia 71, 1089–1096.

Nakamura, S., Shimoda, C., 1954. Studies on antibiotic substance oryzacidin, produced by *Aspergillus oryzae*. Part 5. Existance of b-nitropropionic acid. J. Agric. Chem. Soc. Japan 28, 909–913.

Nevalainen, H., Suominen, P., Taimisto, K., 1994. On the safety of *Trichoderma reesei*. J. Biotechnol. 37, 193–200.

Nielsen, K.F., Thrane, U., 2001. Fast methods for screening of trichothecenes in fungal cultures using gas chromatography-tandem mass spectrometry. J. Chromatogr. A 929, 75–87.

Nielsen, K.F., Gravesen, S., Nielsen, P.A., Andersen, B., Thrane, U., Frisvad, J.C., 1999. Production of mycotoxins on artificially and naturally infested building materials. Mycopathologia 145, 43–56.

Nuehring, L.P., Rowland, G.N., Harrison, L.R., Cole, R.J., Dorner, J.W., 1985. Cyclopiazonic acid mycotoxicosis in the dog. Am. J. Vet. Res. 46, 1670–1676.

Ogawa, A., Morita, Y., Tanaka, T., Sakiyama, T., Nakanishi, K., 1995. Production of kojic acid from *Aspergillus oryzae* var. *oryzae* by membrance-surface liquid culture. Biotechnol. Tech. 9, 153–156.

Orth, R., 1977. Mycotoxins of *Aspergillus oryzae* strains for use in the food industry as starters and enzyme producing molds. Ann. Nutr. Alim. 31, 617–624.

Palmgren, M.S., Lee, L.S., 1986. Separation of mycotoxin-containing sources in grain dust and determination of their mycotoxin potential. Environ. Health Perspect 66, 105–108.

Pariza, M.W., Johnson, E.A., 2001. Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. Reg. Toxicol. Pharmacol. 33, 173–186.

Paul, R.J., 1998. The role of phospholamban and SERCA3 in regulation of smooth muscle-endothelial cell signaling mechanisms: evidence from gene-ablated mice. Acta Physio. Scand. 164, 589–597.

Penel, A.J., Kosikowsi, F.V., 1990. Beta-nitropropionic acid production by *A. oryzae* in selected high protein and carbohydrate-rich foods. J. Food Protect. 53, 321–323.

Peraica, M., Radic, B., Lucic, A., Pavlovic, M., 1999. Toxic effects of mycotoxins In humans. Bull. World Health Organ. 77, 754–766.

Peraica, M., Dominjan, A.M., 2001. Contamination of food with mycotoxins and human health. Arh. Hig. Rada. Toksikol. 52, 23–35.

Peterson, S.W., Ito, Y., Horn, B.W., Goto, T., 2001. *Aspergillus bombycis*, a new aflatoxigenic species and genetic variation in its sibling species, *A. nomius*. Mycologia 93, 689–703.

Pier, A.C., Richard, J.L., 1992. Mycoses and mycotoxicoses of animals caused by aspergilli. In: Bennett, J.W., Klich, M.A. (Eds.), Aspergillus, Biology and Industrial Applications. Butterworth-Heinemann, Boston, pp. 233–248.

Pier, A.C., Belden, E.L., Ellis, J.A., Nelson, E.W., Maki, L.R., 1989. Effects of cyclopiazonic acid and aflatoxin singly and in combination on selected clinical, pathological and immunological responses of guinea pigs. Mycopathologia 105, 135–142.

Pitt, J.I., 2000. Toxigenic fungi: which are important? Med. Mycol. 38 (Suppl. 1), 17–22.

Rebuffat, S., Prigent, Y., Auvin-Guette, C., Bodo, B., 1991. Tricholongins BI and BII, 19-residue peptaibols from *Trichoderma longibrachiatum*. Solution structure from two-dimensional NMR spectroscopy. Eur. J. Biochem. 201, 661–674.

Roehr, M., Kubicek, C.P., Kominek, J., 1992. Industrial acids and other small molecules. In: Bennett, J.W., Klich, M.A. (Eds.), Aspergillus, Biology and Industrial Applications. Butterworth-Heinemann, Boston, pp. 91–132.

Ruijter, G.J., van de Vondervoort, P.J., Visser, J., 1999. Oxalic acid production by *Aspergillus niger*: an oxalate-non-producing mutant produces citric acid at pH5 and in the presence of manganese. Microbiology 145 (Pt 9), 2569–2576.

Samson, R.A., 1994. Taxonomy—current concepts of *Aspergillus* systematics. In: Smith, J.E. (Ed.), Aspergillus. Plenum Press, New York, pp. 1–22.

Schuster, E., Dunn-Coleman, N., Frisvad, J.C., van Dijck, P.W.M., 2002. On the safety of *Aspergillus niger*—a review. Appl. Microbiol. Biotechnol. 59, 426–435.

Scott, P.M., 2002. Methods of analysis for ochratoxin A. Adv. Exp. Med. Biol. 504, 117–134.

Shank, R.C., Wogan, G.N., Gibson, J.B., 1972. Dietary aflatoxins and human liver cancer. I. Toxigenic moulds in foods and foodstuffs of tropical South-east Asia. Food Cosmet. Toxicol. 10, 51–60.

Shimoda, C., 1951. An antibiotic substance, oryzacidin, against sake-putrefying bacteria, produced by *Aspergillus oryzae*. I. Isolation of oryzacidin. J. Agr. Chem. Soc. Japan 25, 254–260.

Smedsgaard, J., 1997. Micro-scale extraction procedure for standardized screening of fungal metabolite production in cultures. J. Chromatogr. A760, 264–270.

Speth, C., Rambach, G., Lass-Florl, C., Wurzner, R., Gasque, P., Mohsenipou, I., Dierich, M.P., 2000. Culture supernatants of patient-derived *Aspergillus* isolates have toxic and lytic activity towards neurons and glial cells. FEMS Immunol. Med. Microbiol. 29, 303–313.

Stanley, A.F., 1944. Aspergillin, a stable antibacterial substance of high potency by a species of *Aspergillus*. Aus. J. Sci. 6, 151–152.

Stefanini, M., Adamis, D.M., Soardi, F., Horace, J.F., Marin, H.M., Mele, R., 1959. Purification of aspergillin O. Lancet 1959-II, 443–444.

Suda, S., Curtis, R.W., 1966. Antibiotic properties of malformin. Appl. Microbiol. 14, 475–476.

Takahashi, N., Curtis, R.W., 1961. Isolation and characterization of malformin. Plant Physiol. 36, 30–336.

Tanaka, H., Wang, P., Yamada, O., Tamura, T., 1966. Yellow pigments of *Aspergillus niger* and *Asp. awamori*. I. Isolation of aurasperone A and related pigments. Agr. Biol. Chem. 30, 107–113.

TePaske, M.R., Gloer, J.B., Wicklow, D.T., Dowd, P.F., 1989. Tubingensin A: an antiviral carbazole alkaloid from the sclerotia of *Aspergillus tubingensis*. J. Org. Chem. 54, 4743–4746.

Teren, J., Varga, J., Hamari, Z., Rinyu, E., Kevei, F., 1996. Immunochemical detection of ochratoxin A in black *Aspergillus* strains. Mycopathologia 134, 171–176.

Tobie, W.C., 1946. Aspergillin, a name misapplied to several different antibiotics. Nature 158, 709.

Udagawa, S., Muroi, T., Kurata, H., Sekita, S., Yoshihira, K., Natoru, S., Umeda, M., 1979. The production of chaetoglobosins, sterigmatocystin, o-methylsterigmatocystin, and chaetocin by *Chaetomium* spp. and related fungi. Can. J. Microbiol. 25, 170–177.

Urbano, G.R., Taniwaki, M.H., Leitao, M.F., Vicentini, M.C., 2001. Occurrence of ochratoxin A-producing fungi in raw Brazilian coffee. J. Food Prot. 64, 1226–1230.

Varoglu, M., Crews, P., 2000. Biosynthetically diverse compounds from a saltwater culture of sponge-derived *Aspergillus niger*. J. Nat. Prod. 63, 41–443.

Vicente, M.F., Cabello, A., Platas, G., Basilio, A., Diez, M.T., Dreikorn, S., Giacobbe, R.A., Onishi, J.C., Meinz, M., Kurtz, M.B., Rosenbach, M., Thompson, J., Abruzzo, G., Flattery, A., Kong, L., Tsipouras, A., Wilson, K.E., Pelaez, F., 2001. Antimicrobial activity of ergokonin A from *Trichoderma longibrachiatum*. J. Appl. Microbiol. 91, 806–813.

Walker, R., 2002. Risk assessment of ochratoxin: current views of the European Scientific Committee on Food, the JECFA and the Codex Committee on Food Additives and Contaminants. Adv. Exp. Med. Biol. 504, 249–255.

Watson, A.J., Fuller, L.J., Jeenes, D.J., Archer, D.B., 1999. Homologs of aflatoxin biosynthesis genes and sequence of aflR in *Aspergillus oryzae* and *Aspergillus sojae*. Appl. Environ. Microbiol. 65, 307–310.

Watts, R., Dahiya, J., Chandhary, K., Tauro, P., 1988. Isolation and characterization of a new antifungal metabolite of *Trichoderma reesei*. Plant Soil 107, 81–84.

Wei, D.L., Jong, S.C., 1986. Production of aflatoxins by strains of the *Aspergillus flavus* group maintained in ATCC. Mycopathologia 93, 19024.

Wicklow, D.T., 1983. Taxonomic features and ecological significance of sclerotia. In: Southern Cooperative Series Bulletin, vol. 79. Auburn University, Auburn, pp. 6–12.

Wicklow, D.T., 1984a. Adaptation in wild and domesticated yellow-green aspergilli. In: Kurata, H., Uero, Y. (Eds.), Toxigenic Fungi–Their Toxins and Health Hazard. Elsevier, Amsterdam, pp. 78–86.

Wicklow, D.T., 1984b. Conidium germination rate in wild and domesticated yellow-green aspergilli. Appl. Environ. Microbiol. 47, 299–300.

Wilson, B.J., Wilson, C.H., 1961. Oxalate formation in moldy feedstuffs as a possible factor in livestock toxic disease. Am. J. Vet. Res. 91, 961–968.

Yamamoto, I., Hemmi, T., Yamano, T., 1969. Sesquiterpenoid antibiotics from molds. Takeda Kenkyusho Nempo 28, 69–75.

Ye, G.S., Fields, M.L., 1989. Cellulolytic enzyme production by three fungi grown in a ground corn cob medium. J. Food Prot. 52, 248–251.

Yokoyama, K., Wang, L., Miyaji, M., Nishimura, K., 2001. Identification, classification and phylogeny of the *Aspergillus* section *Nigri* inferred from mitochodrial cytochrome *b* gene. FEMS Microbiol. Lett. 200, 241–246.

Youssef, M.S., Abo-Dahab, N.F., Abou-Seidah, A.A., 2000. Mycobiota and mycotoxin contamination of dried raisins in Egypt. Afr. J. Mycol. Biotech. 8, 69–86.

Yukioka, M., Winnick, T., 1966. Biosynthesis of malformin in washed cells of *Aspergillus niger*. Biochim. Biophys. Acta 119, 614–623.

Bohn, L.

1038

Journal of Zhejiang University SCIENCE B
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Review:

Phytate: impact on environment and human nutrition. A challenge for molecular breeding*

Lisbeth BOHN[1], Anne S. MEYER[2], Søren K. RASMUSSEN[†‡1]

([1]*Department of Agricultural Sciences, Faculty of Life Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871, Frederiksberg C, Denmark*)

([2]*Department of Chemical Engineering, Technical University of Denmark, DK-2800, Kgs. Lyngby, Denmark*)

[†]E-mail: skr@life.ku.dk

Received Feb. 5, 2008; revision accepted Feb. 18, 2008

Abstract: Phytic acid (PA) is the primary storage compound of phosphorus in seeds accounting for up to 80% of the total seed phosphorus and contributing as much as 1.5% to the seed dry weight. The negatively charged phosphate in PA strongly binds to metallic cations of Ca, Fe, K, Mg, Mn and Zn making them insoluble and thus unavailable as nutritional factors. Phytate mainly accumulates in protein storage vacuoles as globoids, predominantly located in the aleurone layer (wheat, barley and rice) or in the embryo (maize). During germination, phytate is hydrolysed by endogenous phytase(s) and other phosphatases to release phosphate, inositol and micronutrients to support the emerging seedling. PA and its derivatives are also implicated in RNA export, DNA repair, signalling, endocytosis and cell vesicular trafficking. Our recent studies on purification of phytate globoids, their mineral composition and dephytinization by wheat phytase will be discussed. Biochemical data for purified and characterized phytases isolated from more than 23 plant species are presented, the dephosphorylation pathways of phytic acid by different classes of phytases are compared, and the application of phytase in food and feed is discussed.

Key words: Phytase, Phytic acid, Iron bioavailability, Antinutritional factor, Purple acid phosphatase, Cereal
doi:10.1631/jzus.B0710640 **Document code:** A **CLC number:** X5

INTRODUCTION

A complete understanding of the biosynthesis of phytic acid (PA) in plants based on a single model species cannot be achieved and comparative studies are needed. Even if the same core set of inositol phosphate kinases (Ipks) should exist in the plant kingdom, the coordination of biosynthesis, translocation, site of accumulation and storage vary among species. This is exemplified by the fact that in wheat, barley and rice the majority of phytate accumulates in the aleurone cells and only minor amounts in the embryo. The distribution of phytate is just opposite in the maize seeds, which means that within relatively closely related grasses different control points exist. PA biosynthesis initiates shortly after flowering and it accumulates during development until seed maturation and desiccation. During this period of plant development changes in growth conditions occurs: rain, drought, high temperature and pathogens. Individual inositolphosphate kinases accept a broad range of substrates and it is also evident that rice and barley Ipks have phosphatase and isomerase activity. These multiple activities provide degrees of freedom for controlling and fine tuning the PA biosynthesis and accumulation, but it also poses a challenge for molecular breeding strategies, because mutations in one kinase gene can be compensated for by other activities.

During the past years, attention has been focused on PA as an antinutritional factor in the diet of humans because of their inability to utilize phytate. The low bioavailability of the minerals bound in the PA can lead to deficiencies in human populations where

‡ Corresponding author

* Project supported by the Danish Agency for Science, Technology and Innovation, Copenhagen, Denmark and HarvestPlus

staples like wheat, rice and maize are the major or the only source of nutrition. In the case of livestock production, excretion of PA can lead to accumulation of P in soil and water, and subsequently to eutrophication of fresh water streams and near coastal seawaters. Low-PA mutant seeds can potentially reduce these problems. Many low PA mutants have been produced in most of the important crop. However, many examples indicate that random mutations seem to affect PA biosynthesis throughout the plant life cycle and not just in the seeds. At present, little is known about the impact of these mutations on agronomical important quality parameters such as stress response or disease susceptibility of plants. Therefore methods acting exclusively on the biosynthesis of PA are needed to produce sustainable low PA seeds.

NUTRITIONAL ASPECTS

Micronutrient malnutrition and distribution

The main micronutrient deficiencies in the world involve Fe, Zn and Vitamin A (WHO, 2002). The three factors are closely related and, in the correct combination, the uptake of one will enhance the uptake of another, and especially fats or proteins from meat will enhance uptake of all of them (Gibson *et al.*, 2006; Lonnerdal, 2000; Storcksdieck *et al.*, 2007). In developing countries plant foods are the major staples of the diet. Particularly in diets based on unrefined cereals or legumes the bioavailability of several micronutrients, such as Ca, Fe, Zn, I and some vitamins can be quite low, causing metabolic disorders related to these nutritional factors. Consequently, improving the nutritional value of this type of foods would improve the nutritional status of the entire population. The transgenic rice line "Golden Rice" is a good example of how the effect of extra β-carotene can decrease Vitamin A deficiency (Zimmermann and Hurrell, 2002). Unfortunately, similar work has not yet been pursued in wheat, the staple cereal of developed countries.

The mineral bioavailability of wheat depends on the cultivar, environment and harvest year. Positive correlations between Zn and Fe have been found in both spring and winter wheat, but these elements were negatively correlated with Mn and P. Furthermore, genotype seems to influence Fe concentrations (Morgounov *et al.*, 2007), while its content is positively correlated with grain size rather than phytate content, suggesting that ferritin deposits are involved in storage of Fe (Grusak *et al.*, 1999). Zn uptake depends more on geographical location (Morgounov *et al.*, 2007) but positive associations between phytate and zinc have been reported as well as evidence that the position on the spike also influences the composition of the minerals in grain (Liu *et al.*, 2006).

Mineral, phosphorous and phytate content is much higher in the bran than in the whole grain (Guttieri *et al.*, 2003; Iskander and Morad, 1986; Steiner *et al.*, 2007), but also within the bran fractions differences occur. Pearling of wheat has shown that the outer 0~4% layer of wheat has the highest Zn content, whereas the next outer 4%~8% layer has the highest phytase activity, phytate and iron contents. However, the differences are small in the outer 0~12% layer, proving that storage of all of these compounds takes place in the bran (Liu Z.H. *et al.*, 2007).

Factors involved in micronutrient malnutrition outside the grain are, for example, the chemical form of the nutrient in the food matrix, interactions with other compounds or treatment of the food prior to ingestion. Iron bioavailability will be used to exemplify it.

Iron uptake and bioavailability

There are two types of iron in the human diet; both of them are mostly absorbed in the proximal part of the duodenum. Heme iron origins from meat products and consists of iron complexed with the porphyrin ring from either haemoglobin or myoglobin. It only accounts for approximately 10%~20% of dietary iron, but for up to 50% of the iron actually entering the body (Carpenter and Mahoney, 1992). Specific receptors for heme iron on the microvilli of the enterocytes have been identified (Krishnamurthy *et al.*, 2007; Worthington *et al.*, 2001) and the iron is easily absorbed and split from the complex by heme oxygenase inside the cell (Raffin *et al.*, 1974).

The second type of iron is non-heme iron from plants. Unlike heme-iron, its uptake depends on the composition of the meal and other factors in the degradation pathway. Absorption by the enterocytes involves reduction from ferric (Fe^{3+}) to ferrous (Fe^{2+}) iron (Mckie *et al.*, 2001) before co-transport with a proton across the membrane by divalent metal

transporter 1 (DMT1) (Andrews, 1999; Gunshin *et al.*, 1997). Iron is then released into the bloodstream by ferrous ion transporter (FPN) and absorbed by cells through the "transferrin cycle": iron-transferrin is bound by transferrin receptors and internalised by endocytosis. Proton pumps create an acidic environment inside the endosome and iron is released from the transferrin. Iron is now available to the cell either for biologically active compounds or for storage, and the transferrin and transferrin receptors are recycled back to the membrane and the cycle can be repeated (Benito and Miller, 1998).

Beside the dietary factors described in the next section, a very important factor, the constitution of the body, also regulates iron absorption. Humans have no physiologically regulated pathway for iron excretion (Andrews and Schmidt, 2007) but have the primary physiological factors involved in non-heme iron absorption, which are summarised in Table 1.

Dietary factors involved in iron absorption

As previously mentioned, absorption of non-heme iron depends on its surroundings. Most of it is found as ferric iron, which precipitates as iron hydroxide at pH>3 if it is not kept in solution by chelators such as ascorbic acid, peptides or certain sugars and amino acids. In the following, the effects and mechanisms of some of the most influential factors on iron bioavailability will be described.

Vitamins: Ascorbic acid (the active form of Vitamin C) keeps iron available for absorption through several mechanisms. First, it promotes acidic conditions in the stomach and intestines, thereby providing optimal conditions for iron absorption; second, it chelates ferric iron and maintains it in a stable and soluble complex, even at higher pH. Finally it reduces ferric iron to its ferrous form, thereby preventing it from precipitating as ferric hydroxide (Teucher *et al.*, 2004). Vitamin A or β-carotene also enhances iron absorption through formation of soluble iron complexes and to a certain extent it can reverse the effect of several inhibitors such as phytates and polyphenols (Layrisse *et al.*, 2000).

Meat is an important enhancer of the bioavailability of non-heme iron. The "meat factor" is still largely unexplored, but recent findings suggest that it is due to peptides of myosin, generated by pepsin degradation in the gut, which binds and keeps iron in solution (Storcksdieck *et al.*, 2007). Other suggestions to explain the "meat factor" involve sulphydryl groups of e.g., cysteine, to reduce ferric iron to ferrous iron (Mulvihill and Morrissey, 1998), and the induction of gastric juice production by proteins (Carpenter and Mahoney, 1992).

Weaker chelators, such as EDTA, organic acids or amino acids also contribute to iron solubility and these iron salts (and also ferrous sulphate) are some of the most commonly used iron fortificants (Hertrampf and Olivares, 2004; Hurrell *et al.*, 2004; Salovaara *et al.*, 2003b).

Table 1 Physiological factors that influence absorption of non-heme iron

Factor	Comment
Iron status	This is probably the most important factor in regulation of iron absorption. Iron uptake is negatively regulated by the hormone hepcidin, which is produced by the liver and regulated by iron levels, blood oxygen concentration and inflammation (Atanasiu *et al.*, 2007; Nicolas *et al.*, 2002)
Gastric acidity	Keeping the iron soluble through low pH is essential for iron absorption. Gastric juice with pH>2 can barely solubilise iron in bread, for example, as Fe^{2+} is far more soluble at intestinal pH than Fe^{3+} (Salovaara *et al.*, 2003b). Furthermore, the acidity denatures proteins and gives optimal conditions for pepsin, thereby releasing iron from protein complexes and at the same time providing weak chelators to keep the iron soluble. Finally, as iron is co-transported with a proton, a positive pH gradient enhances iron uptake (Bezwoda *et al.*, 1978; Kim *et al.*, 1993; Salovaara *et al.*, 2003b)
Transit time	Gastric emptying slows down in some iron-deficient rats, thereby increasing absorption time in duodenum. This factor is not well proven yet, as other experiments in iron-deficient rats showed no change in transit time (Huebers *et al.*, 1990; Salovaara *et al.*, 2003a)
Mucus secretion	Iron ions must cross a layer of secreted mucus before they reach the microvilli of the mucosal cells. Mucins bind cations in general and can inhibit or enhance iron absorption depending on the composition of minerals in the intestine (Conrad and Umbreit, 2002)
Health	As iron homeostasis is regulated through iron uptake more than excretion, diseases that cause abnormalities in the intestine and stomach can of course affect iron status. Mutations in iron transporters or regulators of their expression also influence iron status in general, but most often the phenotype of these genetic defects will be iron overload of tissues more than anaemia (Anderson *et al.*, 2005; Annibale *et al.*, 2000; Atanasiu *et al.*, 2007)

Contradictory to this, iron uptake is inhibited by strong chelators such as PA and some polyphenols that have a chatechol group in their structure, able to form very stable chelates (Brune *et al.*, 1989; Tuntawiroon *et al.*, 1991). In this group of substances, tannic acid is a particularly strong inhibitor of iron absorption; in vitro experiments have shown that a molar ratio of 1:10 of tannic acid:iron results in 92% inhibition of iron absorption; in comparison, to reach the same result a molar ratio of 10:1 of phytic acid:iron is required (Glahn *et al.*, 2002). However, the type of iron involved in these experiments is an important factor, as $FeSO_4$ has higher solubility than $FeCl_3$ in complexes with PA (Engle-Stone *et al.*, 2005). Ascorbic acid and meat can to some extent reverse the inhibition of iron absorption by PA, but not tannic acid. Furthermore, the combination of tannic acid and fish is even able to decrease iron solubility further.

Other divalent cations, such as Ca^{2+} (Hallberg *et al.*, 1992; Perales *et al.*, 2006), Zn^{2+} (Camara *et al.*, 2007), Co^{2+} and Mn^{2+} (Yeung *et al.*, 2005) competitively inhibit iron absorption, probably because they use the same transporters (the DMT1) to enter the enterocytes, or because they co-precipitate with iron in phytic acid salts. The mode of Ca^{2+}-inhibition is still being debated (Perales *et al.*, 2006; Roughead *et al.*, 2005), as Ca^{2+} seems to have larger impact on heme iron absorption than on non-heme iron absorption, which could be due to mucosal uptake inhibition.

The interactions between inhibitors and enhancers decide the final absorption level of the element in the gut. The sums of the effects are, in general terms, predictable, and several algorithms to predict the percentage of non-heme iron absorption from food are available in literature (Conway *et al.*, 2007).

Despite these cofactors involved in iron uptake, the main inhibitor of Fe absorption is phytic acid, and its chemical properties and functions will be described in the following section.

CHEMISTRY OF PHYTIC ACID

Myo-inositol (1,2,3,4,5,6) hexa*kis*phosphate

Inositol phosphates consist of an inositol ring and at least one phosphate group. Breaking the name into its separate parts describes the exact structure and appearance: the prefix "*myo*" refers to the conformation of the hydroxyl groups on the inositol ring. The nine possible configurations of the inositol ring have been annotated in a number of ways, but the adopted nomenclature is according to the set of rules suggested by Posternak (1965).

The conformation *myo*-inositol thus has one plane of symmetry, going directly from the most left to the most right atom (Fig.1). The D/L-prefixes specify the numbering direction of carbons in the inositol ring, where the D annotates counterclockwise and L clockwise counting, respectively. In general chemistry, numbering of the atoms should always follow the lowest possible route. Confusions regarding *myo*-inositols and enzymes related to them have led the International Union of Pure and Applied Chemistry and the International Union of Biochemistry (IUPAC-IUB, 1989) to recommend that the atoms in the *myo*-inositol ring should always be numbered according to the D configuration. The numbering should be initiated at the atom that is esterified in inositol phospholipids according to Agranoff (1978), using Agranoff's turtle as a reminder. The four limbs and tail of the turtle are coplanar and represent the five equatorial hydroxyl

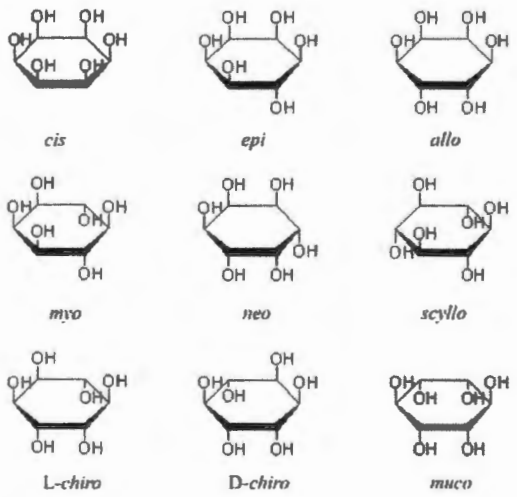


Fig.1 The nomenclature of the nine stereoisomers of inositol (Haworth projections). Seven have a mirror axis in the molecule and numbering of the carbon atoms can be performed either counterclockwise (D) or clockwise (L). Only the *chiro* form has specific D and L conformations (software: ChemDraw 10.0, Cambridgesoft.com)

groups. The turtle's head is erect and represents the axial hydroxyl group. Looking down at the turtle from above, the numbering of the turtle will begin at the right paw and continue past the head to the other limbs, thus numbering the inositol in the counter-clockwise (D) direction (Shears, 2004). This way, the plane of symmetry in the "*myo*" conformation will always go through C2 and C5, and the L (clockwise) configuration will start at the turtle's left paw and the D configuration will start at the turtle's right paw.

The consequence is a simplification of the naming of the enzymes related to the inositol phosphates: any phytase does not automatically produce D/L-*myo*-inositol (1,2,3,4,5) penta*akis*phosphate, thereby obeying the rules of numbering according to the lowest possible route. The phytase more specifically is named according to the D-*myo*-inositol penta*akis*phosphate that indicates initiation point of hydrolysis. According to this set of rules, a 3-phytase will initiate dephosphorylation at the C3 atom and have D-*myo*-inositol (1,2,4,5,6) penta*akis*phosphate as the product. As the enzyme hydrolyses additional phosphate groups from the inositol ring, the numbering of the groups will remain the same regardless of which phosphate is hydrolysed and the configurational counting will not change between the intermediates. Confusions surrounding the enzymes and their products are however not yet entirely resolved. The same enzyme some authors annotate as a 4-phytase, others refer to as a 6-phytase. Here, the IUPAC-IUB recommendations are used and readers should note that a D-6-phytase and an L-4-phytase produce the same product.

Myo-inositol is the major nutritionally relevant form of inositol, and although some of the other stereoisomers also are found in nature, they will not be addressed in this paper.

Myo-inositol (1,2,3,4,5,6) hexa*akis*phosphate has 6 groups of phosphates attached to the inositol ring. Using the prefix "hexa*akis*" instead of "hexa" indicates that the phosphates are not internally connected (Johnson and Tate, 1969) and the compound is consequently a polydentate ligand, which is a chelator that can bind to more than one coordination site of the metal atom. Each of the phosphate groups is esterified to the inositol ring and together they can bind up to 12 protons in total. The acidity of the protons varies from very strong acids to very weak (pK_a up to 9.4) although ionic strength of the solution and temperature influence these values (Brown *et al.*, 1961; Costello *et al.*, 1976; Torres *et al.*, 2005).

Trivial names for D-*myo*-inositol (1,2,3,4,5,6) hexa*akis*phosphate are IP_6, $InsP_6$ or phytic acid (PA). The last two will be used here depending on context and $InsP_1$~$InsP_5$ will also be used regarding lower *myo*-inositol phosphates counted in D configuration unless the prefix L is explicitly added.

In the pH range 0.5~10.5 phytic acid keeps the sterically stable conformation with one axial and five equatorial groups (Fig.2). At higher pH the phytic acid will flip to the reverse conformation with five axial and one equatorial group. A similar phenomenon is detected within the $InsP_5$-group, especially the $InsP_5$s with phosphorylation intact on all three carbon atoms C1, C3 and C5 as these groups are able to form a "chelation cage" that will use a cation to stabilise the otherwise more unstable conformation (Volkmann *et al.*, 2002). For the same reason, crystallisation of PA also favors this conformation (He *et al.*, 2006; Rodrigues-Filho *et al.*, 2005). Lower inositol phosphates keep the stabile 5 eq/1 ax conformation over the entire pH range (Barrientos and Murthy, 1996).

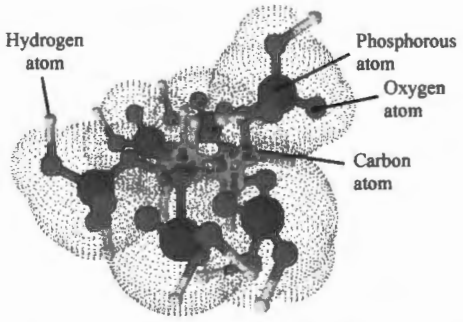


Fig.2 *Myo*-inositol (1,2,3,4,5,6) hexa*akis*phosphate in boat formation showing the 5 equatorial and the 1 axial group. Red oxygen, purple phosphorous and grey hydrogen atoms form phosphate groups on each of the blue carbon atoms that have been numbered according to the IUPAC-IUB recommendation. The special relationships between the atoms have been indicated with dots (software: ChemSketch 10.0, ACDlabs.com)

Phytate

The chelating effect of the phosphate groups causes PA to bind readily to mineral cations, especially to Cu^{2+} and Zn^{2+} which appear to have a high affinity for inositol phosphates. The order of the

ability of the mineral cations to form complexes in vitro with inositol phosphates has been found to be $Cu^{2+}>Zn^{2+}>Cd^{2+}$ for all $InsP_3$~$InsP_6$ at pH 3~7, but binding strength is weaker for the lower inositol phosphates (Persson *et al.*, 1998). Similar binding assays using only phytic acid and none of the lower inositol phosphates show the order $Cu^{2+}>Zn^{2+}>Ni^{2+}>Co^{2+}>Mn^{2+}>Fe^{3+}>Ca^{2+}$ in one study (Vohra *et al.*, 1965) and the order $Zn^{2+}>Cu^{2+}>Co^{2+}>Mn^{2+}>Ca^{2+}$ in another (Maddaiah *et al.*, 1964). Despite of these results, the compositions of minerals in PA stores do not necessarily follow these orders of affinity. Recent finding shows that PA is stored in vivo in complexes not only with these minerals, but to a much larger extents with Mg, Ca and K (Bohn *et al.*, 2007).

In presence of excess phytic acid, formation of soluble complexes between PA and a metal ion displaying 1:1 stoichiometries predominates. However, when metal ions are in excess, an insoluble solid called phytate is formed (Torres *et al.*, 2005). The stoichiometry itself influences the solubility of phytate, as a very low or a very high metal ion:phytic acid ratio increases the solubility of the salt (Bullock *et al.*, 1995; Cheryan *et al.*, 1983; Nolan *et al.*, 1987). However, heterologues precipitation with a combination of more than one type of metal ions seems to be increasing phytate formation. When especially Ca^{2+} is present simultaneously with another metal ion, it increases the proportion of the phytic acid-metal aggregate (Wise and Gilburt, 1983; Simpson and Wise, 1990). In many cases (Mn, Co, Ni, Cu, Sn and Zn), pure phytic acid-metal salt precipitates when the stoichiometry between PA and the ion is between 1:2 and 1:5, but addition of chloride ions to the solution can deprotonise the phytic acid further and form complexes with up to 6 ions per PA molecule (Vasca *et al.*, 2002; Vohra *et al.*, 1965).

The pH is another factor influencing the solubility of phytic acid (Cheryan, 1980). Its Ca, Cd, Zn and Cu salts tend to be soluble at pH lower than 4~5, whereas Mg-phytate is 100% soluble at acid pH up to pH 7.5 (Brown *et al.*, 1961; Nolan *et al.*, 1987). Solubility studies of bran phytate prove that at gastric pH (approximately pH 2), Ca is actually not bound to PA and this component does not contribute to the solubility of the Ca ion (Siener *et al.*, 2001).

Because the resulting phytate precipitate is amorphous and full of co-crystallised water (up to 22 H_2O per PA molecule (Veiga *et al.*, 2006)), the structure of the phytate salts cannot be obtained by crystallisation techniques. Attempts to resolve the structure of phytic acid-Fe^{3+} salt have revealed quite a high binding capacity with almost covalent-strong bonds between P-O-Fe. Precipitation initiates when at least four out of the six phosphate groups, are able to bind with the Fe^{3+}, leading to iron phytate with 2~4 iron atoms per molecule of PA (Mali *et al.*, 2006). In the most idealised version of the salt, the ferric ions will be organised in a grid where each phosphate group is bound to two iron atoms, and each iron atom is bound to three phosphate groups and shared between two PA molecules (Thompson and Erdman, 1982). This is, however, a very constraint structure and in most cases phytic acid will not be completely saturated with ferric irons.

Functions of the *myo*-inositol phosphates

The primary functions of PA in seeds are storage of phosphates as energy source and antioxidant for the germinating seed (Raboy, 2003). Phytate as the mineral bound salt of PA is also an important mineral reserve in seeds, and it is stored in protein storage vacuoles in the aleurone cell layer or the embryo of the seed. Lower inositol phosphates are also involved in stress responses, membrane biogenesis and intracellular signalling (Storcksdieck *et al.*, 2007; Loewus and Murthy, 2000).

Phytic acid accumulates during seed development until the seeds reach maturity and accounts for 60%~90% of total phosphorous content in cereals, legumes, nuts and oil seeds (Lott *et al.*, 2000; 2001). It is however found in most eukaryotic tissues, where it is kept adherent to the cell walls through phosphoinositides, or in complexes with proteins or ions (Torres *et al.*, 2005; Veiga *et al.*, 2006). Phytic acid is found in ten-fold higher concentrations in the brains of rats as in the kidney, indicating that it has great potential outside the plant kingdom (Grases *et al.*, 2001a).

In eukaryotes in general, three main features of PA keep it involved in a number of metabolic processes: its chelating properties and its ability to function as a phosphate donor/acceptor makes it ubiquitous/abundant in numerous cell systems. Moreover, the lower inositol phosphates are involved in a number of cell signalling pathways and finally PA may

consequently act as a precursor of compounds with this function.

One of the best known properties of PA is its antioxidative ability by binding and thereby inactivate Fe ions in solution. This prevents the ferric irons from participating in the Fenton reaction (the formation of the hydroxyl radical ·OH as a consequence of oxidation of Fe^{2+} to Fe^{3+} during reaction of Fe^{2+} with H_2O_2 or peroxides). PA is even better than the in vitro commonly used EDTA (Graf *et al.*, 1984; Wong and Kitts, 2001). These radicals are highly reactive molecules that rapidly and non-specifically react with proteins, lipids or DNA, thereby causing cell injury or cell death. Alzheimer's and Parkinson's diseases, cirrhosis, arthritis and cancer have all been linked with radicals (Benzie, 2003) and PA may therefore be a potential inhibitor of all of these illnesses.

The chelating ability of PA is also used in preservation, where historic ink, made from gallic acid from the tannins and Fe ions, has been used on paper: phytic acid prevents iron catalysed oxidation of the cellulose in the paper, thereby doubling the half-life of the documents (Neevel, 1995). $InsP_5$s with various different combinations of conformations of the phosphate groups are also capable of inhibiting radical formation, as long as the 1,2,3-(equatorial-axial-equatorial) phosphate conformation is kept intact (Hawkins *et al.*, 1993), which has also proven useful in paper preservation (Sala *et al.*, 2006). Quite recently, the ability of PA to inhibit Fe-dependent reactions has also been tested with some success in storage of meat (Stodolak *et al.*, 2007).

In mammalian organisms, PA has been implicated in starch digestibility and blood glucose response (Lee *et al.*, 2006), in the prevention of dystropic calcifications in soft tissues (Grases *et al.*, 2004) and kidney stone formation (Grases *et al.*, 1998; Selvam, 2002), and in the lowering of cholesterol and triglycerides (Jariwalla, 1999; Onomi *et al.*, 2004). PA has also been suggested to be part of a structure that could inhibit transcription of the viral genome from HIV-1 (Filikov and James, 1998), and apparently it has been tested in toothpaste as a tool for preventing plaque formation (Vasca *et al.*, 2002).

At the cellular level, PA or inositol phosphate intermediates are involved in gene regulation, efficient export of mRNA, RNA-editing and DNA repair (York *et al.*, 1999; York, 2006). The lower inositol phosphates such as $Ins(1,4,5)P_3$ take part in cell signalling cascades (Berridge and Irvine, 1989) and pathways leading to versatile functions within Ca^{2+} mobilisation and signalling (Efanov *et al.*, 1997; Larsson *et al.*, 1997). They also contribute to protein folding (Macbeth *et al.*, 2005) and trafficking (Shears, 2004), endo- and exocytosis (Efanov *et al.*, 1997; Saiardi *et al.*, 2002), oocyte maturation (Angel *et al.*, 2002), and cell division and differentiation (Berridge and Irvine, 1989).

The involvement of phytic acid in cancer therapy is also widely discussed (Vucenik and Shamsuddin, 2006) and the potential of PA in cancer treatment are shown in Table 2. As with the previously mentioned functions, most of the effects of phytic acid are related to its chelating abilities, to the lower inositol's involvement in signalling pathways or to their phosphate donor/acceptor capabilities. After intake, phytic acid is dephosphorylated to lower inositol phosphates and these can act as an antioxidant by inhibiting iron mediated oxidative reactions, enhancing immunity by increasing Natural Killer cell function and activity, or stimulate bacterial killing by neutrophils. Furthermore, the compounds can normalise abnormal cell proliferation, induce cell differentiation and apoptosis and inhibit angiogenesis. In addition, inositol phosphates modify Phases I and II metabolising enzymes by causing G0/G1 arrest in cancer cells and modulate oncogene expression and prevent tumor metastasis formation.

It should be noted that although many of these functions of lower inositol phosphates have been shown in vitro, the human small intestines show very little phytase activity (Iqbal *et al.*, 1994). Even though

Table 2 Cancer prevention and treatment functions of phytic acid according to Vucenik and Shamsuddin (2006)

Enhancement	Other effects	Inhibition
Cell differentiation	Antioxidant	Cell proliferation
Apoptosis	Oncogene regulation	Tumor metastasis
Immune function	Regulation of Phases I and II enzymes	Angiogenesis
		Inflammation

mammalian cells are able to synthesize the inositol phosphates themselves (York, 2006), endogenous synthesis of phytic acid is minor (Grases *et al.*, 2001a; 2001b). Using phytic acid in cancer therapy would therefore be dependent on daily intake of phytic acid. This treatment is influenced by the fact that absorption rate of PA itself is low (Grases *et al.*, 2006) and it is suggested that there is a maximum to the uptake in the gut, which again is regulated by PA plasma levels (Grases *et al.*, 2001c; Kemme *et al.*, 2006). The effect of phytic acid in cancer therapy is therefore still under investigation and so far the results have mainly been in connection with colon cancer, where uptake is not a prerequisite.

Regarding the negative functions of phytic acid, the outcome of a homogenous and high PA diet has long been known to be mineral deficiency and malnutrition (McCance and Widdowson, 1949; Cheryan, 1980). The negative effects of PA are consequences of the same chemical features as the potential positive effects. The capability of binding minerals makes phytic acid an antinutritional factor, because the solubility of the phytic acid:metal-complexes are low at the pH of the major part of the intestines. Regarding lower inositol phosphatese, $InsP_5$s are also able to complex cations, whereas inositol phosphates with only three or four phosphate groups attached to the inositol ring do not inhibit mineral absorption independently. They are however able to interact with PA and thereby contribute to the negative mineral absorption (Sandberg *et al.*, 1999). Furthermore, phosphorous in the form of phytic acid is largely unavailable as a nutritional factor to monogastric animals because insufficient degradation capabilities in the gastrointestinal tract prevent the phosphorous from being biologically available. Feeds for pigs and poultry are therefore traditionally supplemented with inorganic phosphate to meet the nutritional requirements for optimal growth of the animals. The excess of phosphorous bond in phytic acid is then excreted through the faeces and spread as manure into the soil. The potential eutrophication of fresh water streams, lakes and near costal areas can then cause cyanobacterial blooms, hypoxia and death of aquatic animals and production of nitrous oxide, a potential greenhouse gas (Vats *et al.*, 2005). In the laboratory, phytic acid in faeces also inhibits polymerase chain reactions (PCR), thereby preventing PCR-based diagnostic tests for detecting microorganisms in livestock (Thornton and Passen, 2004).

Storage of phytic acid

Phytic acid and its co-precipitated cations are stored in electron dense spherical particles named globoids (Pfeffer, 1872). The globoids are localised predominantly in the aleurone layer (wheat and barley) or in the embryo (maize) (Odell *et al.*, 1972). They are compartmentalised inside protein storage vacuoles in the seeds. Protein storage vacuoles in general contain three morphologically distinct regions: a matrix that contains most of the soluble storage proteins, crystalloids composed of proteins yet to be investigated but organised in lattice structure, and globoids of PA or oxalate crystals (Lott, 1980). How many of these regions are present in the seed is species dependent. Protein storage vacuoles of wheat are composed only of matrix proteins and phytate globoids (Morrison *et al.*, 1975) which make the cells highly susceptible to extraction of phytate. Consequently, even a minor rupture of the aleurone cells means release of phytate into the surrounding environment (Antoine *et al.*, 2004). Morrison *et al.*(1975) also reported the detection of a layer of lipid droplets around the wheat "aleurone grain". Membranes surrounding the globoids have been reported in other species such as *Brassicaceae* (Gillespie *et al.*, 2005) and *Solanaceae* (tomato) (Jiang *et al.*, 2001). Certain reports of the phenomenon in wheat has however not been published to date, but the findings of Morrison *et al.*(1975) could be parts of an inner membrane in the protein storage vacuole, which have been broken during staining and cutting of the samples.

The size of the phytate globoids depends on the amount of phytate in the grain. In wildtype (WT) wheat, globoids up to 4 μm in diameter have been detected (Antoine *et al.*, 2004), whereas a low phytic acid (lpa) wheat mutant (Js-12-LPA) with the same amount of P in the grains but lowered phytate concentration has smaller globoids, organised in clusters (Joyce *et al.*, 2005). This phenomenon has also been observed in WT wheat grown under low P conditions (Batten and Lott, 1986) and in lpa mutations in other cereals, such as rice (Liu *et al.*, 2004), maize (Lin *et al.*, 2005) and barley (Ockenden *et al.*, 2004).

The compositions of the globoids seem to be the same for globoids from WT and lpa wheat. Using

scanning transmission electron microscopy-energy-dispersive X-ray analyses on section of wheat grains, P, K, Mg and Ca are found in high concentration in globoids (Joyce *et al.*, 2005), whereas especially Cu and Zn are lowered in lpa compared to WT (Guttieri *et al.*, 2003). Minerals were recently quantified by inductively coupled plasma-mass spectrometry (ICP-MS) of phytate globoids purified from wheat bran (Bohn *et al.*, 2007). Although Cu has high affinity to phytic acid, there is no indication that Cu-phytate globoids are the primary storage facility for this element and K>Mg>Ca>Fe (in concentration order) were found as the main minerals (Bohn *et al.*, 2007). Fe is also found in both lpa and WT wheat, but its distribution is restricted because globoids near the embryo were shown to contain the relatively highest amounts of this element in WT wheat (Lott and Spitzer, 1980).

Dephytinisation and nutrition

The chelating properties of PA not only result in the binding of cations in seeds. When released during food or feed processing or in the gut, PA also binds minerals and makes them unavailable as nutritional factors. Iron and zinc uptake have both been shown to be inhibited when the phytic acid:metal ratio increases above 10:1 (Gharib *et al.*, 2006; Glahn *et al.*, 2002). In human studies, phytic acid has been reported to inhibit absorption of iron, zinc, calcium, magnesium and manganese but surprisingly not copper (Bohn *et al.*, 2004; Davidsson *et al.*, 1995; Egli *et al.*, 2004; Hallberg *et al.*, 1989; Lonnerdal, 1997; Phillippy, 2006; Reddy *et al.*, 1996). Removal or degradation of PA would therefore increase the bioavailability of many cations and the nutritional value of the meal, and several strategies to reduce it are therefore considered.

Milling of cereals removes the phytic acid, but this treatment also removes the major parts of the minerals and dietary fibres and cannot therefore be a nutritional solution to the problem. Similarly, soaking or extracting in aqueous solutions can remove up to two thirds of the PA by the action of endogenous phytase activity, but loss of minerals, water-extractable proteins and vitamins also occurs (Hurrell, 2004). Heat treatments have minor effects (Pontoppidan *et al.*, 2007) usually due to leaching of minerals into the boiling water. Different processing and cooking methodologies for reduction of PA have been compared between wheat variety, and the results have been that if one method is efficient in reduction of PA in one wheat variety, this may not apply for another. Furthermore, the method with the highest phytic acid reduction (germination for 48 h) still only reduced its content by up to 40% (Masud *et al.*, 2007). Avoiding PA formation in the first place or catalysing its degradation by the use of PA hydrolysing enzymes would therefore be more beneficial approaches to dephytinisation.

Reducing phytate content through lpa mutants have been attempted through knock-out of genes involved in PA biosynthesis. With the only exception of one barley mutant accumulating Fe, the mineral composition does not dramatically change in lpa mutants, indicating that there is no direct link between mineral distribution and phytic acid biosynthesis (Joyce *et al.*, 2005; Liu J.C. *et al.*, 2004; Liu K. *et al.*, 2007; Ockenden *et al.*, 2004). Chicks fed lpa barley or corn, respectively excrete 33% less phosphate and they show enhanced growth and bone structure (Jang *et al.*, 2003) as compared to chicks on a normal diet. Similar results have been reported for growing pigs (Veum *et al.*, 2002) and rats (Poulsen *et al.*, 2001) on lpa barley diet. Still, almost the same results on growth performance can be achieved by fortifying with inorganic phosphorous instead of using lpa mutants; the main difference between lpa diets and phosphorous fortified diets is therefore the higher phosphorous excretion from the latter. In human studies, Fe, Ca and Zn uptake increased when meals were based on lpa maize (Adams *et al.*, 2002; Hambidge *et al.*, 2005; Mendoza *et al.*, 1998) and all in all this would be a reasonable approach to increase the bioavailability of micronutrients.

Due to the involvement of the lower inositol phosphates in plant cell metabolism, production of the perfect lpa mutant has however turned out to be quite a challenge. Since many kinases and tranferases are involved in the synthesis of phytic acid (Josefsen *et al.*, 2007), the mutants often find ways to circumvent the knocked out pathway. Furthermore, the yield or germination ability is affected if PA content is reduced more than 50% (Raboy, 2007), thereby making this approach unattractive from an economic perspective. The best result so far is the maize lpa1 mutant, which is mutated in an embryo-specific ATP-

binding cassette (ABC)-tranporter and it is able to hold up to 90% reduction of PA without compromising seed viability. The effect on mineral distribution in this mutant is yet to be evaluated (Shi *et al.*, 2007).

Instead of blocking its biosynthesis, an attempt to reduce PA in wheat products has been performed by introducing the *Aspergillus niger* phytase gene *phy*A into a wheat variety by particle bombardments of immature wheat embryos (Holm *et al.*, 2002). The concerted action of wheat phytase and *Aspergillus* phytase has proven to be an efficient way of increasing the rate of PA degradation in transgenic wheat flour, although experiments in food or feed applications have not been reported yet.

The most successful dephytinisations so far involve endogenous enzymatic activity during germination, but this is a species dependent phenomenon where some plants are more sensitive to the treatment than others. Wheat, barley and rye all have high phytase activity in the grain, whereas maize, millet and sorghum have low initial phytase activity that increase rapidly after germination (Egli *et al.*, 2002). Adding exogenous phytase to food and feed is therefore under investigation in many forms and the next section will present the general classes of phytases and some of the applications.

PHYTASES

Definition

The term phytase (*myo*-inositol (1,2,3,4,5,6) hexa*kis*phosphate phosphohydrolase) is defined as a class of phosphatases with the in vitro capability to release at least one phosphate from PA, thereby releasing phosphate and lowering inositol phosphates and potentially chelated minerals.

The earliest reports of a phytase activity are from the blood of calves (McCollum and Hart, 1908), and rice bran (Suzuki *et al.*, 1907), indicating from its discovery, that this enzyme is found in diverse organisms. Later discoveries have also found phytases in bacteria, yeast and fungi. As previously mentioned, most monogastric animals, including humans, lack the enzyme in their digestive track, making PA hydrolysis in the gut dependent on mucosal or bacterial enzymes or on non-enzymatic hydrolysis by gastrointestinal acidity. The number of enzymes described as phytases has increased rapidly over the years and details regarding the enzymatic properties can be found in several reviews (Haefner *et al.*, 2005; Oh *et al.*, 2004; Vats and Banerjee, 2004).

IUPAC-IUBMB (the International Union of Pure and Applied Chemistry and the International Union of Biochemistry and Molecular Biology) currently acknowledges three classes of phytase enzymes, which initiate the dephosphorylation of PA at different positions on the inositol ring, and produce different isomers of the lower inositol phosphates. Within each class of phytase structural differences can be found, and not all enzymes within a certain class hydrolyze phosphate from PA through the same mechanism. Similarly, the enzymes can be grouped as acidic, neutral or alkaline phosphatases depending on the optimum pH of activity. All phytases however have pronounced stereo specificity and a strong preference for equatorial phosphate groups over axial groups (Lei and Porres, 2003).

EC 3.1.3.8: the 3-phytases

The largest group of phytases to date contains the 3-phytases (EC 3.1.3.8), which in general are found in fungi and bacteria. Structurally, most of the 3-phytases show homology to β-propeller phosphatase (BPP), or histidine acid phosphatases (HAP).

BPPs are tightly bound to three Ca ions and need two adjacent phosphate groups to bind to the "cleavage site" and to the "affinity site" before hydrolysis can occur. The end product has been suggested to be inositol-triphosphate—either Ins(1,3,5)P_3 or Ins(2,4,6)P_3 (Kerovuo *et al.*, 2000; Shin *et al.*, 2001), but most recent data give evidence that Ins(2,4,6)P_3 is sole the end product (Oh *et al.*, 2006), confirming the equatorial preference of most phytases.

Most bacterial, fungal and plant phytases belong to the HAPs. Within this structural classification, there are two phytase subgroups: Some show broad substrate specificity but low specific activity for PA, whereas others have narrow substrate specificities but high specific activity for PA. All members of the HAP class share two conserved active site motifs, RHGXRXP and HD (van Etten *et al.*, 1991), and hydrolyse metal-free phytate in the acidic pH-range. However, they do not necessarily share any additional regions of sequence similarity (Maugenest *et al.*,

1997). HAPs can initiate hydrolysis of phytic acid on either the C3 or the C6 position of the inositol ring and produce *myo*-inositol monophosphate (in particular Ins(2)P due to its axial position) as the final product (Greiner and Carlsson, 2006; Mullaney and Ullah, 2003; Oh *et al*., 2006).

The catalytic mechanism of hydrolysis by HAPs is proposed to be as follows: the histidine residue in the conserved motif is used to make a nucleophilic attack on the carbon of interest, and an aspartic acid in the C-terminal of the enzyme stabilises the leaving group by acting as a proton donor (Ostanin and van Etten, 1993; Ostanin *et al*., 1992). A water molecule is consumed in the hyrolysis of the intermediate, but the phospho-histidine interaction is stable enough for crystallisation, as it has been done e.g., with a heat stable HAP phytase from *Aspergillus fumigatus* (Xiang *et al*., 2004).

A well studied phytase from the class of 3-phytases is isolated from baker's yeast, *Saccharomyces cerevisiae*. This phytase is extracellular and its expression of it can be induced when grown in a medium containing $InsP_6$ as the sole phosphorous source (Andlid *et al*., 2004). The extracellular yeast 3-phytase has been studied intensively as its potential during baking is obvious (Turk *et al*., 1996). Unfortunately, the endogenous phytase of yeast is repressed by the concentrations of phosphate in the dough during leavening (Andlid *et al*., 2004). Constitutive expression of yeast phytase is possible by deletion of genes expressing negative regulators (Veide and Andlid, 2006), but recent investigations have to a large extent advanced to focusing on the use of baker's yeast as host for heterologues expression of microbial phytases due to their higher thermostability (Kaur *et al*., 2007).

The sequential dephosphorylation of phytic acid by an *S. cerevisiae* phytase is depicted in Fig.3. Not all subproducts have been confirmed yet, but the major pathway is from phytic acid over Ins(1,2,4,5,6)P_5 through Ins(1,2,5,6)P_4 and Ins(1,2,6)P_3 to probably Ins(1,2)P_2. End product is Ins(2)P (Andlid *et al*., 2004; Greiner and Alminger, 2001).

EC 3.1.3.72: the 5-phytase

Only a single 5-phytase (EC 3.1.3.72) has been detected so far. This alkaline phytase from lily pollen is interesting, because it is the only one in the family of phytases that initiates hydrolysis with an attack of a phosphate group in the plane of symmetry (the "turtle's tail"), thereby creating yet another symmetrical compound as shown in Fig.4 (Barrientos *et al*., 1994).

The pollen phytase shows highest activity at pH 8.0 and temperature 55 °C (Jog *et al*., 2005). It has the conformation of an HAP phytase, but with the exception of the active site, the amino acid sequence homology is higher towards multiple inositol polyphosphate phosphatase (MINPP) from humans or rats (Mehta *et al*., 2006).

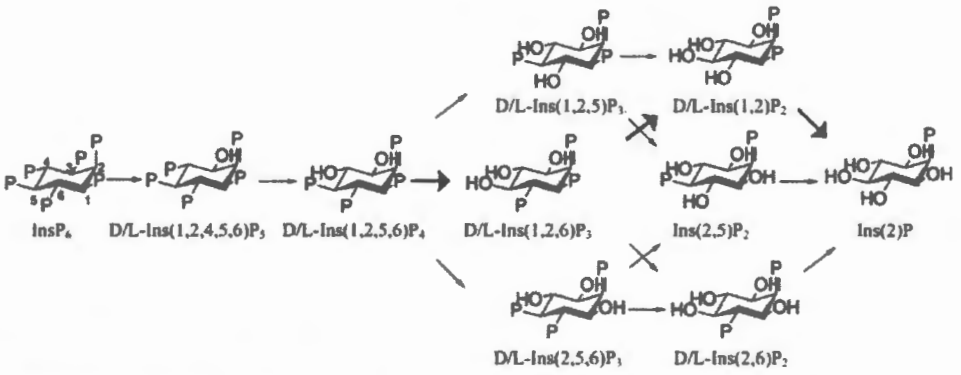


Fig.3 Dephosphorylation of phytic acid by the 3-phytase from *Saccharomyces cerevisiae*. Hydrolysis of phytic acid initiates on C3 of the inositol ring and the adjacent phosphates are removed consecutively. Fat arrows indicate major pathway of dephosphorylation, narrow arrows indicate possible pathways that have not been excluded (modified from Greiner *et al*., 2001a). End product is Ins(2)P. In this figure, P represents inorganic phosphate and the rest of the molecules have been omitted for clarity (Software: ChemSketch 10.0)

Fig.4 Dephosphorylation of phytic acid by the 5-phytase from lily pollen. Hydrolysis of phytic acid initiates on C5 of the inositol ring and the adjacent phosphates are removed consecutively. End product is Ins(1,2,3)P_3 (modified from Barrientos *et al.*, 1994). In this figure, P represents inorganic phosphate and the rest of the molecules have been omitted for clarity (software: ChemDraw Ultra 10.0)

EC 3.1.3.26: the 4/6-phytases

The 4/6-phytases (EC 3.1.3.26) act on the carbon atom next to C5 of the inositol ring. The official name should be 4-phytase, but traditionally it has been called a 6-phytase. Several structurally different phytases are found in this group: The purple acid phosphatase (PAP), the ADP phosphoglycerate phosphatase (related to EC 3.1.3.28), as previously mentioned an HAP-class is also involved, and again the acid phosphatase (related to EC 3.1.3.2).

Plant phytases act preferentially at the C6 carbon, and are 6-phytases. In general, the 4/6-phytases are the most active in weak acidic environments (pH 4~6) with a temperature optimum in the range 40~60 °C. They are usually 50~70 kD and have Michaelis-Menten constants (K_m) in the range of 10^{-5}~10^{-4} mol/L phytic acid. There are exceptions, such as the rather large phytase from tomato roots (164 kD (Li *et al.*, 2007)) and the highly active phytase from wheat (K_m 0.5 μmol/L phytic acid (Nakano *et al.*, 1999)). Also, some alkaline phytases have been purified, such as the previously mentioned 5-phytase from lily pollen, legume seeds and the phytase from *Typha latifolia* (cattail) pollen (Hara *et al.*, 1985; Jog *et al.*, 2005; Scott, 1991). Their pH optima lie at pH 8. A list of characterised plant phytases are found in Table 3.

Wheat phytase

Activity of wheat phytases (EC 3.1.3.26) were first reported in (Posternak and Posternak, 1929), where an aqueous extract of wheat bran was used to investigate optically active *myo*-inositol polyphosphate esters that were produced during the degradation of phytate. Attempts to characterise the enzymes were performed by Collatz and Bailey (1921), and Kolobkowa (1936). Both groups found 55 °C to be the optimum temperature for wheat phytase, as a result that was confirmed along with optimum pH of 5.15 (Peers, 1953). Despite these fairly similar results it should however be noted that none of the groups managed to purify the enzymes to homogeneity and that it required additionally 20 years before a pure phytase from wheat was identified.

The number of phytases in wheat is still under investigation. Two enzymes, Phy1 and Phy2, have previously been purified from wheat bran (Lim and Tate, 1973) and two isozymes with the N-terminal amino acid sequence EPAXTLTGPSRPV have also been purified (Nakano *et al.*, 1999). Based on amino acid sequence and masses of tryptic peptides, a third enzyme with homology to PAP phytases was cDNA cloned from wheat and its homologue from barley (Rasmussen *et al.*, 2007). Recently four cDNAs encoding for MINPPs were also cloned. At least two of these show in vitro phytase activity and they are probably expressed during late seed development and germination (Dionisio *et al.*, 2007).

Although a PAP phytase has also been found in soybean (Hegeman and Grabau, 2001), wheat phytase is mainly used as model for characterisation of this type of phytase. It has been documented to be inhibited when the phytate is complexed with Al^{3+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Ag^{+}, Hg^{+} or Zn^{2+}, but not salts of Ca^{2+}, Mg^{2+} and Mn^{2+} unless some of the inhibiting ions are present in the solution as well. Organic acids, such as citric acid or oxalate, can to a certain extent circumvent this inhibition, but in too high doses they themselves become inhibitory factors (Nagai and Funahashi, 1962; Tang *et al.*, 2006).

The exact structure of PAP phytase has not been elucidated yet, but PAPs usually contain a Fe^{3+}-Me^{2+} centre in their active site, where Me stands for divalent Fe, Mn or Zn (Olczak *et al.*, 2003; Strater *et al.*, 1995). Five conserved regions containing seven negatively charged amino acids stabilise this structure (Fig.5). The PAPs catalyse the hydrolysis of activated phosphoric acid esters and anhydrides in the pH range from 4 to 7 (Vincent *et al.*, 1992) through an interaction between the Me^{2+} and the substrate followed by a

nucleophilic attack on the phosphate group by a Fe^{3+}-coordinated hydroxide ion (Klabunde *et al.*, 1996). Three histidines in the conserved regions stabilise the transition state.

Some similar properties are displayed by the investigated wheat phytases (Table 3). All the enzymes characterised so far have been most active at weak acidic to neutral pH. Optimum activity lies between pH 4.5 and 7.2 and there are several reports of an enzyme with the highest activity around pH 6. This suggests that they all are acid phosphatases. Temperature optimum is in the range of 45 °C (Bohn *et al.*, 2007; Nakano *et al.*, 1999) to 65 °C (Dionisio *et al.*, 2007) and there is general agreement concerning inhibition by approximately 3 mmol/L phosphate of some types of phytases but not others. Inconsistencies in the characterisations primarily regard the activities of the enzymes. The K_m vary between 0.48 μmol/L and 830 μmol/L phytic acid and the velocities at maximal concentrations of substrates (V_{max}) vary from 127 to 230 μmol Pi/(mg·min) (Bohn *et al.*, 2007; Nakano *et al.*, 1999).

The sequential dephosphorylation of PA by wheat phytase has been studied by several groups (Bohn *et al.*, 2007; Lim and Tate, 1973; Nakano *et al.*, 2000; Tomlinson and Ballou, 1962), and it has been determined that it acts through both C6 and C3 to yield *myo*-inositol (Fig.6). This is also the case when isolated phytate globoids are used as the substrate, although the activity of the wheat phytase decreases approximately 30% on globoids as compared to when acting on pure PA (Bohn *et al.*, 2007).

Table 3 Phytases purified from plants and their properties. The lower part of the table describes the properties of cereal phytases; the top part of the table shows examples of other plant phytases in alphabetical order. If more than one phytase is referred to, the individual characteristics of the enzymes are separated with semicolon. Blank space means no value reported

Phytase source	pH	Temp. (°C)	K_m (mmol/L)	M (kD)	Reference
Buttercup squash	4.8	48		67	Goel and Sharma, 1979
Canola seed	4.5~5	50	0.36; 0.25	70	Kim and Eskin, 1987
Faba beans	5	50	0.148	65	Greiner *et al.*, 2001b
Hazel seed	5		0.162	72	Andriotis and Ross, 2003
Legume seeds	8				Scott, 1991
Lily pollen	8	55	0.081	88	Jog *et al.*, 2005
Lupin seeds	5.0	50	0.08; 0.3; 0.13	57~64	Greiner, 2002
Mung beans	7.5	57	0.65	160	Mandal *et al.*, 1972
Navy beans	5.3	50	0.018		Lolas and Markakis, 1977
Peanut	5	55		22	Gonnety *et al.*, 2007
Rapeseed	5.2	50			Mahajan and Dua, 1997
Scallion leaves	5.5	51	0.2		Phillippy, 1998
Soybean seeds	4.5~4.8; 4.5~5	55; 58	0.05; 0.061	119; 72~130	Gibson and Ullah, 1988; Hegeman and Grabau, 2001
Sunflower	5.2	55	0.29		Agostini and Ida, 2006
Tomato roots	4.3	45	0.038	164	Li *et al.*, 1997
Typha latifolia pollen	8		0.017		Hara *et al.*, 1985
Barley	5; 6	45; 55	0.072; 0.19	67	Greiner *et al.*, 2000
Maize	5	55	0.02; 0.03; 0.04; 0.117	71; 76	Hubel and Beck, 1996; Laboure *et al.*, 1993
Oat	5.0	38	0.030	67	Greiner and Alminger, 1999
Rice	4.4; 4.6	40	0.17; 0.09	66; 61	Hayakawa *et al.*, 1989
Rye	6	45	0.3	67	Greiner *et al.*, 1998
Spelt	6	45	0.4	68	Konietzny *et al.*, 1994
Wholemeal wheat	5.15	55	0.3		Peers, 1953
Wheat bran	5		0.49		Nagai and Funahashi, 1962
Wheat bran	5.6; 7.2		0.02; 0.2	47	Lim and Tate, 1973
Wheat bran	6; 5.5	45; 50	0.0005; 0.0008	68; 66	Nakano *et al.*, 1999
Crude extract wheat	6	45	0.83	65	Bohn *et al.*, 2007

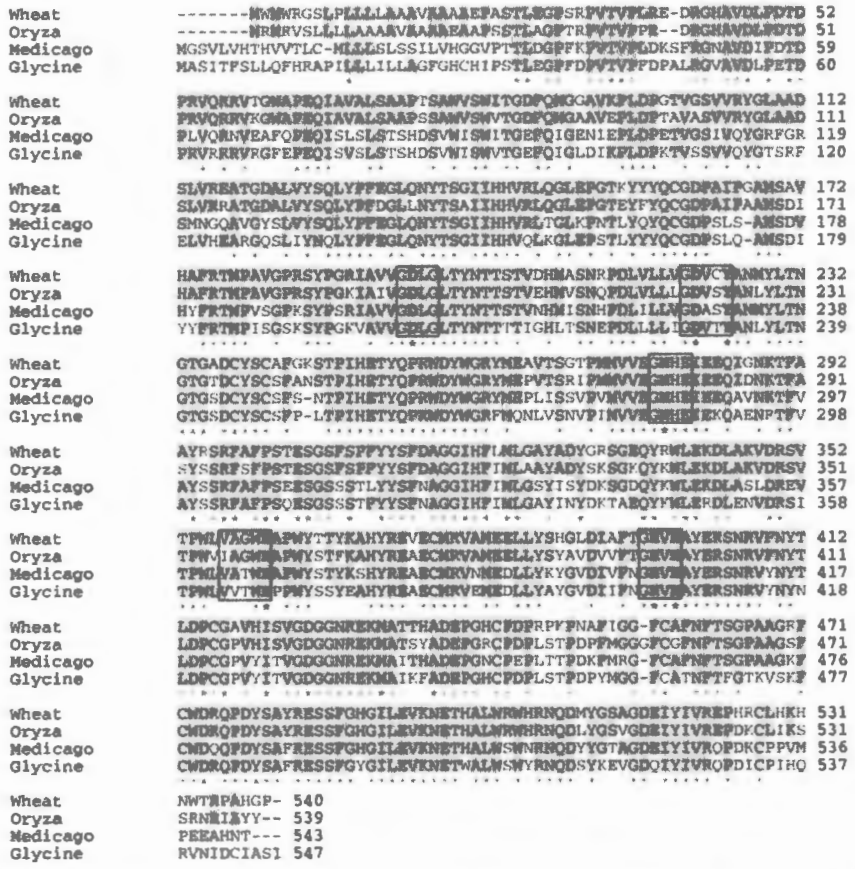


Fig.5 Alignment of wheat phytase to related PAPs in other plants: *Orysa sativa*, *Medicago truncatula* and *Glycine max*. The five conserved regions (black boxes) contain the seven invariant amino acids (bold) involved in coordination of the metals in the binuclear center (Klabunde *et al.*, 1995). Asterisks (*) mark invariant amino acid residue between the aligned sequences and numbers to the right are residue numbers

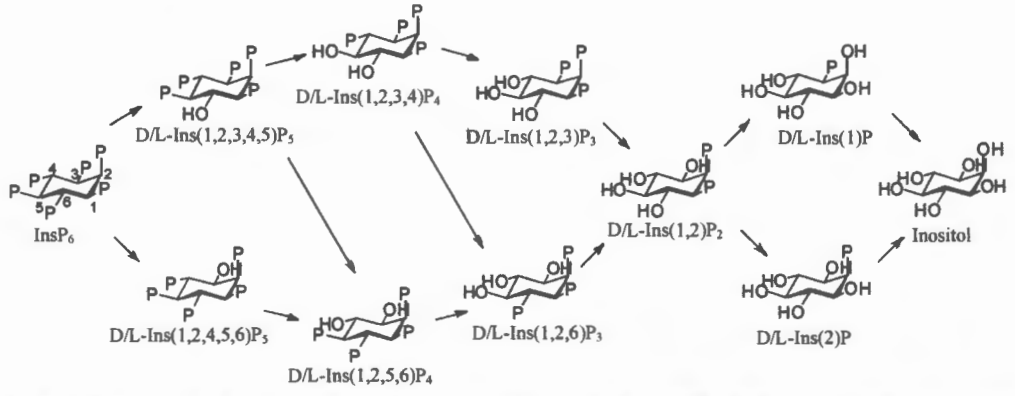


Fig.6 Dephosphorylation of phytic acid by 3/6-wheat phytase according to most recent publications. Hydrolysis of phytic acid initiates on C6 or C3 of the inositol ring (Bohn *et al.*, 2007) and the adjacent phosphates are removed consecutively. End product is inositol (Nakano *et al.*, 2000). In this figure, P represents inorganic phosphate and the rest of the molecules have been omitted for clarity (software: ChemDraw)

PHYTASE IN APPLICATIONS

Degradation of PA and the release of phosphorous and minerals have as previously described been of great interest to human and animal nutritionists as well as ecologists. The development of the most optimal method for applying phytase into food and feed is an on-going process with multiple pathways. The first commercially available phytase was from *Aspergillus niger* (NatuphosTM, BASF) in 1991, but now several phytases are on the market, from e.g., *Peniophora lycii* (RonozymeTM, DSM/Novozymes), *Escherichia coli* (QuantumTM, Diversa/Syngenta) and *Schizosaccharomyces pombe* (PhyzymeTM, Diversa/Danisco). The major problem in production of plant phytases is that a cost-effective and efficient production of the enzymes is yet to be developed. The higher pH and thermal stability of microbial phytases compared to plant phytases have made the microbial phytases more investigated for industrial purposes.

Production of phytase in transgenic plants

The first hurdle in the use of phytase as means for decreasing PA concentration is the production of phytase. In planta production of phytases can be used to reduce the amount of PA in feed for animals, but one of the problems in this regard is the stability of the enzyme. In wheat and barley the WT *Aspergillus fumigatus* phytase has been expressed (Brinch-Pedersen *et al.*, 2003) as well as a heat stable engineered phytase. Although the native *Aspergillus* phytase had high regeneration abilities after heat treatment, it was found that high temperature stability was a more reliable approach to generating low phytic acid feed (Brinch-Pedersen *et al.*, 2006).

In several experiments it has been confirmed that "biofarming" of the phytase is a cost-effective approach to its production. Native *Aspergillus ficuum* phytase has been expressed in tobacco, alfalfa and potato leaves. The most common approach is to use the Cauliflower Mosaic Virus (CaMV) 35S promoter for the construct, and the results are enzymes with almost the same characteristics as the fungal phytase, although minor changes in pH optima and sizes have been observed (Ullah *et al.*, 1999; 2002; 2003). Similar experiments with a heat stable *A. fumigatus* phytase expressed in tobacco leaves and *Pichia pastoris* have also shown great potential. Especially regarding feed production, the relatively high heat resistance of the *A. fumigatus* enzyme is an important asset because the enzyme will then be able to withstand the elevated temperatures employed during feed pelleting processes (Wang *et al.*, 2007).

Purification of the phytase is not always necessary for applications: a transgenic strain of *Bacillus mucilaginosus*, a rhizosphere soil organism able to express high phytase activity extracellularly and degrade PA in the soil, has proven to be able to promote tobacco growth and increase phosphorous content in the plant, thereby potentially limiting eutrophication (Li *et al.*, 2007). Of more nutritional relevance are experiments performed on pigs that were fed fresh rice leaves expressing yeast phytase. The leaves were ground and mixed with a grain-based diet, and PA was degraded using up to twelve weeks old leaves, indicating that the yeast phytase is quite resistant to denaturation when expressed in rice (Hamada *et al.*, 2006).

Phytase as feed additive

Exogenous phytase in feed has multiple benefits, mainly in increasing mineral, phosphorous and energy uptake and thereby decreasing the necessity to fortify the fodder with these substances. The increased availability of phytic acid phosphorous at the same time decreases phosphorous excretion and hence reducing the phosphate load in water supplies in regions with intensive rearing of animals. Comparison of 4 commercially available phytases as fortifiers of pigs feed revealed that none of them satisfied all of the criteria of an ideal phytase for feed production, such as resistance to denaturation under extreme temperatures and pH (Boyce and Walsh, 2006). Nonetheless, supplemental microbial phytase increased P availability by 38%, 12% and 15% in pig diets containing maize, wheat and triticale, respectively (Dungelhoef *et al.*, 1994), and up to 60% reduction in manure P due to phytase supplementation has been reported (Nahm, 2002). Experiments with growing pigs have also shown that the Zn supply can be reduced to approximately 1/3 of the otherwise required amount in a diet based on maize and soybean when microbial phytase is added (Revy *et al.*, 2006). Furthermore, average daily energy intake from feed actually increases with the addition of phytase, making it necessary to reduce the amount of feed offered

to the pigs in order to prevent them from producing too much fat in the muscle tissue (Brady *et al.*, 2003; Revy *et al.*, 2006).

In broiler chickens, supplementing with exogenous phytase has reduced the excretion of endogenous amino acids, calcium, sodium, phytate phosphorus and sialic acid significantly (Cowieson *et al.*, 2004; Nahm, 2002). As in pigs, increased weight gain from a phytase supplemented diet is also reported for broilers. It has been predicted that in both cases this response will be declining with time, due to improvements in animal strains, feeds and management techniques (Selle and Ravindran, 2007).

Exchanging a meat based protein-rich diet with a lower cost plant protein diet would be desired by the industry of aquaculture. However, fish in general have rather short gastrointestinal tracts, and are therefore quite sensitive to the inhibited micronutrient utilisation. Dephytinisation of the plant material is consequently an important prerequisite to this application. The effects on phosphate utilization and growth of fish using phytase treated fodder are to date inconsistent and species-related. Some fish have a basic environment (pH 8) in the gastrointestinal tract which does not correspond to the conditions for optimal phytase activity, and although the potential in dephytinisation is there, for once the acidic microbial phytases may not be the first choice of enzyme (Cao *et al.*, 2007).

Another approach to the degradation of PA by monogastric animals is to create transgenic livestock. A transgenic pig that constitutively secretes microbial phytase from their salivary glands has been generated and it shows up to 75% reduction in phosphorous excretion (Golovan *et al.*, 2001a; 2001b). Furthermore, its requirements for inorganic phosphorous supplementation are decreased to almost zero. Similarly, another group has experimented with expressing an avian MINPP phytase in chickens. This approach would overcome public scepticism towards "foreign" proteins in the food (Ward, 2001) and still decrease phosphorous demands of the transgenic line (Cho *et al.*, 2006).

Phytase as food additive

Degradation of PA during breadmaking has been known to effect mineral bioavailability for many years (Mollgaard, 1946). Several breadmaking procedures designed to diminish the phytate content have therefore been reported. These include the addition of commercial phosphoesterases from wheat (phytase or phosphatase) to whole wheat flour (Knorr *et al.*, 1981) and the activation of the naturally occurring phytase by soaking and malting the grain.

Phytase shows potential as a breadmaking improver, with two main advantages: first, the nutritional improvement produced by decreasing phytate content, and second, all the benefits produced by α-amylase addition (increase in bread volume and improvement in crumb texture) can be obtained by adding phytase, which releases calcium and thereby promotes the activation of endogenous α-amylase (Haros *et al.*, 2001a). The changes in pH values during leavening of bread have been measured to approximately 0.2 pH-units. The pH values ranged between 6.3 and 6.1 in yeast-doughs (Leenhardt *et al.*, 2005) and 5.6 and 5.4 in sourdoughs (Haros *et al.*, 2001b). Wheat phytase has an optimal pH of 6.0 (Bohn *et al.*, 2007), and its activity diminishes markedly as the pH is moved more than 0.5 pH-units from optimum. Conversely, *Aspergillus niger* phytase has two pH optima, one at 5.0 and the other at 2.5~3.0 (Turk and Sandberg, 1992). Therefore, as the pH decreases along the leavening, higher phytase activity is observed using the *Aspergillus* phytase (Haros *et al.*, 2001b). Enhancing the degradation by using an organic acid such as citric acid to acidify the dough is a possibility, although leavening for at least 2 h is required for maximal phytate hydrolysis by phytase (Porres *et al.*, 2001). Consequently, degradation of PA in sourdoughs with long leavening times, such as rye bread, is preferable, and its degradation is almost completed by endogenous phytase in this type of bread, leaving $InsP_3$ as the dominating inositol phosphate (Nielsen *et al.*, 2007). Unless they are able to complex with higher inositol phosphates, $InsP_3$s do not inhibit iron uptake in humans (Brune *et al.*, 1992; Sandberg *et al.*, 1999) and the rye bread should therefore contain bioavailable iron for consumption.

However, although the non-heme iron absorption in humans almost doubles from wheat bread rolls treated with fungal phytase compared to bread untreated with phytase (Sandberg *et al.*, 1996), one should be aware that a minor reduction of phytic acid is not enough to maintain iron status in the long run. Whole grain bread, where dephytinisation is not

completed even after long leavening (Nielsen *et al.*, 2007), should therefore not be consumed to all main meals (Bach Kristensen *et al.*, 2005) to avoid constant mineral uptake inhibition.

Iron absorption from porridges based on flours from rice, wheat, maize, oat, sorghum and wheat-soy flour blend have been tested on humans. The results show that phytate degradation improves iron absorption from cereal porridges prepared with water but not with milk, and that addition of ascorbic acid actually is a better tool for enhancing iron absorption in baby food than addition of phytase. Adding amylase to the porridge in combination with phytase makes the solution more liquid, and probably because of this, the absorption of Fe increases another 3-fold (Hurrell *et al.*, 2003).

Interestingly enough, phytate in tofu is actually considered a positive feature, since phytate inhibits chrystal formation of calcium oxalate—also known as kidney stones. In general, oxalate-rich soy foods also contain higher concentrations of phytate, but most commercially available tofu is categorised as low oxalate/phytate food. Anderson and Wolf (1995) published a review on the changes in phytate concentration related to the processing of soy beans, and the most recent research showed that soy flour is the type of soy-bean product containing the highest amount of phytate (Al-Wahsh *et al.*, 2005).

In summary, phytases have potential for improving mineral bioavailability in food applications. Due to the higher stability of fungal phytases, these would probably be the most efficient in degradation of phytic acid, and the activity of e.g., *Aspergillus niger* would even be increased in the acidic environment in the stomach. One should however note that in bread applications with long leavening time, exogenous phytase is not necessarily required for phytate degradation, unless whole grains are added to the mixture.

References

Adams, C.L., Hambidge, M., Raboy, V., Dorsch, J.A., Sian, L., Westcott, J.L., Krebs, N.F., 2002. Zinc absorption from a low-phytic acid maize. *American Journal of Clinical Nutrition*, **76**(3):556-559.

Agostini, J.D., Ida, E.I., 2006. Partially characterization and application of phytase extracted from germinated sunflower seeds. *Pesquisa Agropecuaria Brasileira*, **41**(6): 1041-1047.

Agranoff, B.W., 1978. Textbook errors—Cyclitol confusion. *Trends in Biochemical Sciences*, **3**(12):N283-N285.

Al-Wahsh, I.A., Horner, H.T., Palmer, R.G., Reddy, M.B., Massey, L.K., 2005. Oxalate and phytate of soy foods. *Journal of Agricultural and Food Chemistry*, **53**(14): 5670-5674. [doi:10.1021/jf0506378]

Anderson, G.J., Frazer, D.M., Mckie, A.T., Vulpe, C.D., Smith, A., 2005. Mechanisms of haem and non-haem iron absorption: Lessons from inherited disorders of iron metabolism. *Biometals*, **18**(4):339-348. [doi:10.1007/s10534-005-3708-8]

Anderson, R.L., Wolf, W.J., 1995. Compositional changes in trypsin-inhibitors, phytic acid, saponins and isoflavones related to soybean processing. *Journal of Nutrition*, **125**(3):S581-S588.

Andlid, T.A., Veide, J., Sandberg, A.S., 2004. Metabolism of extracellular inositol hexaphosphate (phytate) by *Saccharomyces cerevisiae*. *International Journal of Food Microbiology*, **97**(2):157-169. [doi:10.1016/j.ijfoodmicro.2004.04.016]

Andrews, N.C., 1999. The iron transporter DMT1. *International Journal of Biochemistry and Cell Biology*, **31**(10): 991-994. [doi:10.1016/S1357-2725(99)00065-5]

Andrews, N.C., Schmidt, P.J., 2007. Iron homeostasis. *Annual Review of Physiology*, **69**(1):69-85.

Andriotis, V.M.E., Ross, J.D., 2003. Isolation and characterisation of phytase from dormant *Corylus avellana* seeds. *Phytochemistry*, **64**(3):689-699. [doi:10.1016/S0031-9422(03)00415-1]

Angel, R., Tamim, N.M., Applegate, T.J., Dhandu, A.S., Ellestad, L.E., 2002. Phytic acid chemistry: Influence on phytin-phosphorus availability and phytase efficacy. *Journal of Applied Poultry Research*, **11**(4):471-480.

Annibale, B., Capurso, G., Martino, G., Grossi, C., le Fave, G., 2000. Iron deficiency anaemia and *Helicobacter pylori* infection. *International Journal of Antimicrobial Agents*, **16**(4):515-519. [doi:10.1016/S0924-8579(00)00288-0]

Antoine, C., Lullien-Pellerin, V., Abecassis, J., Rouau, X., 2004. Effect of wheat bran ball-milling on fragmentation and marker extractability of the aleurone layer. *Journal of Cereal Science*, **40**(3):275-282. [doi:10.1016/j.jcs.2004.08.002]

Atanasiu, V., Manolescu, B., Stoian, I., 2007. Hepcidin—central regulator of iron metabolism. *European Journal of Haematology*, **78**(1):1-10. [doi:10.1111/j.1600-0609.2006.00772.x]

Bach Kristensen, M., Tetens, I., Alstrup Jorgensen, A.B., Dal Thomsen, A., Milman, N., Hels, O., Sandstrom, B., Hansen, M., 2005. A decrease in iron status in young healthy women after long-term daily consumption of the recommended intake of fibre-rich wheat bread. *European Journal of Nutrition*, **44**(6):334-340. [doi:10.1007/s00394-004-0529-4]

Barrientos, L., Scott, J.J., Murthy, P.P.N., 1994. Specificity of hydrolysis of phytic acid by alkaline phytase from lily

pollen. *Plant Physiology*, **106**(4):1489-1495. [doi:10.1104/pp.106.4.1489]

Barrientos, L.G., Murthy, P.P.N., 1996. Conformational studies of *myo*-inositol phosphates. *Carbohydrate Research*, **296**(1-4):39-54. [doi:10.1016/S0008-6215(96)00250-9]

Batten, G.D., Lott, J.N.A., 1986. The influence of phosphorus-nutrition on the appearance and composition of globoid crystals in wheat aleurone cells. *Cereal Chemistry*, **63**(1):14-18.

Benito, P., Miller, D., 1998. Iron absorption and bioavailability: An updated review. *Nutrition Research*, **18**(3):581-603. [doi:10.1016/S0271-5317(98)00044-X]

Benzie, I.F., 2003. Evolution of dietary antioxidants. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology*, **136**(1):113-126. [doi:10.1016/S1095-6433(02)00368-9]

Berridge, M.J., Irvine, R.F., 1989. Inositol phosphates and cell signalling. *Nature*, **341**(6239):197-205. [doi:10.1038/341197a0]

Bezwoda, W., Charlton, R., Bothwell, T., Torrance, J., Mayet, F., 1978. Importance of gastric hydrochloric-acid in absorption of non-heme food iron. *Journal of Laboratory and Clinical Medicine*, **92**(1):108-116.

Bohn, L., Josefsen, L., Meyer, A.S., Rasmussen, S.K., 2007. Quantitative analysis of phytate globoids isolated from wheat bran and characterization of their sequential dephosphorylation by wheat phytase. *Journal of Agricultural and Food Chemistry*, **55**(18):7547-7552. [doi:10.1021/jf071191t]

Bohn, T., Davidsson, L., Walczyk, T., Hurrell, R.F., 2004. Phytic acid added to white-wheat bread inhibits fractional apparent magnesium absorption in humans. *American Journal of Clinical Nutrition*, **79**(3):418-423.

Boyce, A., Walsh, G., 2006. Comparison of selected physicochemical characteristics of commercial phytases relevant to their application in phosphate pollution abatement. *Journal of Environmental Science and Health Part A-Toxic/Hazardous Substances & Environmental Engineering*, **41**(5):789-798. [doi:10.1080/10934520600614397]

Brady, S.M., Callan, J.J., Cowan, D., McGrane, M., O'Doherty, J.V., 2003. Effect of two microbial phytases on the performance and nutrient retention on grower-finisher pigs fed barley-maize-soyabean meal-based diets. *Irish Journal of Agricultural and Food Research*, **42**(1):101-117.

Brinch-Pedersen, H., Hatzack, F., Sorensen, L.D., Holm, P.B., 2003. Concerted action of endogenous and heterologous phytase on phytic acid degradation in seed of transgenic wheat (*Triticum aestivum* L.). *Transgenic Research*, **12**(6):649-659. [doi:10.1023/B:TRAG.0000005113.38002.e1]

Brinch-Pedersen, H., Hatzack, F., Stoger, E., Arcalis, E., Pontopidan, K., Holm, P.B., 2006. Heat-stable phytases in transgenic wheat (*Triticum aestivum* L.): Deposition pattern, thermostability, and phytate hydrolysis. *Journal of Agricultural and Food Chemistry*, **54**(13):4624-4632. [doi:10.1021/jf0600152]

Brown, E.C., Heit, M.L., Ryan, D.E., 1961. Phytic acid—Analytical investigation. *Canadian Journal of Chemistry-Revue Canadienne de Chimie*, **39**(6):1290-1297. [doi:10.1139/v61-163]

Brune, M., Rossander, L., Hallberg, L., 1989. Iron-absorption and phenolic compounds—Importance of different phenolic structures. *European Journal of Clinical Nutrition*, **43**(8):547-558.

Brune, M., Rossanderhulten, L., Hallberg, L., Gleerup, A., Sandberg, A.S., 1992. Iron-absorption from bread in humans—Inhibiting effects of cereal fiber, phytate and inositol phosphates with different numbers of phosphate groups. *Journal of Nutrition*, **122**(3):442-449.

Bullock, J.I., Duffin, P.A., Nolan, K.B., Smith, T.K., 1995. Effect of phytate on the in-vitro solubility of Al^{3+}, Ca^{2+}, Hg^{2+} and Pb^{2+} as a function of pH at 37 °C. *Journal of the Science of Food and Agriculture*, **67**(4):507-509. [doi:10.1002/jsfa.2740670413]

Camara, F., Barbera, R., Amaro, M.A., Farre, R., 2007. Calcium, iron, zinc and copper transport and uptake by Caco-2 cells in school meals: Influence of protein and mineral interactions. *Food Chemistry*, **100**(3):1085-1092. [doi:10.1016/j.foodchem.2005.11.010]

Cao, L., Wang, W.M., Yang, C.T., Yang, Y., Diana, J., Yakupitiyage, A., Luo, Z., Li, D.P., 2007. Application of microbial phytase in fish feed. *Enzyme and Microbial Technology*, **40**(4):497-507. [doi:10.1016/j.enzmictec.2007.01.007]

Carpenter, C.E., Mahoney, A.W., 1992. Contributions of heme and nonheme iron to human nutrition. *Critcal Reviews in Food Science and Nutrition*, **31**(4):333-367.

Cheryan, M., 1980. Phytic acid interactions in food systems. *CRC Critical Reviews in Food Science and Nutrition*, **13**(4):297-335.

Cheryan, M., Anderson, F.W., Grynspan, F., 1983. Magnesium-phytate complexes—Effect of pH and molar ratio on solubility characteristics. *Cereal Chemistry*, **60**(3):235-237.

Cho, J., Choi, K., Darden, T., Reynolds, P.R., Petitte, J.N., Shears, S.B., 2006. Avian multiple inositol polyphosphate phosphatase is an active phytase that can be engineered to help ameliorate the planet's "phosphate crisis". *Journal of Biotechnology*, **126**(2):248-259. [doi:10.1016/j.jbiotec.2006.04.028]

Collatz, F., Bailey, C., 1921. The activity of phytase as determined by the specific conductivity of phytin-phytase solutions. *The Journal of Industrial and Engineering Chemistry*, **13**(4):317-321. [doi:10.1021/ie50136a017]

Conrad, M.E., Umbreit, J.N., 2002. Pathways of iron absorption. *Blood Cells Molecules and Diseases*, **29**(3):336-355. [doi:10.1006/bcmd.2002.0564]

Conway, R.E., Powell, J.J., Geissler, C.A., 2007. A food-group based algorithm to predict non-heme iron absorption.

International Journal of Food Sciences and Nutrition, **58**(3):29-41. [doi:10.1080/09637480601121250]

Costello, A.J.R., Glonek, T., Myers, T.C., 1976. P-31 nuclear magnetic resonance-pH titrations of myoinositol hexaphosphate. *Carbohydrate Research*, **46**(2):159-171. [doi:10.1016/S0008-6215(00)84287-1]

Cowieson, A.J., Acamovic, T., Bedford, M.R., 2004. The effects of phytase and phytic acid on the loss of endogenous amino acids and minerals from broiler chickens. *British Poultry Science*, **45**(1):101-108. [doi:10.1080/00071660410001668923]

Davidsson, L., Almgren, A., Juillerat, M.A., Hurrell, R.F., 1995. Manganese absorption in humans—The effect of phytic acid and ascorbic-acid in soy formula. *American Journal of Clinical Nutrition*, **62**(5):984-987.

Dionisio, G., Holm, P.B., Brinch-Pedersen, H., 2007. Wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) multiple inositol polyphosphate phosphatases (MINPPs) are phytases expressed during grain filling and germination. *Plant Biotechnology Journal*, **5**(2):325-338. [doi:10.1111/j.1467-7652.2007.00244.x]

Dungelhoef, M., Rodehutscord, M., Spiekers, H., Pfeffer, E., 1994. Effects of supplemental microbial phytase on availability of phosphorus contained in maize, wheat and triticale to pigs. *Animal Feed Science and Technology*, **49**(1-2):1-10. [doi:10.1016/0377-8401(94)90076-0]

Efanov, A.M., Zaitsev, S.V., Berggren, P.O., 1997. Inositol hexakisphosphate stimulates non-Ca^{2+}-mediated and primes Ca^{2+}-mediated exocytosis of insulin by activation of protein kinase C. *Proceedings of the National Academy of Sciences of the United States of America*, **94**(9):4435-4439. [doi:10.1073/pnas.94.9.4435]

Egli, I., Davidsson, L., Juillerat, M.A., Barclay, D., Hurrell, R.F., 2002. The influence of soaking and germination on the phytase activity and phytic acid content of grains and seeds potentially useful for complementary feeding. *Journal of Food Science*, **67**(9):3484-3488. [doi:10.1111/j.1365-2621.2002.tb09609.x]

Egli, I., Davidsson, L., Zeder, C., Walczyk, T., Hurrell, R., 2004. Dephytinization of a complementary food based on wheat and soy increases zinc, but not copper, apparent absorption in adults. *Journal of Nutrition*, **134**(5):1077-1080.

Engle-Stone, R., Yeung, A., Welch, R., Glahn, R., 2005. Meat and ascorbic acid can promote Fe availability from Fe-phytate but not from Fe-tannic acid complexes. *Journal of Agricultural and Food Chemistry*, **53**(26):10276-10284. [doi:10.1021/jf0518453]

Filikov, A.V., James, T.L., 1998. Structure-based design of ligands for protein basic domains: Application to the HIV-1 Tat protein. *Journal of Computer-Aided Molecular Design*, **12**(3):229-240. [doi:10.1023/A:1007949625270]

Gharib, A.G., Mohseni, S.G., Mohajer, M., Gharib, M., 2006. Bioavailability of essential trace elements in the presence of phytate, fiber and calcium. *Journal of Radioanalytical and Nuclear Chemistry*, **270**(1):209-215. [doi:10.1007/s10967-006-0330-3]

Gibson, D.M., Ullah, A.H.J., 1988. Purification and characterization of phytase from cotyledons of germinating soybean seeds. *Archives of Biochemistry and Biophysics*, **260**(2):503-513. [doi:10.1016/0003-9861(88)90475-4]

Gibson, R.S., Perlas, L., Hotz, C., 2006. Improving the bioavailability of nutrients in plant foods at the household level. *Proceedings of the Nutrition Society*, **65**(2):160-168. [doi:10.1079/PNS2006489]

Gillespie, J., Rogers, S.W., Deery, M., Dupree, P., Rogers, J.C., 2005. A unique family of proteins associated with internalized membranes in protein storage vacuoles of the Brassicaceae. *Plant Journal*, **41**(3):429-441.

Glahn, R.P., Wortley, G.M., South, P.K., Miller, D.D., 2002. Inhibition of iron uptake by phytic acid, tannic acid, and ZnCl2: Studies using an in vitro digestion/Caco-2 cell model. *Journal of Agricultural and Food Chemistry*, **50**(2):390-395. [doi:10.1021/jf011046u]

Goel, M., Sharma, C.B., 1979. Multiple forms of phytase in germinating cotyledons of cucurbita-maxima. *Phytochemistry*, **18**(12):1939-1942. [doi:10.1016/S0031-9422(00)82707-7]

Golovan, S.P., Meidinger, R.G., Ajakaiye, A., Cottrill, M., Wiederkehr, M.Z., Barney, D.J., Plante, C., Pollard, J.W., Fan, M.Z., Hayes, M.A., Laursen, J., Hjorth, J.P., Hacker, R.R., Phillips, J.P., Forsberg, C.W., 2001a. Pigs expressing salivary phytase produce low-phosphorus manure (errata). *Nature Biotechnology*, **19**(10):979. [doi:10.1038/nbt1001-979]

Golovan, S.P., Meidinger, R.G., Ajakaiye, A., Cottrill, M., Wiederkehr, M.Z., Barney, D.J., Plante, C., Pollard, J.W., Fan, M.Z., Hayes, M.A., Laursen, J., Hjorth, J.P., Hacker, R.R., Phillips, J.R., Forsberg, C.W., 2001b. Pigs expressing salivary phytase produce low-phosphorus manure. *Nature Biotechnology*, **19**(8):741-745. [doi:10.1038/90788]

Gonnety, J.T., Niamke, S., Meuwiah, F.B., N'guessan Kouadio, E.J., Kouame, L.P., 2007. Purification, kinetic properties and physicochemical characterization of a novel acid phosphatase (AP) from germinating peanut (*Arachis hypogaea*) seed. *Italian Journal of Biochemistry*, **56**(2):149-157.

Graf, E., Mahoney, J.R., Bryant, R.G., Eaton, J.W., 1984. Iron-catalyzed hydroxyl radical formation—Stringent requirement for free iron coordination site. *Journal of Biological Chemistry*, **259**(6):3620-3624.

Grases, F., Garcia-Ferragut, L., Costa-Bauza, A., 1998. Development of calcium oxalate crystals on urothelium: Effect of free radicals. *Nephron*, **78**(3):296-301. [doi:10.1159/000044939]

Grases, F., Simonet, B.M., Prieto, R.M., March, J.G., 2001a. Phytate levels in diverse rat tissues: Influence of dietary phytate. *British Journal of Nutrition*, **86**(2):225-231.

Grases, F., Simonet, B.M., Prieto, R.M., March, J.G., 2001b. Variation of InsP(4), InsP(5) and InsP(6) levels in tissues and biological fluids depending on dietary phytate. *Journal of Nutrional Biochemistry*, **12**(10):595-601. [doi:10.1016/S0955-2863(01)00178-4]

Grases, F., Simonet, B.M., Vucenik, I., Prieto, R.M., Costa-Bauza, A., March, J.G., Shamsuddin, A.M., 2001c. Absorption and excretion of orally administered inositol hexaphosphate (IP6 or phytate) in humans. *Biofactors*, **15**(1):53-61.

Grases, F., Perello, J., Prieto, R.M., Simonet, B.M., Torres, J.J., 2004. Dietary myo-inositol hexaphosphate prevents dystrophic calcifications in soft tissues: A pilot study in Wistar rats. *Life Sciences*, **75**(1):11-19. [doi:10.1016/j.lfs.2003.11.030]

Grases, F., Costa-Bauza, A., Perello, J., Isern, B., Vucenik, I., Valiente, M., Munoz, J.A., Prieto, R.M., 2006. Influence of concomitant food intake on the excretion of orally administered myo-inositol hexaphosphate in humans. *Journal of Medicinal Food*, **9**(1):72-76. [doi:10.1089/jmf.2006.9.72]

Greiner, R., 2002. Purification and characterization of three phytases from germinated lupine seeds (*Lupinus albus* var. Amiga). *Journal of Agricultural and Food Chemistry*, **50**(23):6858-6864. [doi:10.1021/jf025619u]

Greiner, R., Alminger, M.L., 1999. Purification and characterization of a phytate-degrading enzyme from germinated oat (*Avena sativa*). *Journal of the Science of Food and Agriculture*, **79**(11):1453-1460. [doi:10.1002/(SICI)1097-0010(199908)79:11<1453::AID-JSFA386>3.3.CO;2-I]

Greiner, R., Alminger, M.L., 2001. Stereospecificity of *myo*-inositol hexa*kis*phosphate dephosphorylation by phytate-degrading enzymes of cereals. *Journal of Food Biochemistry*, **25**(3):229-248. [doi:10.1111/j.1745-4514.2001.tb00736.x]

Greiner, R., Carlsson, N.G., 2006. *Myo*-Inositol phosphate isomers generated by the action of a phytate-degrading enzyme from *Klebsiella terrigena* on phytate. *Canadian Journal of Microbiology*, **52**(8):759-768. [doi:10.1139/W06-028]

Greiner, R., Konietzny, U., Jany, K.D., 1998. Purification and properties of a phytase from rye. *Journal of Food Biochemistry*, **22**(2):143-161. [doi:10.1111/j.1745-4514.1998.tb00236.x]

Greiner, R., Jany, K.D., Alminger, M.L., 2000. Identification and properties of *myo*-inositol hexa*kis*phosphate phosphohydrolases (*Phytases*) from barley (*Hordeum vulgare*). *Journal of Cereal Science*, **31**(2):127-139. [doi:10.1006/jcrs.1999.0254]

Greiner, R., Alminger, M.L., Carlsson, N.G., 2001a. Stereospecificity of *myo*-inositol hexa*kis*phosphate dephosphorylation by a phytate-degrading enzyme of baker's yeast. *Journal of Agricultural and Food Chemistry*, **49**(5):2228-2233. [doi:10.1021/jf0100090]

Greiner, R., Muzquiz, M., Burbano, C., Cuadrado, C., Pedrosa, M.M., Goyoga, C., 2001b. Purification and characterization of a phytate-degrading enzyme from germinated faba beans (*Vicia faba* var. Alameda). *Journal of Agricultural and Food Chemistry*, **49**(5):2234-2240. [doi:10.1021/jf0100806]

Grusak, M.A., Pearson, J.N., Marentes, E., 1999. The physiology of micronutrient homeostasis in field crops. *Field Crops Research*, **60**(1-2):41-56. [doi:10.1016/S0378-4290(98)00132-4]

Gunshin, H., Mackenzie, B., Berger, U.V., Gunshin, Y., Romero, M.F., Boron, W.F., Nussberger, S., Gollan, J.L., Hediger, M.A., 1997. Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature*, **388**(6641):482-488. [doi:10.1038/41343]

Guttieri, M., Bowen, D., Dorsch, J.A., Raboy, V., Souza, E., 2003. Identification and characterization of a low phytic acid wheat. *Crop Science*, **44**(2):418-424.

Haefner, S., Knietsch, A., Scholten, E., Braun, J., Lohscheidt, M., Zelder, O., 2005. Biotechnological production and applications of phytases. *Applied Microbiology and Biotechnology*, **68**(5):588-597. [doi:10.1007/s00253-005-0005-y]

Hallberg, L., Brune, M., Rossander, L., 1989. Iron-absorption in man—ascorbic-acid and dose-dependent inhibition by phytate. *American Journal of Clinical Nutrition*, **49**(1):140-144.

Hallberg, L., Rossander-Hulten, L., Brune, M., Gleerup, A., 1992. Calcium and iron absorption: Mechanism of action and nutritional importance. *European Journal of Clinical Nutrition*, **46**(5):317-327.

Hamada, A., Yamaguchi, K., Harada, M., Horiguchi, K., Takahashi, T., Honda, H., 2006. Recombinant, rice-produced yeast phytase shows the ability to hydrolyze phytate derived from seed-based feed, and extreme stability during ensilage treatment. *Bioscience Biotechnology and Biochemistry*, **70**(6):1524-1527. [doi:10.1271/bbb.60039]

Hambidge, K.M., Krebs, N.F., Westcott, J.L., Sian, L., Miller, L.V., Peterson, K.L., Raboy, V., 2005. Absorption of calcium from tortilla meals prepared from low-phytate maize. *American Journal of Clinical Nutrition*, **82**(1):84-87.

Hara, A., Ebina, S., Kondo, A., Funaguma, T., 1985. A new type of phytase from pollen of Typha-Latifolia l. *Agricultural and Biological Chemistry*, **49**(12):3539-3544.

Haros, M., Rosell, C.M., Benedito, C., 2001a. Fungal phytase as a potential breadmaking additive. *European Food Research and Technology*, **213**(4-5):317-322. [doi:10.1007/s002170100396]

Haros, M., Rosell, C.M., Benedito, C., 2001b. Use of fungal phytase to improve breadmaking performance of whole wheat bread. *Journal of Agricultural and Food Chemistry*, **49**(11):5450-5454. [doi:10.1021/jf010642l]

Hawkins, P.T., Poyner, D.R., Jackson, T.R., Letcher, A.J., Lander, D.A., Irvine, R.F., 1993. Inhibition of

iron-catalyzed hydroxyl radical formation by inositol polyphosphates—A possible physiological function for myoinositol hexakisphosphate. *Biochemical Journal*, **294**:929-934.

Hayakawa, T., Toma, Y., Igaue, I., 1989. Purification and characterization of acid-phosphatases with or without phytase activity from rice bran. *Agricultural and Biological Chemistry*, **53**(6):1475-1483.

He, Z.Q., Honeycutt, C.W., Zhang, T.Q., Bertsch, P.M., 2006. Preparation and FT-IR characterization of metal phytate compounds. *Journal of Environmental Quality*, **35**(4): 1319-1328. [doi:10.2134/jeq2006.0008]

Hegeman, C.E., Grabau, E.A., 2001. A novel phytase with sequence similarity to purple acid phosphatases is expressed in cotyledons of germinating soybean seedlings. *Plant Physiology*, **126**(4):1598-1608. [doi:10.1104/pp.126.4.1598]

Hertrampf, E., Olivares, M., 2004. Iron amino acid chelates. *International Journal for Vitamin and Nutrition Research*, **74**(6):435-443. [doi:10.1024/0300-9831.74.6.435]

Holm, P.B., Kristiansen, K.N., Pedersen, H.B., 2002. Transgenic approaches in commonly consumed cereals to improve iron and zinc content and bioavailability. *Journal of Nutrition*, **132**(3):514S-516S.

Hubel, F., Beck, E., 1996. Maize root phytase—Purification, characterization, and localization of enzyme activity and its putative substrate. *Plant Physiology*, **112**(4): 1429-1436.

Huebers, H.A., Csiba, E., Josephson, B., Finch, C.A., 1990. Iron-absorption in the iron-deficient rat. *Blut.*, **60**(6): 345-351. [doi:10.1007/BF01737850]

Hurrell, R.F., 2004. Phytic acid degradation as a means of improving iron absorption. *International Journal for Vitamin and Nutrition Research*, **74**(6):445-452. [doi:10.1024/0300-9831.74.6.445]

Hurrell, R.F., Reddy, M.B., Juillerat, M.A., Cook, J.D., 2003. Degradation of phytic acid in cereal porridges improves iron absorption by human subjects. *American Journal of Clinical Nutrition*, **77**(5):1213-1219.

Hurrell, R.F., Lynch, S., Bothwell, T., Cori, H., Glahn, R., Hertrampf, E., Kratky, Z., Miller, D., Rodenstein, M., Streekstra, H., Teucher, B., Turner, E., Yeung, C.K., Zimmermann, M.B., 2004. Enhancing the absorption of fortification iron—A SUSTAIN Task Force report. *International Journal for Vitamin and Nutrition Research*, **74**(6):387-401. [doi:10.1024/0300-9831.74.6.387]

Iqbal, T.H., Lewis, K.O., Cooper, B.T., 1994. Phytase activity in the human and rat small intestine. *Gut*, **35**(9): 1233-1236. [doi:10.1136/gut.35.9.1233]

Iskander, F.Y., Morad, M.M., 1986. Multielement determination in wheat and bran. *Journal of Radioanalytical and Nuclear Chemistry-Letters*, **105**(3):151-156. [doi:10.1007/BF02162964]

IUPAC-IUB, 1989. Numbering of atoms in myo-inositol. Recommendations 1988. Nomenclature Committee of the International Union of Biochemistry. *Biochem. J.*, **258**(1):1-2.

Jang, D.A., Fadel, J.G., Klasing, K.C., Mireles, A.J.Jr, Ernst, R.A., Young, K.A., Cook, A., Raboy, V., 2003. Evaluation of low-phytate corn and barley on broiler chick performance. *Poultry Science*, **82**(12):1914-1924.

Jariwalla, R.J., 1999. Inositol hexaphosphate (IP6) as an anti-neoplastic and lipid-lowering agent. *Anticancer Research*, **19**(5A):3699-3702.

Jiang, L.W., Phillips, T.E., Hamm, C.A., Drozdowicz, Y.M., Rea, P.A., Maeshima, M., Rogers, S.W., Rogers, J.C., 2001. The protein storage vacuole: A unique compound organelle. *Journal of Cell Biology*, **155**(6):991-1002. [doi:10.1083/jcb.200107012]

Jog, S.P., Garchow, B.G., Mehta, B.D., Murthy, P.P.N., 2005. Alkaline phytase from lily pollen: Investigation of biochemical properties. *Archives of Biochemistry and Biophysics*, **440**(2):133-140. [doi:10.1016/j.abb.2005.05.029]

Johnson, L.F., Tate, M.E., 1969. Structure of phytic acids. *Canadian Journal of Chemistry*, **47**(1):63-73. [doi:10.1139/v69-008]

Josefsen, L., Bohn, L., Sørensen, M.B., Rasmussen, S.K., 2007. Characterization of a multifunctional inositol phosphate kinase from rice and barley belonging to the ATP-grasp superfamily. *Gene*, **397**(1-2):114-125. [doi:10.1016/j.gene.2007.04.018]

Joyce, C., Deneau, A., Peterson, K., Ockenden, I., Raboy, V., Lott, J.N.A., 2005. The concentrations and distributions of phytic acid phosphorus and other mineral nutrients in wild-type and low phytic acid Js-12-LPA wheat (*Triticum aestivum*) grain parts. *Canadian Journal of Botany-Revue Canadienne de Botanique*, **83**(12):1599-1607. [doi:10.1139/b05-128]

Kaur, P., Kunze, G., Satyanarayana, T., 2007. Yeast phytases: Present scenario and future perspectives. *Critical Reviews in Biotechnology*, **27**(2):93-109. [doi:10.1080/07388550701334519]

Kemme, P.A., Schlemmer, U., Mroz, Z., Jongbloed, A.W., 2006. Monitoring the stepwise phytate degradation in the upper gastrointestinal tract of pigs. *Journal of the Science of Food and Agriculture*, **86**(4):612-622. [doi:10.1002/jsfa.2380]

Kerovuo, J., Rouvinen, J., Hatzack, F., 2000. Analysis of *myo*-inositol hexakisphosphate hydrolysis by *Bacillus* phytase: Indication of a novel reaction mechanism. *Biochemical Journal*, **352**(3):623-628. [doi:10.1042/0264-6021:3520623]

Kim, H., Eskin, N.A.M., 1987. Canola phytase—isolation and characterization. *Journal of Food Science*, **52**(5): 1353-1354. [doi:10.1111/j.1365-2621.1987.tb14080.x]

Kim, Y.J., Carpenter, C.E., Mahoney, A.W., 1993. Gastric-acid production, iron status and dietary phytate alter enhancement by meat of iron-absorption in rats. *Journal of Nutrition*, **123**(5):940-946.

Klabunde, T., Strater, N., Krebs, B., Witzel, H., 1995. Structural relationship between the mammalian Fe(III)-Fe(II) and the Fe(III)-Zn(II) plant purple acid-phosphatases. *Febs Letters*, **367**(1):56-60. [doi:10.1016/0014-5793(95)00536-I]

Klabunde, T., Strater, N., Frohlich, R., Witzel, H., Krebs, B., 1996. Mechanism of Fe(III)-Zn(II) purple acid phosphatase based on crystal structures. *Journal of Molecular Biology*, **259**(4):737-748. [doi:10.1006/jmbi.1996.0354]

Knorr, D., Watkins, T.R., Carlson, B.L., 1981. Enzymatic reduction of phytate in whole wheat breads. *Journal of Food Science*, **46**(6):1866-1869. [doi:10.1111/j.1365-2621.1981.tb04506.x]

Kolobkowa, E.B., 1936. Investigation of phytase from wheat flour. *Biochimija/Akademija Nauk SSSR*, (1): 512-524 (in German).

Konietzny, U., Greiner, R., Jany, K.D., 1994. Purification and characterization of a phytase from spelt. *Journal of Food Biochemistry*, **18**(3):165-183. [doi:10.1111/j.1745-4514.1994.tb00495.x]

Krishnamurthy, P., Xie, T., Schuetz, J.D., 2007. The role of transporters in cellular heme and porphyrin homeostasis. *Pharmacology and Therapeutics*, **114**(3):345-358. [doi:10.1016/j.pharmthera.2007.02.001]

Laboure, A.M., Gagnon, J., Lescure, A.M., 1993. Purification and characterization of a phytase (*myo*-inositol-hexa*kis*phosphate phosphohydrolase) accumulated in maize (*Zea mays*) seedlings during germination. *Biochemical Journal*, **295**:413-419.

Larsson, O., Barker, C.J., Sjoholm, A., Carlqvist, H., Michell, R.H., Bertorello, A., Nilsson, T., Honkanen, R.E., Mayr, G.W., Zwiller, J., Berggren, P.O., 1997. Inhibition of phosphatases and increased Ca^{2+} channel activity by inositol hexakisphosphate. *Science*, **278**(5337):471-474. [doi:10.1126/science.278.5337.471]

Layrisse, M., Garcia-Casal, M.N., Solano, L., Baron, M.A., Arguello, F., Llovera, D., Ramirez, J., Leets, I., Tropper, E., 2000. New property of vitamin a and beta-carotene on human iron absorption: Effect on phytate and polyphenols as inhibitors of iron absorption. *Archivos Latinoamericanos de Nutricion*, **50**(3):243-248.

Lee, S.H., Park, H.J., Chun, H.K., Cho, S.Y., Cho, S.M., Lillehoj, H.S., 2006. Dietary phytic acid lowers the blood glucose level in diabetic KK mice. *Nutrition Research*, **26**(9):474-479. [doi:10.1016/j.nutres.2006.06.017]

Leenhardt, F., Levrat-Verny, M.A., Chanliaud, E., Remesy, C., 2005. Moderate decrease of pH by sourdough fermentation is sufficient to reduce phytate content of whole wheat flour through endogenous phytase activity. *Journal of Agricultural and Food Chemistry*, **53**(1):98-102. [doi:10.1021/jf049193q]

Lei, X.G., Porres, J.M., 2003. Phytase enzymology, applications, and biotechnology. *Biotechnology Letters*, **25**(21):1787-1794. [doi:10.1023/A:1026224101580]

Li, M.G., Osaki, M., Honma, M., Tadano, T., 1997. Purification and characterization of phytase induced in tomato roots under phosphorus-deficient conditions. *Soil Science and Plant Nutrition*, **43**(1):179-190.

Li, X., Wu, Z.Q., Li, W.D., Yan, R.X., Li, L., Li, J., Li, Y.H., Li, M.G., 2007. Growth promoting effect of a transgenic Bacillus mucilaginosus on tobacco planting. *Applied Microbiology and Biotechnology*, **74**(5):1120-1125. [doi:10.1007/s00253-006-0750-6]

Lim, P.E., Tate, M.E., 1973. Phytases. 2. Properties of phytase fractions F1 and F2 from wheat bran and myoinositol phosphates produced by fraction F2. *Biochimica et Biophysica Acta*, **302**(2):316-328.

Lin, L., Ockenden, I., Lott, J.N.A., 2005. The concentrations and distribution of phytic acid-phosphorus and other mineral nutrients in wild-type and low phytic acid1-1 (lpa1-1) corn (*Zea mays* L.) grains and grain parts. *Canadian Journal of Botany-Revue Canadienne de Botanique*, **83**(1):131-141. [doi:10.1139/b04-146]

Liu, J.C., Ockenden, I., Truax, M., Lott, J.N.A., 2004. Phytic acid-phosphorus and other nutritionally important mineral nutrient elements in grains of wild-type and low phytic acid (lpa1-1) rice. *Seed Science Research*, **14**(2):109-116. [doi:10.1079/SSR2004160]

Liu, K., Peterson, K.L., Raboy, V., 2007. Comparison of the phosphorus and mineral concentrations in bran and abraded kernel fractions of a normal barley (*Hordeum vulgare*) cultivar versus four low phytic acid isolines. *Journal of Agricultural and Food Chemistry*, **55**(11):4453-4460. [doi:10.1021/jf0637776]

Liu, Z.H., Wang, H.Y., Wang, X.E., Zhang, G.P., Chen, P.D., Liu, D.J., 2006. Genotypic and spike positional difference in grain phytase activity, phytate, inorganic phosphorus, iron, and zinc contents in wheat (*Triticum aestivum* L.). *Journal of Cereal Science*, **44**(2):212-219. [doi:10.1016/j.jcs.2006.06.001]

Liu, Z.H., Wang, H.Y., Wang, X.E., Zhang, G.P., Chen, P.D., Liu, D.J., 2007. Phytase activity, phytate, iron, and zinc contents in wheat pearling fractions and their variation across production locations. *Journal of Cereal Science*, **45**(3):319-326. [doi:10.1016/j.jcs.2006.10.004]

Loewus, F.A., Murthy, P.P.N., 2000. *Myo*-inositol metabolism in plants. *Plant Science*, **150**(1):1-19. [doi:10.1016/S0168-9452(99)00150-8]

Lolas, G.M., Markakis, P., 1977. Phytase of navy beans (*Phaseolus vulgaris*). *Journal of Food Science*, **42**(4):1094-1097. [doi:10.1111/j.1365-2621.1977.tb12674.x]

Lonnerdal, B., 1997. Effects of milk and milk components on calcium, magnesium, and trace element absorption during infancy. *Physiological Reviews*, **77**(3):643-669.

Lonnerdal, B., 2000. Dietary factors influencing zinc absorption. *Journal of Nutrition*, **130**(5):1378S-1383S.

Lott, J.N.A., 1980. Protein Bodies. *In*: Tolbert, N.E. (Ed.), The Biochemistry of Plants. Academic Press, New York, p.589-623.

Lott, J.N.A., Spitzer, E., 1980. X-Ray-analysis studies of elements stored in protein body globoid crystals of Triticum grains. *Plant Physiology*, **66**(3):494-499.

Lott, J.N.A., Ockenden, I., Raboy, V., Batten, G.D., 2000. Phytic acid and phosphorus in crop seeds and fruits: A global estimate. *Seed Science Research*, **10**(1):11-33.

Lott, J.N.A., Ockenden, I., Raboy, V., Batten, G.D., 2001. Phytic acid and phosphorus in crop seeds and fruits: A global estimate (Errata). *Seed Science Research*, **11**(2):181.

Macbeth, M.R., Schubert, H.L., van Demark, A.P., Lingam, A.T., Hill, C.P., Bass, B.L., 2005. Inositol hexakisphosphate is bound in the ADAR2 core and required for RNA editing. *Science*, **309**(5740):1534-1539. [doi:10.1126/science.1113150]

Maddaiah, V.T., Kurnick, A.A., Reid, B.L., 1964. Phytic acid studies. *Proceedings of the Society for Experimental Biology and Medicine*, **115**(2):391-393.

Mahajan, A., Dua, S., 1997. Nonchemical approach for reducing antinutritional factors in rapeseed (*Brassica campestris* var. Toria) and characterization of enzyme phytase. *Journal of Agricultural and Food Chemistry*, **45**(7):2504-2508. [doi:10.1021/jf9609136]

Mali, G., Sala, M., Arcon, I., Kaucic, V., Kolar, J., 2006. Insight into the short-range structure of amorphous iron inositol hexaphosphate as provided by P-31 NMR and Fe X-ray absorption spectroscopy. *Journal of Physical Chemistry B*, **110**(46):23060-23067. [doi:10.1021/jp0633805]

Mandal, N.C., Biswas, B.B., Burman, S., 1972. Metabolism of inositol phosphates. 3. Isolation, purification and characterization of phytase from germinating mung beans. *Phytochemistry*, **11**(2):495-502. [doi:10.1016/0031-9422(72)80003-7]

Masud, T., Mahmood, T., Latif, A., Sammi, S., Hameed, T., 2007. Influence of processing and cooking methodologies for reduction of phytic acid content in wheat (*Triticum aestivum*) varieties. *Journal of Food Processing and Preservation*, **31**(5):583-594. [doi:10.1111/j.1745-4549.2007.00147.x]

Maugenest, S., Martinez, I., Lescure, A.M., 1997. Cloning and characterization of a cDNA encoding a maize seedling phytase. *Biochemical Journal*, **322**:511-517.

McCance, R.A., Widdowson, E.M., 1949. Phytic acid. *British Journal of Nutrition*, **2**(4):401-403. [doi:10.1079/BJN19480069]

McCollum, E.V., Hart, E.B., 1908. On the occurrence of a phytin-splitting enzyme in animal tissues. *Journal of Biological Chemistry*, **4**(6):497-500.

Mckie, A.T., Barrow, D., Latunde-Dada, G.O., Rolfs, A., Sager, G., Mudaly, E., Mudaly, M., Richardson, C., Barlow, D., Bomford, A., Peters, T.J., Raja, K.B., Shirali, S., Hediger, M.A., Farzaneh, F., Simpson, R.J., 2001. An iron-regulated ferric reductase associated with the absorption of dietary iron. *Science*, **291**(5509):1755-1759. [doi:10.1126/science.1057206]

Mehta, B.D., Jog, S.P., Johnson, S.C., Murthy, P.P.N., 2006. Lily pollen alkaline phytase is a histidine phosphatase similar to mammalian multiple inositol polyphosphate phosphatase (MINPP). *Phytochemistry*, **67**(17):1874-1886. [doi:10.1016/j.phytochem.2006.06.008]

Mendoza, C., Viteri, F.E., Lonnerdal, B., Young, K.A., Raboy, V., Brown, K.H., 1998. Effect of genetically modified, low-phytic acid maize on absorption of iron from tortillas. *American Journal of Clinical Nutrition*, **68**(5):1123-1127.

Mollgaard, H., 1946. On phytic acid, its importance in metabolism and its enzymic cleavage in bread supplemented with calcium. *Biochemical Journal*, **40**(4):589-603.

Morgounov, A., Gomez-Becerra, H.F., Abugalieva, A., Dzhunusova, M., Yessimbekova, M., Muminjanov, H., Zelenskiy, Y., Ozturk, L., Cakmak, I., 2007. Iron and zinc grain density in common wheat grown in Central Asia. *Euphytica*, **155**(1-2):193-203. [doi:10.1007/s10681-006-9321-2]

Morrison, I.N., Kuo, J., Obrien, T.P., 1975. Histochemistry and fine-structure of developing wheat aleurone cells. *Planta*, **123**(2):105-116. [doi:10.1007/BF00383859]

Mullaney, E.J., Ullah, A.H., 2003. The term phytase comprises several different classes of enzymes. *Biochemical and Biophysical Research Communications*, **312**(1):179-184. [doi:10.1016/j.bbrc.2003.09.176]

Mulvihill, B., Morrissey, P.A., 1998. An investigation of factors influencing the bioavailability of non-haem iron from meat systems. *Irish Journal of Agricultural and Food Research*, **37**(2):219-226.

Nagai, Y., Funahashi, S., 1962. Phytase (*myo*-inositol-hexaphosphate phosphohydrolase) from wheat bran. 1. Purification and substrate specificity. *Agricultural and Biological Chemistry*, **26**(12):794-803.

Nahm, K.H., 2002. Efficient feed nutrient utilization to reduce pollutants in poultry and swine manure. *Critical Reviews in Environmental Science and Technology*, **32**(1):1-16. [doi:10.1080/10643380290813435]

Nakano, T., Joh, T., Tokumoto, E., Hayakawa, T., 1999. Purification and characterization of phytase from bran of *Triticum aestivum* L. cv. Nourin #61. *Food Science and Technology Research*, **5**(1):18-23. [doi:10.3136/fstr.5.18]

Nakano, T., Joh, T., Narita, K., Hayakawa, T., 2000. The pathway of dephosphorylation of *myo*-inositol hexakisphosphate by phytases from wheat bran of *Triticum aestivum* L. cv. Nourin #61. *Bioscience Biotechnology and Biochemistry*, **64**(5):995-1003. [doi:10.1271/bbb.64.995]

Neevel, J.G., 1995. Phytate—A potential conservation agent for the treatment of ink corrosion caused by irongall inks. *Restaurator-International Journal for the Preservation of Library and Archival Material*, **16**(3):143-160.

Nicolas, G., Chauvet, C., Viatte, L., Danan, J.L., Bigard, X.,

Devaux, I., Beaumont, C., Kahn, A., Vaulont, S., 2002. The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. *Journal of Clinical Investigation*, **110**(7):1037-1044. [doi:10.1172/JCI200215686]

Nielsen, M.M., Damstrup, M.L., Dal Thomsen, A., Rasmussen, S.K., Hansen, A., 2007. Phytase activity and degradation of phytic acid during rye bread making. *European Food Research and Technology*, **225**(2):173-181. [doi:10.1007/s00217-006-0397-7]

Nolan, K.B., Duffin, P.A., Mcweeny, D.J., 1987. Effects of phytate on mineral bioavailability—Invitro studies on Mg^{2+}, Ca^{2+}, Fe^{3+}, Cu^{2+} and Zn^{2+} (also Cd^{2+}) solubilities in the presence of phytate. *Journal of the Science of Food and Agriculture*, **40**(1):79-85. [doi:10.1002/jsfa.2740400110]

Ockenden, I., Dorsch, J.A., Reid, M.M., Lin, L., Grant, L.K., Raboy, V., Lott, J.N.A., 2004. Characterization of the storage of phosphorus, inositol phosphate and cations in grain tissues of four barley (*Hordeum vulgare* L.) low phytic acid genotypes. *Plant Science*, **167**(5):1131-1142. [doi:10.1016/j.plantsci.2004.06.008]

Odell, B.L., Deboland, A.R., Koirtyohann, S.R., 1972. Distribution of phytate and nutritionally important elements among morphological components of cereal grains. *Journal of Agricultural and Food Chemistry*, **20**(3):718-721. [doi:10.1021/jf60181a021]

Oh, B.C., Choi, W.C., Park, S., Kim, Y.O., Oh, T.K., 2004. Biochemical properties and substrate specificities of alkaline and histidine acid phytases. *Applied Microbiology and Biotechnology*, **63**(4):362-372. [doi:10.1007/s00253-003-1345-0]

Oh, B.C., Kim, M.H., Yun, B.S., Choi, W.C., Park, S.C., Bae, S.C., Oh, T.K., 2006. Ca^{2+}-inositol phosphate chelation mediates the substrate specificity of beta-propeller phytase. *Biochemistry*, **45**(31):9531-9539. [doi:10.1021/bi0603118]

Olczak, M., Morawiecka, B., Watorek, W., 2003. Plant purple acid phosphatases—Genes, structures and biological function. *Acta Biochimica Polonica*, **50**(4):1245-1256.

Onomi, S., Okazaki, Y., Katayama, T., 2004. Effect of dietary level of phytic acid on hepatic and serum lipid status in rats fed a high-sucrose diet. *Bioscience Biotechnology and Biochemistry*, **68**(6):1379-1381. [doi:10.1271/bbb.68.1379]

Ostanin, K., van Etten, R.L., 1993. Asp304 of *Escherichia coli* acid phosphatase is involved in leaving group protonation. *Journal of Biological Chemistry*, **268**(28):20778-20784.

Ostanin, K., Harms, E.H., Stevis, P.E., Kuciel, R., Zhou, M.M., van Etten, R.L., 1992. Overexpression, site-directed mutagenesis, and mechanism of *Escherichia coli* acid phosphatase. *Journal of Biological Chemistry*, **267**(32):22830-22836.

Peers, F.G., 1953. The phytase of wheat. *Biochemical Journal* **53**(1):102-110.

Perales, S., Barbera, R., Lagarda, M.J., Farre, R., 2006. Fortification of milk with calcium: Effect on calcium bioavailability and interactions with iron and zinc. *Journal of Agricultural and Food Chemistry* **54**(13):4901-4906. [doi:10.1021/jf0601214]

Persson, H., Turk, M., Nyman, M., Sandberg, A.S., 1998. Binding of Cu^{2+}, Zn^{2+} and Cd^{2+} to inositol tri-, tetra-, penta, and hexaphosphates. *Journal of Agricultural and Food Chemistry*, **46**(8):3194-3200. [doi:10.1021/jf971055w]

Pfeffer, W., 1872. Investigation of the Protein Bodies and the Importance of Aspargins in Seed Germs. *In*: Pringsheim, N. (Ed.), Annual Science Book of Botany. Verlag von Wilh. Engelmann, Leipzig, p.429-574 (in German).

Phillippy, B.Q., 1998. Purification and catalytic properties of a phytase from scallion (*Allium fistulosum* L.) leaves. *Journal of Agricultural and Food Chemistry*, **46**(9):3491-3496. [doi:10.1021/jf9803177]

Phillippy, B.Q., 2006. Transport of calcium across Caco-2 cells in the presence of inositol hexakisphosphate. *Nutrition Research*, **26**(3):146-149. [doi:10.1016/j.nutres.2006.02.008]

Pontoppidan, K., Pettersson, D., Sandberg, A.S., 2007. The type of thermal feed treatment influences the inositol phosphate composition. *Animal Feed Science and Technology*, **132**(1-2):137-147. [doi:10.1016/j.anifeedsci.2006.03.008]

Porres, J.M., Etcheverry, P., Miller, D.D., Lei, X.G., 2001. Phytase and citric acid supplementation in whole-wheat bread improves phytate-phosphorus release and iron dialyzability. *Journal of Food Science*, **66**(4):614-619. [doi:10.1111/j.1365-2621.2001.tb04610.x]

Posternak, S., Posternak, T., 1929. About the configuration of inactive inosite. *Helv. Chim. Acta/Soc. Chim. Helv.*, **12**:1165-1181 (in French).

Posternak, T., 1965. Cyclitols. Holden-Day, Inc., San Francisco, CA.

Poulsen, H.D., Johansen, K.S., Hatzack, F., Boisen, S., Rasmussen, S.K., 2001. Nutritional value of low-phytate barley evaluated in rats. *Acta Agriculturae Scandinavica Section A-Animal Science*, **51**(1):53-58. [doi:10.1080/090647001300004790]

Raboy, V., 2003. *Myo*-Inositol-1,2,3,4,5,6-hexakisphosphate. *Phytochemistry*, **64**(6):1033-1043. [doi:10.1016/S0031-9422(03)00446-1]

Raboy, V., 2007. The ABCs of low-phytate crops. *Nature Biotechnology*, **25**(8):874-875. [doi:10.1038/nbt0807-874]

Raffin, S.B., Woo, C.H., Roost, K.T., Price, D.C., Schmid, R., 1974. Intestinal absorption of hemoglobin iron-heme cleavage by mucosal heme oxygenase. *Journal of Clinical Investigation*, **54**(6):1344-1352.

Rasmussen, S.K., Johansen, K.S., Sørensen, M.B., 2007. Polynucleotides Encoding Phytase Polypeptides. US Patent 10/275,311(7,186,817).

Reddy, M.B., Hurrell, R.F., Juillerat, M.A., Cook, J.D., 1996. The influence of different protein sources on phytate inhibition of nonheme-iron absorption in humans. *American Journal of Clinical Nutrition*, **63**(2):203-207.

Revy, P.S., Jondreville, C., Dourmad, J.Y., Nys, Y., 2006. Assessment of dietary zinc requirement of weaned piglets fed diets with or without microbial phytase. *Journal of Animal Physiology and Animal Nutrition*, **90**(1-2):50-59. [doi:10.1111/j.1439-0396.2005.00576.x]

Rodrigues-Filho, U.P., Vaz, S., Felicissimo, M.P., Scarpellini, M., Cardoso, D.R., Vinhas, R.C.J., Landers, R., Schneider, J.F., McGarvey, B.R., Andersen, M.L., Skibsted, L.H., 2005. Heterometallic manganese/zinc-phytate complex as a model compound for metal storage in wheat grains. *Journal of Inorganic Biochemistry*, **99**(10):1973-1982. [doi:10.1016/j.jinorgbio.2005.06.014]

Roughead, Z.K., Zito, C.A., Hunt, J.R., 2005. Inhibitory effects of dietary calcium on the initial uptake and subsequent retention of heme and nonheme iron in humans: Comparisons using an intestinal lavage method. *American Journal of Clinical Nutrition*, **82**(3):589-597.

Saiardi, A., Sciambi, C., McCaffery, J.M., Wendland, B., Snyder, S.H., 2002. Inositol pyrophosphates regulate endocytic trafficking. *Proceedings of the National Academy of Sciences of the United States of America*, **99**(22):14206-14211. [doi:10.1073/pnas.212527899]

Sala, M., Kolar, J., Strlic, M., Kocevar, M., 2006. Synthesis of myo-inositol 1,2,3-tris- and 1,2,3,5-tetra*kis*(dihydrogen phosphate)s as a tool for the inhibition of iron-gall-ink corrosion. *Carbohydrate Research*, **341**(7):897-902. [doi:10.1016/j.carres.2006.02.029]

Salovaara, S., Alminger, M.L., Eklund-Jonsson, C., Andlid, T., Sandberg, A.S., 2003a. Prolonged transit time through the stomach and small intestine improves iron dialyzability and uptake in vitro. *Journal of Agricultural and Food Chemistry*, **51**(17):5131-5136. [doi:10.1021/jf0208233]

Salovaara, S., Sandberg, A.S., Andlid, T., 2003b. Combined impact of pH and organic acids on iron uptake by Caco-2 cells. *Journal of Agricultural and Food Chemistry*, **51**(26):7820-7824. [doi:10.1021/jf030177n]

Sandberg, A.S., Hulthen, L.R., Turk, M., 1996. Dietary *Aspergillus niger* phytase increases iron absorption in humans. *Journal of Nutrition*, **126**(2):476-480.

Sandberg, A.S., Brune, M., Carlsson, N.G., Hallberg, L., Skoglund, E., Rossander-Hulthen, L., 1999. Inositol phosphates with different numbers of phosphate groups influence iron absorption in humans. *American Journal of Clinical Nutrition*, **70**(2):240-246.

Scott, J.J., 1991. Alkaline phytase activity in nonionic detergent extracts of legume seeds. *Plant Physiology*, **95**(4):1298-1301.

Selle, P.H., Ravindran, V., 2007. Microbial phytase in poultry nutrition. *Animal Feed Science and Technology*, **135**(1-2):1-41. [doi:10.1016/j.anifeedsci.2006.06.010]

Selvam, R., 2002. Calcium oxalate stone disease: Role of lipid peroxidation and antioxidants. *Urol. Res.*, **30**(1):35-47. [doi:10.1007/s00240-001-0228-z]

Shears, S.B., 2004. How versatile are inositol phosphate kinases? *Biochemical Journal*, **377**(2):265-280. [doi:10.1042/BJ20031428]

Shi, J., Wang, H., Schellin, K., Li, B., Faller, M., Stoop, J.M., Meeley, R.B., Ertl, D.S., Ranch, J.P., Glassman, K., 2007. Embryo-specific silencing of a transporter reduces phytic acid content of maize and soybean seeds. *Nature Biotechnology*, **25**(8):930-937. [doi:10.1038/nbt1322]

Shin, S., Ha, N.C., Oh, B.C., Oh, T.K., Oh, B.H., 2001. Enzyme mechanism and catalytic property of beta propeller phytase. *Structure*, **9**(9):851-858. [doi:10.1016/S0969-2126(01)00637-2]

Siener, R., Heynck, H., Hesse, A., 2001. Calcium-binding capacities of different brans under simulated gastrointestinal pH conditions. In vitro study with Ca-45. *Journal of Agricultural and Food Chemistry*, **49**(9):4397-4401. [doi:10.1021/jf010381f]

Simpson, C.J., Wise, A., 1990. Binding of zinc and calcium to inositol phosphates (phytate) in vitro. *British Journal of Nutrition*, **64**(1):225-232. [doi:10.1079/BJN19900024]

Steiner, T., Mosenthin, R., Zimmermann, B., Greiner, R., Roth, S., 2007. Distribution of phytase activity, total phosphorus and phytate phosphorus in legume seeds, cereals and cereal by-products as influenced by harvest year and cultivar. *Animal Feed Science and Technology*, **133**(3-4):320-334. [doi:10.1016/j.anifeedsci.2006.04.007]

Stodolak, B., Starzynska, A., Czyszczon, M., Zyla, K., 2007. The effect of phytic acid on oxidative stability of raw and cooked meat. *Food Chemistry*, **101**(3):1041-1045. [doi:10.1016/j.foodchem.2006.02.061]

Storcksdieck, S., Bonsmann, G., Hurrell, R.F., 2007. Iron-binding properties, amino acid composition, and structure of muscle tissue peptides from in vitro digestion of different meat sources. *Journal of Food Science*, **72**(1):S019-S029. [doi:10.1111/j.1750-3841.2006.00229.x]

Strater, N., Klabunde, T., Tucker, P., Witzel, H., Krebs, B., 1995. Crystal structure of a purple acid phosphatase containing a dinuclear Fe(III)-Zn(II) active site. *Science*, **268**(5216):1489-1492. [doi:10.1126/science.7770774]

Suzuki, U., Yoshimura, K., Takaishi, M., 1907. About the enzyme "phytase", which splits "anhydro-oxy-methylene diphosphoric acid". *Bulletin of the College of Agriculture, Tokyo Imperial University*, **7**:503-512 (in German).

Tang, J., Leung, A., Leung, C., Lim, B.L., 2006. Hydrolysis of precipitated phytate by three distinct families of phytases. *Soil Biology and Biochemistry*, **38**(6):1316-1324. [doi:10.1016/j.soilbio.2005.08.021]

Teucher, B., Olivares, M., Cori, H., 2004. Enhancers of iron absorption: Ascorbic acid and other organic acids. *International Journal for Vitamin and Nutrition Research*, **74**(6):403-419. [doi:10.1024/0300-9831.74.6.403]

Thompson, D.B., Erdman, J.W., 1982. Structural model for

ferric phytate—Implications for phytic acid analysis. *Cereal Chemistry*, **59**(6):525-528.

Thornton, C.G., Passen, S., 2004. Inhibition of PCR amplification bovine fecal specimens with by phytic acid, and treatment of phytase to reduce inhibition. *Journal of Microbiological Methods*, **59**(1):43-52. [doi:10.1016/j.mimet.2004.06.001]

Tomlinson, R.V., Ballou, C.E., 1962. Myoinositol polyphosphate intermediates in dephosphorylation of phytic acid by phytase. *Biochemistry*, **1**(1):166. [doi:10.1021/bi00907a025]

Torres, J., Dominguez, S., Cerda, M.F., Obal, G., Mederos, A., Irvine, R.F., Diaz, A., Kremer, C., 2005. Solution behaviour of *myo*-inositol hexakisphosphate in the presence of multivalent cations. Prediction of a neutral pentainagnesium species under cytosolic/nuclear conditions. *Journal of Inorganic Biochemistry*, **99**(3):828-840. [doi:10.1016/j.jinorgbio.2004.12.011]

Tuntawiroon, M., Sritongkul, N., Brune, M., Rossanderhulten, L., Pleehachinda, R., Suwanik, R., Hallberg, L., 1991. Dose-dependent inhibitory effect of phenolic-compounds in foods on nonheme-iron absorption in men. *American Journal of Clinical Nutrition*, **53**(2):554-557.

Turk, M., Sandberg, A.S., 1992. Phytate degradation during breadmaking—Effect of phytase addition. *Journal of Cereal Science*, **15**(3):281-294.

Turk, M., Carlsson, N.G., Sandberg, A.S., 1996. Reduction in the levels of phytate during wholemeal bread making; Effect of yeast and wheat phytases. *Journal of Cereal Science*, **23**(3):257-264. [doi:10.1006/jcrs.1996.0026]

Ullah, A.H.J., Sethumadhavan, K., Mullaney, E.J., Ziegelhoffer, T., ustin-Phillips, S., 1999. Characterization of recombinant fungal phytase (*phy*A) expressed in tobacco leaves. *Biochemical and Biophysical Research Communications*, **264**(1):201-206. [doi:10.1006/bbrc.1999.1501]

Ullah, A.H.J., Sethumadhavan, K., Mullaney, E.J., Ziegelhoffer, T., ustin-Phillips, S., 2002. Cloned and expressed fungal *phyA* gene in alfalfa produces a stable phytase. *Biochemical and Biophysical Research Communications*, **290**(4):1343-1348. [doi:10.1006/bbrc.2002.6361]

Ullah, A.H.J., Sethumadhavan, K., Mullaney, E.J., Ziegelhoffer, T., ustin-Phillips, S., 2003. Fungal *phy*A gene expressed in potato leaves produces active and stable phytase. *Biochemical and Biophysical Research Communications*, **306**(2):603-609. [doi:10.1016/S0006-291X(03)01002-7]

van Etten, R.L., Davidson, R., Stevis, P.E., MacArthur, H., Moore, D.L., 1991. Covalent structure, disulfide bonding, and identification of reactive surface and active site residues of human prostatic acid phosphatase. *Journal of Biological Chemistry*, **266**(4):2313-2319.

Vasca, E., Materazzi, S., Caruso, T., Milano, O., Fontanella, C., Manfredi, C., 2002. Complex formation between phytic acid and divalent metal ions: A solution equilibria and solid state investigation. *Analytical and Bioanalytical Chemistry*, **374**(1):173-178. [doi:10.1007/s00216-002-1469-6]

Vats, P., Banerjee, U.C., 2004. Production studies and catalytic properties of phytases (*myo*-inositolhexakisphosphate phosphohydrolases): An overview. *Enzyme and Microbial Technology*, **35**(1):3-14. [doi:10.1016/j.enzmictec.2004.03.010]

Vats, P., Bhattacharyya, M.S., Banerjee, U.C., 2005. Use of phytases (*myo*-inositolhexakisphosphate phosphohydrolases) for combatting environmental pollution: A biological approach. *Critical Reviews in Environmental Science and Technology*, **35**(5):469-486. [doi:10.1080/10643380590966190]

Veide, J., Andlid, T., 2006. Improved extracellular phytase activity in *Saccharomyces cerevisiae* by modifications in the PHO system. *International Journal of Food Microbiology*, **108**(1):60-67. [doi:10.1016/j.ijfoodmicro.2005.10.020]

Veiga, N., Torres, J., Dominguez, S., Mederos, A., Irvine, R.F., Diaz, A., Kremer, C., 2006. The behaviour of *myo*-inositol hexakisphosphate in the presence of magnesium(II) and calcium(II): Protein-free soluble InsP(6) is limited to 49 mu M under cytosolic/nuclear conditions. *Journal of Inorganic Biochemistry*, **100**(11):1800-1810. [doi:10.1016/j.jinorgbio.2006.06.016]

Veum, T.L., Ledoux, D.R., Bollinger, D.W., Raboy, V., Cook, A., 2002. Low-phytic acid barley improves calcium and phosphorus utilization and growth performance in growing pigs. *Journal of Animal Science* **80**(10):2663-2670.

Vincent, J.B., Crowder, M.W., Averill, B.A., 1992. Hydrolysis of phosphate monoesters: A biological problem with multiple chemical solutions. *Trends in Biochemical Sciences*, **17**(3):105-110. [doi:10.1016/0968-0004(92)90246-6]

Vohra, P., Gray, G.A., Kratzer, F.H., 1965. Phytic acid-metal complexes. *Proceedings of the Society for Experimental Biology and Medicine*, **120**(2):447-449.

Volkmann, C.J., Chateauneuf, G.M., Pradhan, J., Bauman, A.T., Brown, R.E., Murthy, P.P.N., 2002. Conformational flexibility of inositol phosphates: Influence of structural characteristics. *Tetrahedron Letters*, **43**(27):4853-4856. [doi:10.1016/S0040-4039(02)00875-4]

Vucenik, I., Shamsuddin, A.M., 2006. Protection against cancer by dietary IP6 and inositol. *Nutrition and Cancer*, **55**(2):109-125. [doi:10.1207/s15327914nc5502_1]

Wang, Y., Gao, X.R., Su, Q., Wu, W., An, L.J., 2007. Expression of a heat stable phytase from Aspergillus fumigatus in tobacco (*Nicotiana tabacum* L. cv. NC89). *Indian Journal of Biochemistry and Biophysics*, **44**(1):26-30.

Ward, K.A., 2001. Phosphorus-friendly transgenics. *Nature Biotechnology*, **19**(5):415-416. [doi:10.1038/88064]

WHO, 2002. Reducing Risks, Promoting Healthy Life. *In*:

World Health Report 2002. World Health Organization, Geneva, Switzerland.

Wise, A., Gilburt, D.J., 1983. Accessibility of trace-metals, co-precipitated with calcium phytate, to soluble chelating agents. *Nutrition Research*, **3**(3):321-324. [doi:10.1016/S0271-5317(83)80080-3]

Wong, P.Y.Y., Kitts, D.D., 2001. An iron binding assay to measure activity of known food sequestering agents: Studies with buttermilk solids. *Food Chemistry*, **72**(2):245-254. [doi:10.1016/S0308-8146(00)00237-5]

Worthington, M.T., Cohn, S.M., Miller, S.K., Luo, R.Q., Berg, C.L., 2001. Characterization of a human plasma membrane heme transporter in intestinal and hepatocyte cell lines. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, **280**(6):G1172-G1177.

Xiang, T., Liu, Q., Deacon, A.M., Koshy, M., Kriksunov, I.A., Lei, X.G., Hao, Q., Thiel, D.J., 2004. Crystal structure of a heat-resilient phytase from *Aspergillus fumigatus*, carrying a phosphorylated histidine. *Journal of Molecular Biology*, **339**(2):437-445. [doi:10.1016/j.jmb.2004.03.057]

Yeung, C.K., Glahn, R.P., Miller, D.D., 2005. Inhibition of iron uptake from iron salts and chelates by divalent metal cations in intestinal epithelial cells. *Journal of Agricultural and Food Chemistry*, **53**(1):132-136. [doi:10.1021/jf049255c]

York, J.D., 2006. Regulation of nuclear processes by inositol polyphosphates. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, **1761**(5-6):552-559. [doi:10.1016/j.bbalip.2006.04.014]

York, J.D., Odom, A.R., Murphy, R., Ives, E.B., Wente, S.R., 1999. A phospholipase C-dependent inositol polyphosphate kinase pathway required for efficient messenger RNA export. *Science*, **285**(5424):96-100. [doi:10.1126/science.285.5424.96]

Zimmermann, M.B., Hurrell, R.F., 2002. Improving iron, zinc and vitamin A nutrition through plant biotechnology. *Current Opinion in Biotechnology*, **13**(2):142-145. [doi:10.1016/S0958-1669(02)00304-X]

Donohue, M.

FD39

Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
Toxicology 219 (2006) 150–155
TOXICOLOGY
www.elsevier.com/locate/toxicol

Characterization of nigerlysin ©, hemolysin produced by *Aspergillus niger*, and effect on mouse neuronal cells in vitro

Maura Donohue[a], Wei Wei[b], Jinfang Wu[b], Nasser H. Zawia[b], Nicholas Hud[c], Victor De Jesus[d], Detlef Schmechel[e], Justin M. Hettick[e], Donald H. Beezhold[e], Stephen Vesper[a,*]

[a] *National Exposure Research Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH, USA*
[b] *Department of Biomedical and Pharmaceutical Sciences, University of Rhode Island, Kingston RI, USA*
[c] *School of Chemistry and Biochemistry, Parker H. Petit Institute of Bioengineering and Biosciences, Atlanta, GA, USA*
[d] *Georgia Tech Research Institute, Georgia Institute of Technology, Atlanta, GA, USA*
[e] *National Institute for Occupational Safety and Health, Health Effects Laboratory Division, Allergy and Clinical Immunology Branch, Morgantown, WV, USA*

Received 12 July 2005; received in revised form 28 October 2005; accepted 13 November 2005
Available online 9 December 2005

Abstract

Aspergillus niger produced a proteinaceous hemolysin, nigerlysin ©when incubated on sheep's blood agar (SBA) at both 23 and 37 °C. Nigerlysin was purified from tryptic soy broth (TSB) culture filtrate and found to have a molecular weight of approximately 72 kDa, with an isoelectric point of 3.45. Nigerlysin is heat stable up to 65 °C but unstable at 75 °C when incubated for 10 min. Circular dichroic analysis revealed that nigerlysin has an alpha helical structure. Exposure of mouse primary cortical neuronal cells to 0.1 $\mu g\,ml^{-1}$ of nigerlysin resulted in the rapid loss of their viability, approximately 50% in 24 h. The IC_{50} is estimated to be 0.037 $\mu g\,ml^{-1}$, or between 0.034 and 0.041 $\mu g\,ml^{-1}$ at the 95% confidence level.

Keywords: *Aspergillus niger*; Nigerlysin; Hemolysin; Neuronal cells

1. Introduction

Aspergillus niger is an opportunistic fungal pathogen (Richardson and Warnock, 2003) and a common indoor contaminant (Summerbell et al., 1992) even in hospitals (Curtis et al., 2005). *A. niger* causes infections of the brain and other organs (Denning, 1998). Infections of the central nervous system occur in 10–20% of all cases of invasive aspergillosis resulting in nearly 100% mortality (Denning, 1998). Culture filtrates from *A. niger* were toxic to human neuroblastoma and microglial cell lines (Speth et al., 2000). However, the toxic protein in this filtrate was not identified. In this study, we suggest that this neurotoxic protein is the hemolysin, nigerlysin.

2. Materials and methods

2.1. Characterization and hemolytic activity of strains of *A. niger*

Strains of *A. niger* used in the study and their sources are shown in Table 1. These strains were grown on potato dextrose agar (PDA) (Becton Dickinson, Sparks, MD) and conidia were

* Corresponding author. Tel.: +1 513 569 7367; fax: +1 513 487 2512.
E-mail address: vesper.stephen@epa.gov (S. Vesper).

0300-483X/$ – see front matter © 2005 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.tox.2005.11.013

Table 1
Aspergillus niger strains and sources, as well as growth and hemolysin production on sheep's blood agar.

Culture collection and number	Environmental source	Hemolysis	
		23 °C	37 °C
ATCC[a] 16888	Fermentation	+	+
EPACC 1[b]	Clinical	+	+
EPACC 10	Air	+	+
EPACC 655	Lake Michigan	+	+
EPACC 685	Dust	+	+
EPACC 733	Ceiling tile	+	+

[a] ATCC, American Type Culture Collection, Rockville, MD.
[b] EPACC, Environmental Protection Agency Culture Collection, Cincinnati, OH.

collected using a sterile cotton tipped swab and placed on two sheep's blood agar plates (SBA) (Becton Dickinson) which were then incubated at either 23 or 37 °C. The plates were observed daily for evidence of growth and hemolysin production.

2.2. Purification of nigerlysin

Nigerlysin was purified using the following procedure. Strain ATCC 16888 was grown on PDA and the conidia recovered. Approximately 1×10^5 spores were added to 500 ml of tryptic soy broth (TSB) (Becton Dickinson). The cultures were incubated at 23 °C for 48 h on an incubator shaker at 100 rpm. Subsequently, the temperature was raised to 35 °C and the incubation continued for an additional 72 h. The fungal mass was then removed by filtering through Whatman 541 filter paper in a Büchner funnel. The recovered filtrate was centrifuged in a Millipore Centricon plus 70 filter apparatus with a MW cut-off of 30 kDa (Millipore, Bedford, MA), following the manufacturer's instructions. The concentrate from the filtration was then subjected to ion exchange chromatography.

DEAE-cellulose (Sigma, St. Louis, MO) was hydrated in 20 mM Tris–HCl, pH 6.5, for 1 h and then poured into a column giving a final bed of 3 cm × 0.5 cm (height × diameter). Then 0.5 ml of the concentrate was introduced on the top of the column. The bed was eluted with 10 ml of the 20 mM Tris–HCl buffer, followed by 10 ml of 0.1 M NaCl in 20 mM Tris–HCl buffer. Fractions of the elution with 0.2 M NaCl in 20 mM Tris–HCl buffer were collected (five drops each) throughout the elution. Ten microliters of each fraction was plated on SBA and hemolysis noted at 24 h.

The five hemolytically active fractions from the ion exchange chromatography were subjected to gel filtration using Sephadex G 100-50 (Sigma, St. Louis, MO) hydrated for 72 h in the running buffer containing 0.2 M NaCl and poured into a chromatography column to give a final bed 24 cm × 0.25 cm (height × diameter). Fractions of five drops each were collected at 1.5 ml h^{-1} using a fraction collector (ISCO, Lincoln, NE). Ten microliters of each fraction was plated on SBA and incubated at 37 °C, and hemolysis noted at 24 h. The process was repeated through a Sephadex G 200 column.

The five most hemolytically active fractions from the gel filtration were combined and dialyzed against sterile type 1 water. The desalted solution was frozen at −80 °C and lyophilized using a Spin Vac (Savant Instruments, Farmingdale, NY) resulting in a lyophilized pellet containing the nigerlysin.

2.3. Test of nigerlysin heat stability

Purified nigerlysin in 20 mM Tris–HCl buffer was incubated at 55, 65, 75 or 85 °C for 10 min then 10 μl aliquots of each was placed on SBA. Lysis of the RBCs was photographed at 24 and 48 h.

2.4. Gel electrophoresis

Methods described by Donohue et al. (2005) were used for native protein electrophoresis, SDS gel electrophoresis and isoelectric focusing (IEF) analysis.

2.5. Mass spectrometry

Samples were analyzed using a PBSIIc matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF MS) operated in positive linear mode (Ciphergen Biosystems Inc., Fremont, CA). Samples were prepared as conventional dried droplets, on a gold sample support, using a-cyano-4-hydroxycinnamic acid (CHCA, 10 mg/ml in acetonitrile/0.1% TFA) as a matrix. All mass spectra were acquired between 0 and 150,000 *m/z* at a laser setting slightly above the threshold for ion production (laser step 140). The spectra were externally calibrated using bovine serum albumin $[M+H]^{2+}$ at *m/z* 33,217 and $[M+H]^{+}$ at *m/z* 66,433. The acceleration voltage was maintained at 20 kV, and a low mass gate was used to reject ions below *m/z* 500.

2.6. Circular dichroic analysis of nigerlysin

Circular dichroic analysis of an aqueous solution of nigerlysin (1 μg μl^{-1}) was carried out at room temperature on a Jasco J-810 CD spectrophotometer (Jasco Inc., Easton, MD). Quartz cells of 5 mm path length were used for measurements in the far (190–280 nm) UV spectra.

2.7. Neuronal tissue culture and exposure to nigerlysin

All animal experiments were performed under a protocol approved by an institutional animal care and use committee (IACUC) of the University of Rhode Island. Mice fetuses (C57BL/6) (Charles River Laboratories, Wilmington, MA, USA) were used to generate primary cortical neuronal cultures. Pregnant mice were sacrificed on Day 15 of gestation. Fetuses were removed and the cortexes were separated from the rest of the brain under the microscope. After removing the meninges, the cortices were placed in 15 ml tube containing 5 ml Hanks'

Balanced Salt Solution (HBSS). The cortices were then dissociated by papain (2 mg ml^{-1}) while incubating in a 37 °C water bath for 15 min. After incubation, the cells were centrifuged at 1000 rpm and the supernatant was aspirated leaving the pellet. Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum was added to triturate with a fire polished Pasteur pipette; centrifuged again and then plating medium was added to the cell pellet. Cells were then counted with trypan blue and plated at a constant density of 6.5×10^4 cells per well, in 96-well pre-coated plates. The plating medium contained 2% B27, 0.5 mM L-glutamine and 25 μM glutamic acid in NEUROBASAL medium (Invitrogen). On Day 4, half of the medium was replaced with fresh medium that did not contain glutamic acid. The cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

Modified NEUROBASAL medium with 2% B27 and 0.5 μM L-glutamine is an optimized serum-free substitute formulated to meet the special requirement of neuronal cells and gives optimal long-term maintenance of the normal phenotype of neural cells without the need of an astrocyte feeder layer. In addition, the low concentration of L-glutamine does not support the survival and growth of glia cells. These conditions are designed to limit glial growth to less than 0.5%, preserving a nearly pure neuronal culture (Brewer et al., 1993).

2.8. Cytotoxicity assays

On Day 7 of culture incubation, the cell suspension in each well was changed to 40 μl of old medium and 40 μl of new medium. Nigerlysin was dissolved in water to give a final concentration of 0.10 μg ml^{-1}, measured with the Micro BCA kit (Pierce, Rockford, IL). The nigerlysin was diluted 200× with the cell medium NEUROBASAL containing 2% B27, 0.5 mM L-glutamine and 1% antibiotic, as working solution. Then 20 μl of the working solution was added to each well.

In the dose response studies, cells in each well were exposed to 0, 0.025, 0.033, 0.05 and 0.1 μg ml^{-1} of nigerlysin for 72 h. For the time course studies, cells in each well were exposed to 0.1 μg ml^{-1} of nigerlysin for 0, 4, 24, 48 and 72 h. Sterile water was added to the NEUROBASAL medium as controls. During exposure, cells were assessed for viability using the MTT (3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) assay, CellTiter 96 Non-Radioactive cell Proliferation Assay (Promega, Madison, WI). Either three or five replicate treatments were assayed at each time point.

3. Results

A. niger is readily isolated from many environments, including lake water, ceiling tiles, indoor air and dust. All strains of *A. niger* in this study grew at 23 °C and at 37 °C on SBA and all produced hemolysis within 4 days (Table 1).

After purification, nigerlysin in a native gel revealed a single band which when place on SBA lysed the blood cells underneath (Fig. 1(A and B)). Under reducing conditions, the purified nigerlysin showed a single silver staining band with a MW of about 72 kDa in an SDS-PAGE gel (Fig. 1(C and D)), in good agreement with MALDI-TOF MS (Fig. 2). The isoelectric point of nigerlysin is 3.45 with four isomers (Fig. 1(E and F)). Nigerlysin is stable at 65 °C for 10 min but unstable at 75 °C for 10 min (Fig. 3). The circular dichroic analysis showed that nigerlysin forms an alpha helix (Fig. 4).

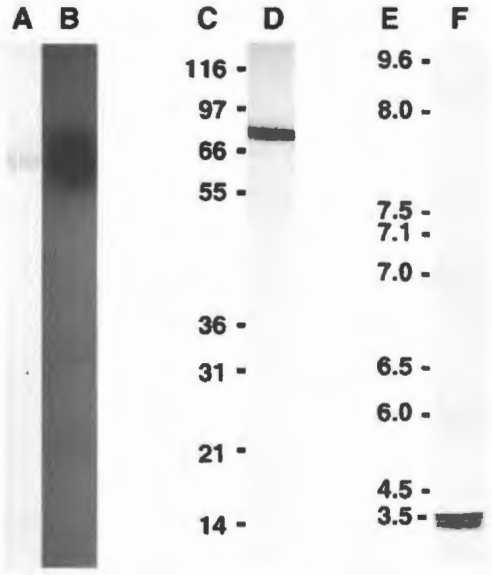


Fig. 1. Characteristics of nigerlysin. Native gel electrophoresis of purified nigerlysin (A); and SBA plate after native nigerlysin gel exposure for 24 h (B); molecular weight standards in kDa (C); for SDS-PAGE of nigerlysin, after treatment with dithiothrietol (D); isoelectric focusing gel standards (E); and nigerlysin (F).

Nigerlyisn is toxic to about 60% of the cultured neuronal cells but about 40% are more resistant (Fig. 5). The loss of viability time course shows that cell death begins

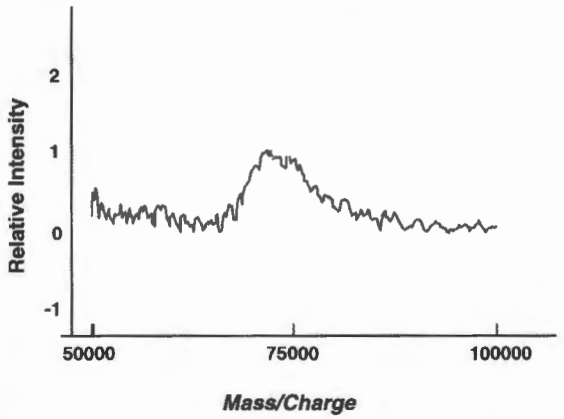


Fig. 2. Matrix-assisted laser desorption/ionization time-of-flight mass spectrum of nigerlysin.

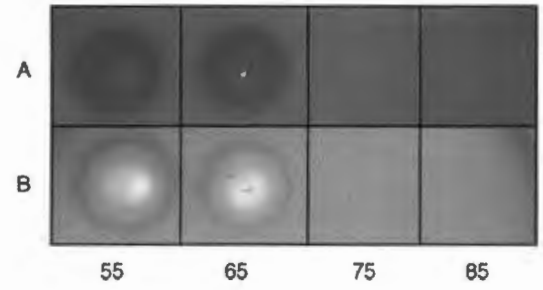


Fig. 3. Test of heat stability of nigerlysin. Purified nigerlysin in 20 mM Tris–HCl buffer was incubated at 55, 65, 75 or 85 °C for 10 min then 10 μl placed on sheep's blood agar and incubated at room temperature for 24 h (A) and 48 h (B).

almost immediately and proceeds rapidly (Fig. 6). Since these are primary neuronal cultures, there is some loss of cell viability (less than 5%) even in the controls. The percent cell viability should be considered relative and not absolute. Robust and differentiated neurons with synaptic connections are seen prior to exposure (Fig. 7(A)) and mostly dead and floating cells are seen post-exposure (Fig. 7(B)).

One-way analysis of variance on the difference in absorbance at 690 nm from 570 nm among the five concentrations, including zero, was performed and one-sided Bonferroni-adjusted *p*-values were used to determine whether survival (which is linearly related to the difference in absorbance) declined significantly from one concentration to the next (four comparisons, including the control). Parametric determination of an IC_{50} using logistic regression in either the raw or log-transformed dose failed to result in an adequate fit to these data, therefore a natural cubic spline interpolation was used. A 95% confidence interval for the IC_{50} was estimated using a boot-strap and Monte Carlo sampling of the original data.

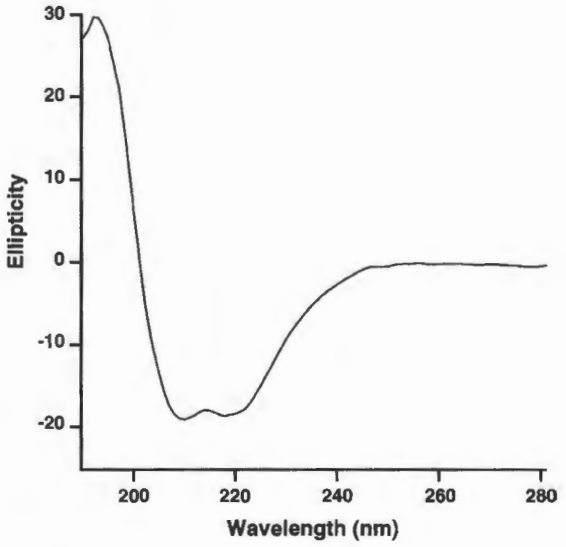


Fig. 4. Circular dichroic analysis of nigerlysin. Circular dichroic analysis of an aqueous solution of nigerlysin (1 μg μl^{-1}) was carried out at room temperature on a Jasco J-810 CD spectrophotometer. Quartz cells of 5 mm path length were used for measurements in the far (190–280 nm) UV spectrum.

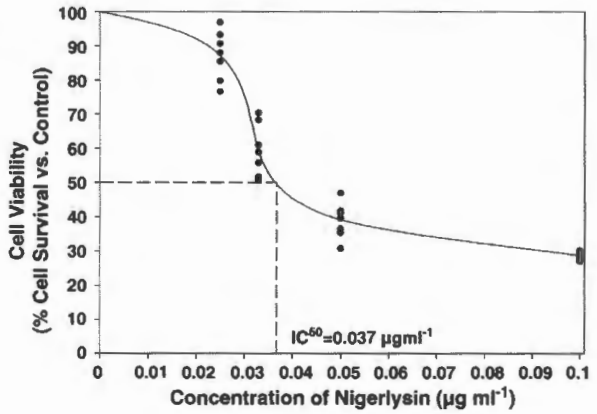


Fig. 5. Dose response (mean percent viability ± standard deviation) of cortical neuronal primary cell cultures following exposure to nigerlysin at different concentrations. Cells were exposed to 0, 0.025, 0.033, 0.05 and 0.1 μg ml^{-1} of nigerlysin for 72 h. Each data point represents the mean and standard deviation of relative cell viability of exposed cells vs. relative cell viability of control cells. (Viability of control cells is greater than 95% during the experiment.)

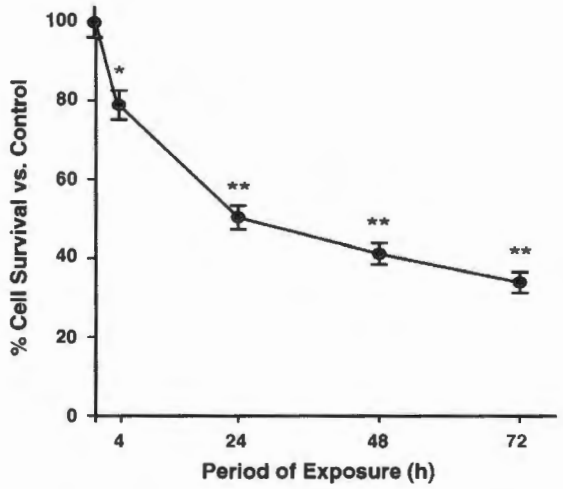


Fig. 6. Time course of toxicity (mean percent viability ± standard deviation) of cortical neuronal primary cell cultures following exposure to 0.1 μg ml^{-1} nigerlysin for 0, 4, 24, 48 and 72 h. Each data point represents relative cell viability of exposed cells vs. relative cell viability of control cells. (Viability of control cells is greater than 95% during the experiment.) Values marked with an asterisk or asterisks are significantly different from their corresponding controls ($^{*}p < 0.05$, $^{**}p < 0.001$).

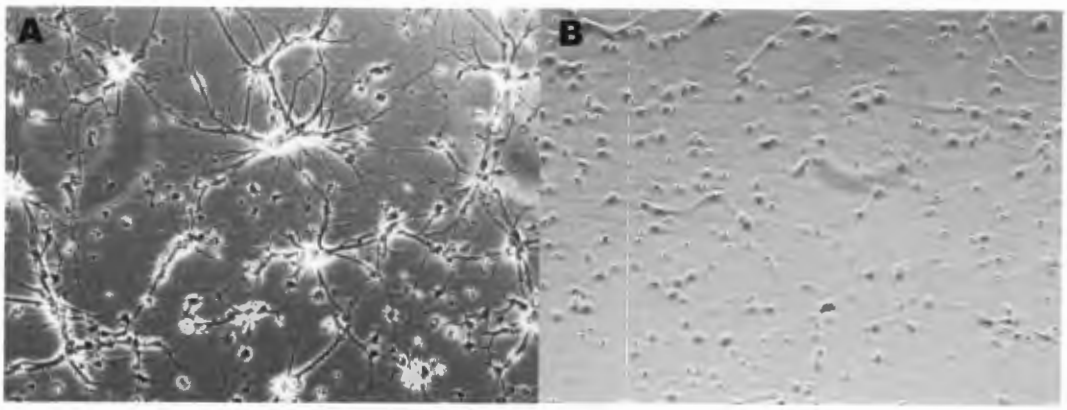


Fig. 7. Appearance of control cortical neuronal cells (A) and cells after exposure to 0.1 $\mu g\,ml^{-1}$ nigerlysin for 72 h (B), approximate magnification 400×.

Survival at the lowest dose level, 0.025 $\mu g\,ml^{-1}$, was significantly lower than that of the control ($p = 0.005$). Survival further declined at 0.033 and 0.05 $\mu g\,ml^{-1}$ compared to the next lower dose, $p < 0.0001$ and $p = 0.017$, respectively. The IC_{50} is estimated to be 0.037 $\mu g\,ml^{-1}$, or between 0.034 and 0.041 $\mu g\,ml^{-1}$ at the 95% confidence level.

4. Discussion

Hemolysins lyse RBCs by creating pores or holes in red blood cell membranes resulting in the release of iron that promotes microbial growth (Bullen, 1981). Many bacterial hemolysins are critical virulence factors (Bhakdi et al., 1996; Cavalieri et al., 1984; Doran et al., 2002; Johnson et al., 1985; Ou Said et al., 1988) The hemolysin produced by *A. fumigatus* (asp-hemolysin) promotes aspergillosis (Ebina et al., 1982). Nigerlysin may also promote opportunistic infections, such as cerebral aspergillosis.

Invasion of the central nervous system by fungi can occur through an anatomically adjacent site like the nasal sinus (Young et al., 1970). Human nasal sinus is colonized by many indoor molds, including *A. niger* (Ponikau et al., 1999). Sinus colonization by *A. niger* may be accompanied by release of nigerlysin and could promote infections of many organs including the brain. Patients with cerebral aspergillosis present with symptoms like fever, altered mental status, headache, hemiplegia and seizures (Hagensee et al., 1994). If nigerlysin can cross the blood brain barrier, it may cause some neurological effects independent of the presence of the fungus in the brain.

In this study we have demonstrated the rapid loss in the viability of primary mouse cortical neuronal cells, after the in vitro exposure to nigerlysin (Fig. 6). The destruction begins quickly and after 24 h, approximately 50% of the cells were no longer viable compared to the controls. If such destruction occurs in humans, then some cognitive effects might be expected.

Human exposure to indoor environmental fungi purportedly causes cognitive dysfunction (Gordon et al., 2004). Since *A. niger* can colonize the human nasal sinuses and potentially produce nigerlysin there, nigerlysin has the potential to enter the central nervous system and damage neurons. Further work is required to determine the effects of nigerlysin on human neuronal cells but earlier work may already have provided some answers.

Speth et al. (2000) demonstrated that a "toxic factor" from *A. niger* filtrates killed cultured human neurons and glial cells. They described this toxic factor as a protein with a MW between 50 and 100 kDa, heat stable to 65 °C but labile at 85 °C, and toxic to neuronal cells. These characteristics match those of nigerlysin and we suggest that their "toxic factor" was probably nigerlysin. If so, then nigerlysin is a toxin similar to other known microbial toxins.

Since nigerlysin has an alpha helical structure, it may behave like the alpha helical bundle toxins, e.g., diphtheria toxin (MW 58 kDa), colicins (MW 60 kDa), δ-endotoxins (MW 70–135 kDa) and *Pseudomonas aeruginosa* exotoxin A (MW 67 kDa) (Parker and Pattus, 1993). The alpha helical structure of nigerlysin differs from the beta-sheet structure of many bacterial hemolysins, such as staphylococcal alpha toxin (Tobkes et al., 1985) and perfringolysin-O (Shimada et al., 1999). Alpha helical toxins damage susceptible cells by creating pores in membranes. It seems likely that nigerlysin has a similar mode of action. Therefore, the common occurrence of *A. niger* in the environment, along with

the toxic characteristics and the stability of nigerlysin, highlight a potential threat to human health.

Acknowledgements

Thanks to Teresa Ruby and Catherine Loizos for preparation of the figures.

Notice: The U.S. Environmental Protection Agency (EPA) through its Office of Research and Development, funded and collaborated in the research described here. It has been subjected to the Agency's peer review and has been approved as an EPA publication. Mention of trade names or commercial products does not constitute endorsement or recommendation by the EPA for use. And the findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute of Occupational Safety and Health.

References

Bhakdi, S., Bayley, H., Valeva, A., Walev, I., Walker, B., Weller, U., Kehoe, M., Palmer, M., 1996. Staphylococcal alpha-toxin, streptolysin-O, and *Escherichia coli* hemolysin prototypes of pore-forming bacterial cytolysins. Arch. Microbiol. 165, 73–79.

Brewer, G.J., Torricelli, J.R., Evege, E.K., Price, P.J., 1993. Optimized survival of hippocampal neurons in B27-supplemented neurobasal, a new serum-free medium combination. J. Neurosci. Res. 35, 567–576.

Bullen, J.J., 1981. The significance of iron in infection. Rev. Infect. Dis. 3, 1127–1138.

Cavalieri, S.J., Bohach, G.A., Snyder, I.S., 1984. *Escherichia coli* α-hemolysin characteristics and probable role in pathogenicity. Microbiol. Rev. 48, 326–343.

Curtis, L., Cali, S., Conroy, L., Baker, K., Ou, C.H., Hershow, R., Norlock-Cruz, F., Scheff, P., 2005. *Aspergillus* surveillance project at a large tertiary-care hospital. J. Hosp. Infect. 59, 188–196.

Denning, D.W., 1998. Invasive aspergillosis. Clin. Infect. Dis. 26, 781–805.

Donohue, M., Chung, Y., Magnuson, M.L., Ward, M., Selgrade, M.J., Vesper, S.J., 2005. Hemolysin, chrysolysin™ from *Penicillium chrysogenum*, promotes inflammatory response. Int. J. Hyg. Environ. Health 208, 279–285.

Doran, K.S., Chang, J.C.W., Benoit, V.M., Eckmann, L., Nizet, V., 2002. Group B streptococcal β-hemolysin/cytolysin promotes invasion of human lung epithelial cells and the release of interleukin-8. J. Infect. Dis. 185, 196–203.

Ebina, K., Yokota, K., Sakaguchi, O., 1982. Studies on the toxin of *Aspergillus fumigatus*. XIV. Relationship between Asp-hemolysin and experimental infection of mice. Jpn. J. Med. Mycol. 23, 246–252.

Gordon, W.A., Cantor, J.B., Johanning, E., Charatz, H.J., Ashman, T.B., Breeze, J.L., Haddad, L., Abramowitz, S., 2004. Cognitive impairment associated with toxigenic fungal exposure: a replication and extension of previous findings. Appl. Neuropsychol. 11, 65–74.

Hagensee, M.E., Bauwens, J.E., Bent, K., Bowden, R.A., 1994. Brain abscess following marrow transplantation: experience at the Fred Hutchinson Cancer Research Center, 1984–1992. Clin. Infect. Dis. 19, 402–408.

Johnson, P., Lindberg, M., Haraldsson, I., Waldstrom, T., 1985. Virulence of *Staphylococcus aureus* in a mouse mastitis model: studies of alpha hemolysin, coagulase and protein A as possible virulence determinants with protoplast fusion and gene cloning. Infect. Immun. 49, 765–769.

Ou Said, A.M., Contrepois, M.G., Vartanian, M.D., Girardeau, J., 1988. Virulence factors and markers in *Escherichia coli* from calves with bacteremia. Am. J. Vet. Res. 49, 1657–1660.

Parker, M.W., Pattus, F., 1993. Rendering a membrane protein soluble in water: a common packing motif in bacterial protein toxins. TIBS 18, 391–395.

Ponikau, J.U., Sherris, D.A., Kern, E.B., Homburger, H.A., Frigas, E., Gaffey, T.A., Roberts, G.D., 1999. The diagnosis and incidence of allergic fungal sinusitis. Mayo Clin. Proc. 74, 877–884.

Richardson, M.D., Warnock, D.W., 2003. Aspergillosis. In: Fungal Infection: Diagnosis and Management, third ed. Blackwell Publishing, Malden, MA, pp. 156–184.

Shimada, Y., Nakamura, M., Naito, Y., Nomura, K., Ohno-Iwashita, Y., 1999. C-terminal amino acid residues are required for the folding and cholesterol binding property of perfringolysin O, a pore-forming cytolysin. J. Biol. Chem. 26, 18536–18542.

Speth, C., Rambach, G., Lass-Florl, C., Wurzner, R., Gasque, P., Mohsenipour, I., Dietrich, M.P., 2000. Culture supernatants of patient-derived *Aspergillus* isolates have toxic and lytic activity towards neurons and glial cells. FEMS Immunol. Med. Microbiol. 29, 303–313.

Summerbell, R.C., Staib, F., Dales, R., Nolard, N., Kane, J., Zwanenburg, H., Burnett, R., Krajdn, S., Fung, D., Leong, D., 1992. Ecology of fungi in human dwellings. J. Med. Vet. Mycol. 30 (Suppl. 1), 279–285.

Tobkes, N., Wallace, B.A., Bayley, H., 1985. Secondary structure and assembly mechanism of an oligomeric channel protein. Biochemistry 24, 1915–1920.

Young, R.C., Bennett, J.E., Vogel, C.L., Carbone, P.P., DeVita, V.T., 1970. Aspergillosis: the spectrum of the disease in 98 patients. Medicine 49, 147–173.

Dvorakova, J.

NO MATERIAL SUBMITTED

FDA-1995-S-0039-0735-AOM0-ETC

Eckhout, W. and De Paepe, M.

A041

REVIEW

Folia Microbiol. 43 (4), 323–338 (1998)

Phytase: Sources, Preparation and Exploitation

J. DVOŘÁKOVÁ

Laboratory of Biotransformation, Institute of Microbiology, Academy of Sciences of the Czech Republic, 142 20 Prague 4, Czech Republic

Received December 29, 1997
Revised version February 9, 1998

ABSTRACT. This review deals with phytase (*myo*-inositol hexakisphosphate phosphohydrolase) and covers microbiological sources, phytase occurrence in plants and animals, its purification, physico-chemical and molecular properties. Protein engineering of phytase and potential enzyme applications are discussed.

CONTENTS

1 Introduction

Phytic acid was discovered as early as in 1872 by Pfeffer and a first note on phytase can be found in the literature in 1907 (Suzuki *et al.*). This paper already suggests this enzyme as a mean for lowering of phytic acid content in rice-bran (by its hydrolysis producing *myo*-inositol and inorganic phosphate). This very first application accounts for the industrial importance of phytase.

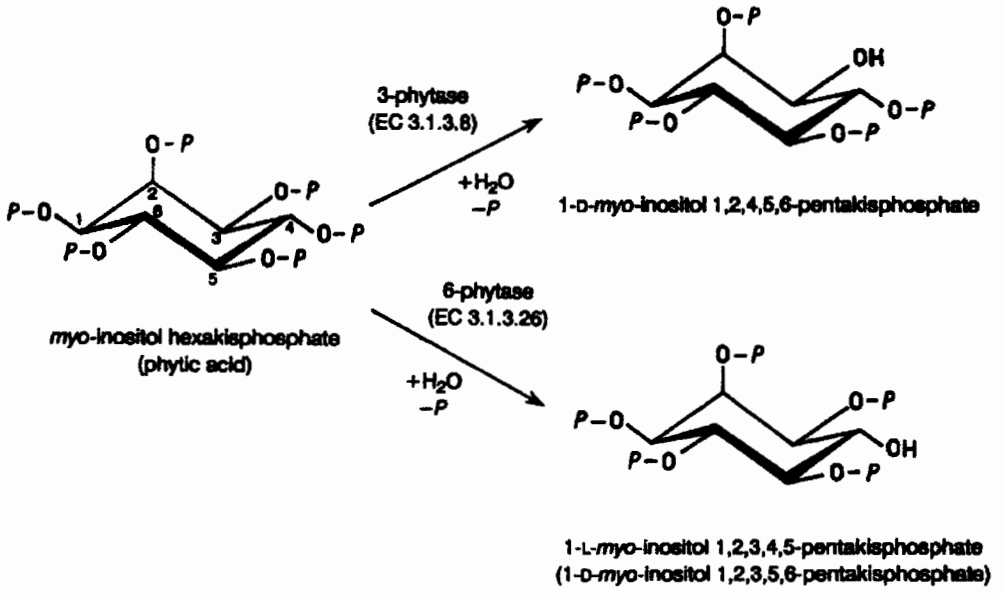
Phytase (*myo*-inositol hexakisphosphate phosphohydrolase) belongs to the group of phosphoric monoester hydrolases; it catalyzes the hydrolysis of *myo*-inositol hexakisphosphate (phytic acid, *myo*-inositol-P_6) to inorganic monophosphate and lower phosphoric esters of *myo*-inositol, or in some cases to free *myo*-inositol.

The *Enzyme Nomenclature Committee of the International Union of Biochemistry* distinguishes two types of phytase: 3-phytase and 6-phytase. This classification is based on the first phosphate group attacked by the enzyme (Scheme 1). 3-Phytase is typical for microorganisms and 6-phytase for plants (Johnson and Tate 1969; Cosgrove 1969; Cosgrove 1970).

myo-Inositol hexakisphosphate is often the major phosphorus stock in plant seeds as 60–80 % of the organically bound phosphorus is present in the form of phytic acid (Posternak 1965; Lolas and Markakis 1977; Asada *et al.* 1969; Erdman 1979). Phytic acid plays other important roles in the plants: (*1*) energy store, (*2*) competition for ATP, (*3*) complexation of multivalent cations and (*4*) regulation of an inorganic phosphate level. For review on inositol phosphates see Cosgrove (1980) and Billington (1993).

On the other hand, as a component of all plant seeds phytic acid is considered to be an antinutritive part of human and animal diets because (*1*) it forms complexes with some multivalent metal

ions and thus interferes with the assimilation of important trace metals, (*2*) it binds to proteins and makes them more resistant to proteolytic digestion, and (*3*) phytate phosphorus is poorly available to monogastrics (because of phytase absence). Utilization of phytate phosphorus and increase of feed and food nutritive value can be well achieved by dietary phytase supplementation (Simell *et al.* 1989; Newman 1991).



Scheme 1.

Besides *myo*-inositol hexakisphosphate also lower phosphoric esters of *myo*-inositol (mono-, bis- and trisphosphate) occur frequently in the nature. They are important constituents of phospholipids in animal and plant tissues (Cason and Anderson 1938) and play a crucial role in signalling processes (Michell 1975; Berridge and Irvine 1984; Samanta *et al.* 1993; Dasgupta *et al.* 1996). These discoveries and subsequent intensive investigation of the cell signalling system caused increased the interest in and requirement for inositol phosphates. Billington (1993) exhaustively reviewed the current knowledge of chemical synthesis and biological significance of these compounds.

Phytase plays an important role in biochemistry of inositol phosphates and has been intensively studied. Now the interest of investigators has concentrated mostly to genetically increasing the production of this commercially interesting enzyme, especially of microbial extracellular phytases suitable for large-scale production.

Due to its selectivity, phytase can be used not only as feed additive but also for selective phytate hydrolysis leading to lower *myo*-inositol phosphates (Phillippy *et al.* 1987; *Wakamoto Pharmaceuticals* 1988; *Brocades* 1991*a*; *Mitsui Toatsu Chemicals* 1992; Blatný *et al.* 1994; Dvořáková *et al.* 1996). It thus draws an attention of both academic and industrial research. One of the most comprehensive review about phytase — mostly from plant sources — including purification, assay, and properties was written by Irwing (1980). Since then new knowledge has been accumulated which we aim to include into this review.

2 Sources and production of phytase

Phytase is widespread in nature, occurring in plants, microorganisms, as well as in some animals.

2.1 Microbial sources of phytase

Microbial phytase activity was most frequently detected in fungi. Shieh and Ware (1968) tested over 2000 microorganisms isolated from soil. Extracellular phytase activity was observed in only 30 isolates. All phytase producers were filamentous fungi, 28 of them belonged to the genus *Aspergillus*, one species belonging to *Penicillium* and on to *Mucor*. Phytase from the *A. niger* group was the most

active. Most isolates produced only intracellular phytase. Also other screenings (Howson and Davies 1983; Volfová *et al.* 1994) confirmed *A. niger* strains to be the best producers of extracellular phytase while bacterial cultures produced only intracellular enzyme.

Only *Bacillus subtilis* (Powar and Jagannathan 1967) and *B. subtillis* var. *natto* (Shimizu 1992; *Zenkoku Nogyo Kyodo* 1994) were shown to produce extracellular phytase.

The results of Shirai *et al.* (1994) on the ability of lactic acid bacteria to degrade phytic acid were ambiguous (coprecipitation of phytate and protein occurred as the pH of the medium decreased due to lactic acid production, indicating, that not only phytase activity may be responsible for phytase loss).

Phytase production by yeasts was also studied. *Saccharomyces cerevisiae* was found to produce phytase (Nayini and Markakis 1984) and further *Candida tropicalis*, *Torulopsis candida*, *Debaryomyces castelii*, *Kluyveromyces fragilis* and *Schwanniomyces castellii* were able to hydrolyze phytate (Lambrechts *et al.* 1992). *S. castellii*, which showed the highest extracellular phytase activity, was chosen for the further study (Segueilha *et al.* 1992; Lambrechts *et al.* 1993).

The published sources of microbial phytase are summarized in Table I.

Table I. Microbial sources of phytase

Phytase source	Localization[a]	Reference
Aerobacter aerogenes	IN	Greaves *et al.* 1967
Citrobacter freundii	IN	Delucca *et al.* 1992
Bacillus subtilis	EX	Powar and Jagannathan 1982
B. subtilis var. *natto*	EX	Shimizu 1992
Klebsiella sp.	IN	Shah and Parekh 1990
K. aerogenes	IN	Tambe *et al.* 1994
Escherichia coli	IN	Greiner *et al.* 1993
Aspergillus sp.	EX	Shieh and Ware 1968
Penicillium sp.	EX	Shieh and Ware 1968
Penicillium caseoicolum	EX	*Amano-Pharm* 1995
Mucor sp.	EX	Shieh and Ware 1968
A. niger NRRL 3135	EX	Shieh *et al.* 1969
A. niger	EX	Skowronski 1978; Volfová *et al.* 1994
A. terreus	EX	Yamada *et al.* 1968
A. carneus	EX	Ghareib 1990
A. oryzae	EX	Shimizu 1993
A. carbonarius	EX	Al Asheh and Duvnjak 1994*a–c*
A. fumigatus	EX	Pasamontes *et al.* 1997
Rhizopus oligosporus	EX	Sutardi and Buckle 1988
Neurospora sp.	EX	*Ichibiki* 1995
Candida tropicalis	EX	Lambrechts *et al.* 1992
Torulopsis candida	EX	Lambrechts *et al.* 1992
Debaryomyces castelii	EX	Lambrechts *et al.* 1992
Kluyveromyces fragilis	EX	Lambrechts *et al.* 1992
K. oxytoca MO-3	IN	Jareonkitmongkol *et al.* 1997
K. terrigena	IN	Greiner *et al.* 1997
Schwanniomyces castelii	EX	Lambrechts *et al.* 1992
Saccharomyces cerevisiae	EX	Nayini and Markakis 1984
Paramecium sp.	IN	Freund *et al.* 1992; van der Kaay and van Haastert 1995

[a]IN – intracellular, EX – extracellular.

2.2 *Production of microbial phytase*

Production of extracellular fungal phytase was induced by a limiting concentration of inorganic phosphate in the growth medium. A higher phosphate concentration inhibited phytase synthesis and this phenomenon was generally observed in all fungal producers (Shieh and Ware 1968). Regulation of the *Aspergillus niger* var. *ficuum* NRRL 3135 (further only *A. niger* NRRL 3135) phytase production was first described by Shieh *et al.* (1969) and the culture medium was subsequently optimized (Gibson 1987;

Han and Gallagher 1987; Han *et al.* 1987). Kujawski and Zyla (1992) observed a correlation of the ability of *A. niger* spp. to produce citric acid by the solid surface method with its ability to synthesize intracellular phytase and acid phosphatase. Extracellular *A. niger* phytase was produced during active cell growth (Volfová *et al.* 1994, 1997). Chelius and Wodzinski (1994) increased extracellular *A. niger* NRRL 3135 phytase activity by a method involving irradiation of cell suspension.

In contrast to the above mentioned fungal phytase, extracellular *B. subtillis* phytase was induced by phytate in a cultivation medium. It required Ca^{2+} ions for its activity (Powar and Jagannathan 1982). Also cytoplasmatic phytase from *Klebsiella terrigena* is induceable in the presence of phytate under carbon limitation (Greiner *et al.* 1997).

The activity of intracellular *E. coli* phytase was remarkably increased in the stationary phase and under anaerobic conditions (Greiner and Jany 1992; Greiner *et al.* 1993).

2.3 Plant phytase

Phytase occurs very frequently in the plant kingdom. Its activity was detected in many plant species such as wheat, rye, barley, pea, bean, soybean, maize, rice, white mustard, potato, radish, lettuce, spinach, grass, lilly pollen, *etc.*

A rapid increase of phytase activity was monitored in plant seeds during their germination (Chen and Pan 1977). Generally, it is assumed that during seed germination phytate, after decomposition by phytase, is utilized in the form of phosphate and inositol (Asada *et al.* 1969). Some authors ascribed the rise in phytase activity to *de novo* phytase synthesis during germination (Satirana and Bianchetti 1967; Mandal and Biswas 1970; Meyer *et al.* 1971) while others attribute it to a rise of an already existing phytase activity (Eastwood *et al.* 1969; Eastwood and Laidman 1971). Nevertheless, it was concluded that seeds contain both constitutive and germination-inducible phytases (Nayni and Markakis 1986).

Laboure *et al.* (1993) purified and characterized phytase from germinating maize seedlings, and cDNA coding for this phytase was cloned (Maugenest *et al.* 1997). This would allow the isolation of corresponding genes and the study of their regulation during germination.

2.4 Animal phytase

The first report on animal phytase in calf liver and blood was given by McCollum and Hart (1908). However, further search for mammal blood phytase was unsuccessful; phytase was detected only in the blood of lower vertebrates — birds, reptiles, fishes, batrachians, sea turtle (Rapoport *et al.* 1941).

Because intact phytate coming from cereals has adverse nutritional consequences for animals including man, the presence of phytase in the gastrointestinal tract of various animals was investigated. Phytate hydrolysis in the rat intestine was first noted by Patwardhan (1937) and attributed to phytase. Subsequently, other authors observed phytase activity in the intestine of pig and cow (Spitzer and Phillips 1945), sheep (Oto 1960). Pileggi (1959), Maddaiah *et al.* (1964) and Williams *et al.* (1985) attributed the intestinal phytase activity to the action of nonspecific alkaline phosphatases in the intestine. However, Bitar and Reinhold (1972) partially purified from the rat, chicken, calf and human intestines phytase different from the intestinal alkaline phosphatase. A suggestion that rat phytase and phosphatase from the small intestine are different enzymes was supported also by Rao and Ramakrishnan (1985) and Yang *et al.* (1991*a*,*b*) who purified phytase from the rat intestinal mucosa. However, intestinal phytase did not appear to be of a great significance in rats (Williams and Taylor 1985). An about 30 times lower phytase activity was found in the human small intestine as compared to that of rat. The highest activity was in the duodenum and the lowest one in the ileum. The normal human small intestine has a limited ability to digest undegraded phytates (Iqbal *et al.* 1994). It does not seem to play a significant role in phytate digestion, but dietary phytase may be an important factor in phytate hydrolysis (Frolich 1990).

The ruminants probably digest phytate through the action of phytase produced by the microbial flora in the rumen. The anaerobic gut fungi and bacteria are present in the microflora of ruminants and are responsible for the primary colonization of plant material within the rumen. The inorganic phosphate produced by splitting of phytate is utilized by both the microbial flora and ruminant host.

3 Purification and properties of phytase

3.1 Purification

Purification of phytase includes common biochemical techniques such as ammonium sulfate fractionation (Gibbins and Norris 1963), gel chromatography (Skowronski 1978; Ullah and Dischinger 1990; Dvořáková *et al.* 1997), ion-exchange (Nagai and Funahashi 1962; Lim and Tate 1973; Skowronski 1978; Ullah 1988*a*; Dvořáková *et al.* 1997) and affinity (Ullah 1988*a*) chromatography, and RP HPLC (Ullah 1988*b*).

3.2 Temperature and pH stability and optima

Phytases are generally thermostable enzymes active in a broad pH range. Extremly thermostable phytase from *A. fumigatus* able to withstand temperatures up to 100 °C over a period of 20 min with a loss only 10 % of the initial enzymic activity, was reported by Pasamontes *et al.* (1997). Most microbial phytases, especially those of fungal origin, show the main pH optimum between 4.5–5.5; some bacterial ones have a pH optimum at 6.5–7.5. Plant seeds phytases have been described to have usually pH optimum between 4.0–5.6. Recently, alkaline phytases having a pH optimum at 8 were extracted by a non-ionic detergent from legume seeds (Scott 1991). Another alkaline phytase with a pH optimum at 8 was found in mature lily pollen (Hara *et al.* 1985; Scott and Loewus 1986). pH and temperature optima of phytases from various sources are given in Table II.

Table II. Temperature and pH optima of some published phytases

Phytase source	pH	Temperature, °C	Reference
Aspergillus niger NRRL 3135	2.2; 5.0–5.5	58	Ullah and Gibson 1987
A. niger IIIAn/8	2.7; 5.5	–	Skowronski 1978
A. niger 92	5.0	55	Dvořáková *et al.* 1997
A. oryzae	5.5	50	Shimizu 1993
A. terreus	4.5	70	Yamada *et al.* 1968
A. carneus	5.6	40	Ghareib 1990
A. carbonarius	4.7	53	Al Asheh and Duvnjak 1994*c*
Rhizopus oligosporus	4.5	55	Sutardi and Buckle 1988
Klebsiella sp.	6.0	37	Shah and Parekh 1990
K. aerogenes[a]	4.5	–	Tambe *et al.* 1994
K. aerogenes[b]	5.2	–	Tambe *et al.* 1994
K. oxytoca MO-3	5.0–6.0	55	Jareonkitmongkol *et al.* 1997
K. terrigena	5.0	58	Greiner *et al.* 1997
Penicillium caseoicolum	3.0	45	*Amano Pharmaceuticals* 1995
Neurospora sp.	5.0–6.0	60	*Ichibiki* 1995
Aerobacter aerogenes	4.0–5.0	45–50	Greaves *et al.* 1967
Citrobacter freundii	2.7; 5.0	52	Delucca *et al.* 1992
Escherichia coli	4.5	55	Greiner and Jany 1992
Bacillus subtilis	7.0–7.5	–	Powar and Jagannathan 1982
B. subtilis var. *natto*	6.0–6.5	60	Shimizu 1992
Schwanniomyces castelii	4.4	77	Segueilha *et al.* 1992
Wheat bran	5.0	–	Nagai and Funahashi 1962
Typha latifolia pollen	8.0	–	Hara *et al.* 1985
Soybean seeds	4.5–4.8	55	Gibson and Ullah 1988
Legume seeds	8.0	–	Scott 1991
Maize (germinated)	4.8	55	Laboure *et al.* 1993
Rat intestine	7.0–7.5	–	Bitar and Reinhold 1972
Human intestine	7.4	–	Bitar and Reinhold 1972
Chicken intestine	8.3	–	Bitar and Reinhold 1972
Calf intestine	8.6	–	Bitar and Reinhold 1972

[a]700 kDa. [b]10–13 kDa.

3.3 *Substrate specificity and kinetic parameters*

Phytases usually show a broad substrate specificity with the highest affinity for phytate. Only a few phytases have been described as highly specific for phytic acid — the phytase produced by *B. subtilis* (Powar and Jagannathan 1982) and alkaline phytase isolated from the lily pollen (Barrientos *et al.* 1994). The K_m values of some published phytases are given in Table III. Excess substrate causes inhibition of the enzyme. Ullah (1988*b*) found that *myo*-inositol-P_6 concentration exceeding 2 mmol/L was inhibitory. The product of the reaction — inorganic monophosphate — acts also as a weak inhibitor with K_i = 1.9 mmol/L.

Table III. K_m values of some published phytases for *myo*-inositol-P_6 (phytate), sodium salt

Phytase source	K_m mmol/L	Reference
Aspergillus niger IIIAn/8	0.48	Skowronski 1978
A. niger NRRL3135	0.04	Ullah and Gibson 1987
A. niger 92	0.44	Dvořáková *et al.* 1997
A. niger	0.39	Simell *et al.* 1989
A. carbonarius	0.35	Al Asheh and Duvnjak 1994c
Rhizopus oligosporus	0.15	Sutardi and Buckle 1988
Escherichia coli	0.13	Greiner and Jany 1992
Bacillus subtilis	0.04	Powar and Jagannathan 1982
B. subtilis var. *natto*	0.50	Shimizu 1992
Citrobacter freundii	0.25	Delucca *et al.* 1992
Klebsiella aerogenes[a]	0.06	Tambe *et al.* 1994
K. aerogenes[b]	0.11	Tambe *et al.* 1994
K. terrigena	0.30	Greiner *et al.* 1997
Schwanniomyces castelii	0.04	Segueilha *et al.* 1992
Phaseolus vulgaris (bean)	0.15	Gibbins and Norris 1963
Wheat bran	0.49	Nagai and Funahashi 1963
Typha latifolia pollen	0.02	Hara *et al.* 1985
Soybean seeds	0.05	Gibson and Ullah 1988
Maize	0.12	Laboure *et al.* 1993
Rat intestine	0.21	Yang *et al.* 1991*a*

[a]700 kDa. [b]10-13 kDa.

Phytase from *A. niger* NRRL 3135 was several times immobilized. Ullah and Cummins (1987) as well as Ullah and Phillippy (1988) immobilized phytase on fractogel, Ullah and Cummins (1988) used for phytase immobilization glutaraldehyde-activated silicate, and Dischinger and Ullah (1992) immobilized this enzyme onto cross-linked agarose. Kinetic characterization of the immobilized enzyme was performed and reaction products were characterized.

3.4 *Dephosphorylation of* myo-*inositol-*P_6 *by phytase*

As already mentioned, there are two classes of phytases, 6-phytase occurring in plants and 3-phytase found in fungi. The 3-phytase hydrolyzes the phosphate esters at the D-3 position and 6-phytase at the L-6 (or D-4) position. This is probably the main difference between microbial and plant phytases. However, alkaline phytase discovered and isolated from lily (*Lilium longiflorum*) pollen (Scott and Loewus 1986; Baldi *et al.* 1988) started to hydrolyze phytic acid at position D-5 with inositol 1,2,3-trisphosphate as the final product (Barrientos *et al.* 1994). Acid phytases are able to catalyze phytate dephosphorylation to lower *myo*-inositol phosphates (multiple isomers of IP_4, IP_3 and IP_2) by alternative pathways of IP_5 hydrolysis (Lim and Tate 1973), sometimes yielding free *myo*-inositol, whereas alkaline phytase is unable to catalyze dephosphorylation of IP_3 (Barrientos *et al.* 1994).

3.5 *Molecular properties*

Only a few phytases were purified to such homogeneity that their molar mass could be estimated. Table IV records the published molar mass of phytase including a number of subunits.

Table IV. Published molar mass of phytases from various sources

Phytase source	*M* kDa	Number of subunits	Reference
Aspergillus niger IIIAn/8	200	1	Skowronski 1978
A. niger NRRL3135	85–100	1	Ullah and Gibson 1987
A. niger 92	100	1	Dvořáková *et al.* 1997
A. terreus	214	6	Yamada *et al.* 1972
A. niger	80	1	Simell *et al.* 1989
Penicillium caseoicolum	60–81	1	*Amano Pharmaceuticals* 1995
Escherichia coli	42	1	Greiner *et al.* 1993
Bacillus subtilis	36.5	1	Powar and Jagannathan 1982
B. subtilis var. *natto*	36–38	1	Shimizu 1992
Klebsiella aerogenes	700 and 10–13	1	Tambe *et al.* 1994
K. terrigena	40	1	Greiner *et al.* 1997
Schwanniomyces castelii	490	1 + 3	Segueilha *et al.* 1992
Soybean seeds	119	2	Gibson and Ullah 1988
Maize	76	2	Laboure *et al.* 1993
Rat intestine	70 and 90	2	Yang *et al.* 1991*b*

An enzyme produced by *A. niger* NRRL 3135 is nowadays the most intensively studied among all phytases. *A. niger* NRRL 3135 native phytase with a molar mass of 85 kDa was found to be 27 % glycosylated. It contains a substantial proportion of N-linked mannose chains and galactose (Ullah 1988*b*). Amino acid analysis revealed a high content of aspartic acid, serine and threonine (Ullah 1988*a*). Ullah and Dischinger (1990) published the procedures for peptide mapping and purification of enzymically cleaved peptides generated from phytase. The complete primary structure of phytase was elucidated by Edman sequencing. The deglycosylated molecule is a 441-residue protein of 48.5 kDa, consisting of 37 % non-polar, 42 % polar, 11.5 % acidic, and 9.5 % basic amino acids (Ullah and Dischinger 1993*a*).

Chemical mapping of its active site demonstrated a sensitive Arg residue in this region (Ullah *et al.* 1991). Further investigation of *A. niger* var. *ficuum* phytase by a site-directed mutagenesis indicated that His and Trp are involved in the formation of the active catalytic centre, and it was concluded that Arg^{58}, Arg^{62}, His^{59} and Trp^{25} are the target residues (Ullah and Dischinger 1992). The function of disulfide bonds in *A. niger* var. *ficuum* phytase was showen to play an important role in the folding of fungal phytase and they are necessary for the enzymic structure and activity (Ullah and Mullaney 1996).

It is necessary to note that the *A. niger* NRRL 3135 phytase (EC 3.1.3.8) mentioned above has two pH optima (5.0 and 2.5), and was denoted phytase A (PhyA) by van Hartingsveldt *et al.* (1993). Another enzyme, originally referred to as pH 2.5 acid phosphatase (EC 3.1.3.2) by Ullah and Cummins (1987), was recently characterized as a potent phytase having a pH optimum at 2.5 (non-active at pH 5; Ullah and Phillippy 1994). At present, this enzyme is termed phytase B (PhyB); its primary structure was determined by Edman degradation and gene cloning (Ehrlich *et al.* 1993). The PhyB active site was investigated by Ullah and Dischinger (1993*b*). Comparison of amino acid sequences of PhyA and PhyB and two secreted yeast acid phosphatases (from *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*) showed an extensive homology and conservation of residues (Ullah and Dischinger 1995). The same sequences were also identified in bacterial and mammalian acid phosphatases (van Etten *et al.* 1991; Ostanin *et al.* 1992). Two different fungal PhyA phytases from *A. terreus* 9A-1 and *Myceliophtora thermophila* showed 60 % and 48 % identity, respectively, to the PhyA of *A. niger* (Mitchell *et al.* 1997). Also amino acid sequences of both fungi *Emericella nidulans* and *Talaromyces thermophilus* exhibited high identity to known phytases. Twenty one amino acids were identified to be conserved in fungal PhyA phytases and to be a part of the residues forming the substrate pocket (Pasamontes *et al.* 1997). On the other hand the amino acid sequence of the maize seedling phytase deduced from cDNA appears to be very different from those of *A. niger* phytase, however, a homologous region of 33 amino acids — probably the acceptor site for phosphate — was detected (Maugenest *et al.* 1997).

The crystal structure of PhyB from *A. niger* var. *ficuum* was successfully elucidated by Kostrewa *et al.* (1997). The enzyme has an α/β domain similar to that of rat acid phosphatase and an α domain with a new fold.

4 Molecular genetics

Due to the interest in the use of fungal phytase in feed applications, a program with the aim to improve its cost-effective industrial production has been initiated.

A strategy for the production of recombinant 3-phytase in *Aspergillus* sp. suggested by Conneely (1992) involved cloning of the phytase gene from *e.g. Aspergillus niger* var. *ficuum* or *Saccharomyces cerevisiae*, insertion of the gene into *Aspergillus* sp. using a multicopy expression vector (for the expression of multiple copies of the gene), and isolation of the recombinant phytase from the medium. Later, van den Hondel *et al.* (1994) published the general strategy for the overproduction of fungal proteins in *Aspergillus* sp., especially in *A. niger* and *A. oryzae*, comprising (*1*) isolation of the gene of the protein of interest, (*2*) generation of strains with multiple copies of the gene, (*3*) providing the gene with well characterized, strong constitutive or regulated gene expression signals and generation of strains containing multiple copies of such expression cassetes.

The phytase gene can be isolated by DNA hybridization with an oligonucleotide, or by PCR (*Brocades* 1991*a*).

Mullaney *et al.* (1991) isolated and identified, using the phytase amino acid sequence, a recombinant clone containing part of the *A. niger* var. *ficuum* phytase gene. Subsequently, the genes for PhyA (van Hartingsveldt *et al.* 1993) and for PhyB (Ehrlich *et al.* 1993) were cloned and sequenced. Expression of multiple copies of *phyA* in the transformants resulted in more than tenfold higher phytase levels as compared with the wild type strain. It was shown that the expression of *phyA* was controlled at the level of mRNA accumulation in response to inorganic phosphate levels (van Hartingsveldt *et al.* 1993).

With the aim to improve the cost-effective phytase production, the gene *phy* from *A. niger* var. *awamori* ALKO243 encoding phytase was also cloned and sequenced; reintroduction of the cloned *phy* gene resulted in several-fold overproduction of phytase (Piddington *et al.* 1993). However, as the expression of this gene is strongly phosphate repressed (Han and Gallagher 1987), the simple gene dosage increase is not sufficient for raising the enzyme production to a level relevant for industrial application. It was suggested that more significant overexpression may be achieved by replacement with strong, phosphate-resistant promoters (Piddington *et al.* 1993).

Intensive genetic investigation and strong interest of industry in phytase yielded in a number of patents in the last years. Vectors comprising a DNA sequence encoding 3-phytase preferably derived from *A. niger* var. *ficuum* or *A. niger* were expressed in *A. niger* var. *ficuum*, *A. niger*, *A. awamori*, *A. oryzae*, *Trichoderma resei*, *Mucor miehei*, *Kluyveromyces lactis*, *S. cerevisiae*, *B. subtilis* and *B. licheniformis*, and the enzyme was secreted from the host cells (*Brocades* 1991*a*). Further, the phytase gene from *A. niger* var. *ficuum* was fused with expression signals of the *P. chrysogenum* gene and the plasmid pPYH01 was constructed (*Brocades* 1993). A DNA sequence encoding *A. niger* phytase was also expressed in *A. foetidus* (Berka *et al.* 1995*a*) and in *A. japonicus* var. *aculeatus* (Berka *et al.* 1995*b*). To achieve the overproduction of synergistic phytate degrading enzymes, DNA sequences encoding both phytase and acid phosphatase were cloned and expressed in *A. niger* (*Panlabs*, *Alko* 1994) and in *Trichoderma* sp. host cells (*Alko* 1994).

Two- to six-fold increase in phytase activity was obtained by Moore *et al.* (1995) after *S. cerevisiae* and *A. niger* acid phosphatase expression in *A. oryzae*. They used the enzyme for phytic acid removal from a feedstuff.

Patents were issued describing an expression system to enhance secretion of exoproteins including phytase in Gram-positive bacteria, *e.g. Bacillus subtilis*, *B. alkalophilus*, *B. amyloliquefaciens*, *B. coagulans*, *B. circulans*, *B. lautus* and *B. thuringiensis* (*Finnish Nat. Publ. Health Inst.* 1994). Phillippy and Mullaney (1997) expressed the gene (*phyA*) for *A. niger* phytase with optimum at pH 5.5 and 2.2 in *E. coli* under control of the T7*lac* promoter, however, recombinant phytase displayed a single pH optimum at pH 5.1, and was irreversibly denaturated at pH 2.0.

Phytase production is also included in the new concept in plant biotechnology using transgenic plant engineering for pharmaceutical protein and industrial enzyme production (Pen *et al.* 1994; Whitelam 1995; Day 1996). Seeds obtained from transgenic plants containing enhanced levels of a heterologous enzyme are used to catalyze the desired reactions (*Brocades* 1991*c*). The method was developed to produce transgenic plants with an enhanced level of 3-phytase using transformation of plant host with an expression vector containing fungal phytase DNA — preferably obtained from *A. niger* NRRL 3135, *A. niger*, *A. awamori* and *A. nidulans* — operably linked to regulatory sequences (*Brocades* 1991*b*).

The phytase and acid phosphatase genes from *Aspergillus* spp., especially *A. niger* var. *ficuum*, *A. niger* or *A. terreus*, were expressed in transgenic plants with the aim to obtain a thermostable synergistic enzyme mixture for phytic acid hydrolysis to improve feedstuff digestibility (*Avewe* 1994).

Active phytase from *A. niger* was engineered in tobacco (*Nicotiana tabacum*) seeds using a binary vector pMOG413 containing a chimeric phytase gene encoding the tobacco pathogen-related S signal peptide (Cornelissen *et al.* 1986) responsible for protein secretion (Sijmons *et al.* 1991), and cDNA fragment encoding the mature *A. niger* phytase gene (van Hartingsveldt *et al.* 1993). Phytase was expressed as 1 % of the soluble protein in mature seeds of transgenic plants (Pen *et al.* 1993, 1994).

Recently, Verwoerd *et al.* (1995) reported a high-level accumulation of biologically active phytase from *A. niger* in transgenic tobacco leaves. Phytase cDNA was constitutively expressed in transgenic tobacco plants and secretion of the enzyme to the extracellular fluid was established using the above mentioned signal sequence from the tobacco pathogen-related protein S. Biologically active phytase was localized extracellularly and accumulated in leaves up to 14.4 % of total soluble protein during plant maturation (Verwoerd *et al.* 1995). Li *et al.* (1997) inserted phytase gene from *A. niger* into soybean transformation plasmids under control of constitutive and seed specific promotors, and with a plant signal sequence. Soybean cell cultures secreted after that an active recombinant phytase.

5 Application

There are two main areas where phytase can be used. The first one is phytate elimination in feed and food industries, and the second one a preparation of special *myo*-inositol phosphates as tools for biochemical investigation.

The main reason for removal of phytate from food and feed is its undigestibility for monogastric animals including man (Simell *et al.* 1989). Undegraded phytate together with part of additional inorganic phosphate passing through the intestinal tract is excreted and causes environmental problems. The most important problem of today is discharging of phosphates into water streams leading to eutrophization, especially in areas with intensive livestock production (mostly chickens and pigs).

A further reason for phytate removal is complexation of multivalent metal ions which interferes with the assimilation of important trace metals such as Fe, Zn and Ca by humans and animals, causing their deficiency.

Phytate also inhibits digestive enzymes (α-amylase, pepsin and trypsin), and makes proteins more resistant to proteolytic digestion. This may restrict the use of protein preparations in food industry.

All the above problems can be solved by phytate hydrolysis using phytase (Tamminga 1990; Whitaker 1990; *Brocades* 1991*a*–*c*; Lyons 1992; Zyla 1992; Sanders 1993; Souppe 1995).

5.1 Feed application

Phytase, releasing phosphate from phytate, reduces the need of feed supplementation by phosphorus. Degradation of *myo*-inositol phosphates yields also *myo*-inositol, an important growth factor, and trace minerals become available. This improves the digestibility and therefore the nutritive value of the feed, and lowers the level of excreted phosphorus.

There are two basic ways how to use phytase in feeds. The first possibility is replacement of inorganic phosphorus supplementation with phytase. However, as the reaction conditions (pH, temperature, moisture, incubation time) in the animal stomach or intestine are not optimal, the second method of phytase use — feed pretreatment — becomes more attractive (Simell *et al.* 1989).

The possibility of increasing of phytase activity in the seedlings was considered using genetic material encoding phytases (*Brocades* 1991*a*–*c*).

The *Alko Co.* (Finland) as well as *Alltech* (USA) and *BASF* (USA) started the industrial scale production of phytase marketed under the names Finase, Allzyme Phytase, and Natuphos, respectively, and successfully utilized it in feed applications. Finase added to a corn–soybean pig diet converted approximately one-third of the unavailable phosphorus to an available form (Cromwell *et al.* 1993). In a similar way, experiments with Allzyme and Natuphos addition to a pig and chicken diet indicated also that phytase is efficacious in improving the bioavailability of phytate phosphorus for pigs (Cromwell *et al.* 1995*a*,*b*; Yi *et al.* 1996; O'Quinn *et al.* 1997) and broilers (Yi *et al.* 1997).

Not only experiments with pigs (Simons *et al.* 1990; Jongbloed *et al.* 1992; Lei *et al.* 1993*a*,*b*; Cromvell *et al.* 1993; Young *et al.* 1993; Ketaren *et al.* 1993; Pallauf 1994; Han *et al.* 1997; Murry *et al.*

1997) but also trials with chickens (Simons *et al.* 1990; Richter 1993; Edwards 1993; Perney *et al.* 1993; Young *et al.* 1993; Broz *et al.* 1994; Roberson and Edwards 1994; Biehl 1995; Kornegay *et al.* 1996; Qian *et al.* 1996, 1997; Biehl and Baker 1997; Zobač *et al.* 1997) confirmed the possibility to replace inorganic phosphorus supplementation by the use of microbial phytase in a phytate-rich diet for monogastric animals.

Besides increased phytate phosphorus availability, also enhanced digestibility of amino acids (Ketaren 1993; Mroz *et al.* 1994), and increased utilization of calcium (Mroz *et al.* 1994), magnesium (Pallauf *et al.* 1994) and zinc (Lei *et al.* 1993c; Pallauf *et al.* 1994; Roberson and Edwards 1994; Adeola *et al.* 1995) by pigs, chickens (Sebastian *et al.* 1996; Yi *et al.* 1996) and rats (Rimbach and Pallauf 1992, 1993; Rimbach *et al.* 1995) fed phytase-supplemented corn–soybean meal were observed. On the other hand, calcium in the diet greatly reduced the efficiency of phytase supplements (Sandberg 1993; Lei *et al.* 1994; Qian *et al.* 1996). Effects of *A. niger* phytase supplements on the digestibility and utilization of dietary phosphorus originating from plants were studied in experiments with rainbow trout and found positive (Rodehutscord *et al.* 1995).

Canola meal, used as a feedstuff for livestock and fowl, was successfully dephytinizated by *A. niger* NRRL 3135 in a solid-state fermentation (Nair and Duvnjak 1990; Nair *et al.* 1991; Ebune *et al.* 1995). Similarly, *A. niger* var. *ficuum*, *A. niger* (Zyla and Koreleski 1993) and *A. carbonarius* phytases (Al Asheh and Duvnjak 1994*a,b*, 1995*a,b*) were used to reduce the phytic acid content in rape and canola meals in solid-state fermentation processes aiming at increasing phytate phosphorus bioavailability and the nutritive value of the feed. Segueilha *et al.* (1993) removed phytic acid in wheat bran and glandless cotton flour using phytase from the yeast *S. castelii*.

Larsen (1993) achieved dephytinization of a rat diet high in both plant phytate and phytase by its wetting. The phytate content in feed decreased progressively with the time of soaking, and the absorption and retention of minerals and trace elements were significantly improved in balance studies.

5.2 *Food application*

Increased dietary consumption of cereal fibers, legumes and soy protein isolates results in an increased intake of phytate. Vegetarians eating mostly whole grain products and extruded cereals, elderly people consuming unbalanced food with a lot of cereals, people in undeveloped countries who eat unleavened bread, and babies which eat soy based infant formulas take in large amounts of phytate (Simell *et al.* 1989).

Since phytate binds to proteins, protein isolates from soy beans are rich in phytate. Simell *et al.* (1989) used Finase S phytase for the preparation of phytate-free soy protein isolates being more soluble at low pH (pH 3) than the control protein. The Finase phytase can also be used in the production of phytate-free soybean milk. Anno *et al.* (1985) eliminated phytate from soybean milk before its consumption using wheat phytase from *Sigma*, and Khare *et al.* (1994) successfully used immobilized wheat phytase for hydrolysis of soy milk phytate.

Cereal phytate from raw cereals is largely digested by humans, apparently because of a dietary phytase. When the dietary phytase is inactivated during extrusion cooking, the phytate digestion is very poor. Undigested phytate in the small intestine negatively affects the absorption of zinc, magnesium and iron. For improvement of mineral availability, degradation of inositol hexakis- and pentakisphosphates is essential (Sandberg *et al.* 1988). Addition of *A. niger* phytase to the flour containing wheat bran increased iron absorption in humans (Sandberg *et al.* 1996). The use of phytase, very easily integrated into the existing process, was suggested for producing low-phytin bread (Simell *et al.* 1989).

Phytic acid has, however, also positive effects. It exerts an antineoplastic effect in animal models of both colon and breast carcinomas. The presence of undigested phytate in the colon may protect it against the development of colonic carcinoma (Igbal *et al.* 1994).

5.3 *Preparation of* myo-*inositol phosphates*

The increasing interest in inositol phosphates and phospholipids which play a pivotal role in transmembrane signalling and mobilization of calcium from intracellular reserves resulted in the need of various inositol phosphate preparations (Billington 1993). Siren (1986) described the preparation of D-*myo*-inositol 1,2,6-trisphosphate, D-*myo*-inositol 1,2,5-trisphosphate, L-*myo*-inositol 1,3,4-trisphosphate and *myo*-inositol 1,2,3-trisphosphate by enzymic hydrolysis of phytic acid using *S. cerevisiae* phytase. Also a method for production of inositol 1,4,5-trisphosphate (calcium immobilizing substance) was developed using 6-phytase or alkaline phosphatase to hydrolyze phytic acid or its salts (*Wakamoto Pharmaceuticals* 1988). Greiner and Konietzny (1996) prepared inositol 1,2,3,4,5-pentakisphosphate,

inositol 2,3,4,5-tetrakisphosphate, inositol 2,4,5-trisphosphate and inositol 2,5-bisphosphate using immobilized phytase from *E. coli*. Inositol phosphate derivatives can be used as enzyme stabilizers (Siren 1986), enzyme substrates for metabolic investigation, as enzyme inhibitors and therefore potential drugs, and as chiral building blocks (Laumen and Ghisalba 1994).

Chemical syntheses of inositol phosphates were summarized by Billington (1993). They very often include protection and deprotection steps, and are performed at extreme temperatures and pressures. Phytase, converting phytic acid to lower *myo*-inositol phosphate derivatives or free *myo*-inositol and inorganic phosphate, was suggested for the industrial production of inositol or inositol phosphates (*Brocades* 1991*a*; *Mitsui Toatsu Chemicals* 1992). Among the advantages of enzymic hydrolysis belong its stereospecificity and mild reaction conditions.

6 Conclusions and future prospects

Phytase, which plays an important role in biochemistry of inositol phosphates, has been both extensively and intensively studied. A lot of sources of this enzyme were subsequently discovered. The physico-chemical and biochemical properties of some of the described phytases seem to be well known, especially the phytase produced by *Aspergillus* spp. Investigation in the field of molecular genetics yielded the possibility to overproduce this enzyme. This program aiming at improving the industrial production of phytase on a cost-effective level, will continue because the efforts to use phytase in feed and food industries have been successful. Also the preparation of inositol phosphates using phytase to hydrolyze phytic acid seems to be interesting. As lower inositol phosphates and phospholipids play important role in transmembrane cell signalling and calcium mobilization from intracellular stock, the investigation of the potential role of phytase in this mechanism would be interesting.

The author would like to thank Dr. O. Volfová for many discussions and to Dr. M. Novák for critical reading of the manusript. The support of grant no. 203/69/1267 from the *Grant Agency of the Czech Republic* is gratefully acknowledged.

REFERENCES

ADEOLA O., LAWRENCE B.V., SUTTON A.L., CLINE T.R.: Phytase-induced changes in mineral utilization in zinc-supplemented diets for pigs. *J.Anim.Sci.* **73**, 3384–3391 (1995).

AL ASHEH S., DUVNJAK Z.: Effect of glucose concentration on the biomass and phytase productions and the reduction of the phytic acid content in canola meal by *Aspergillus carbonarius*. *Biotechnol.Prog.* **10**, 353–359 (1994*a*).

AL ASHEH S., DUVNJAK Z.: The effect of surfactants on the phytase production and the reduction of the phytic acid content in canola meal by *Aspergillus carbonarius* during a solid-state fermentation process. *Biotechnol.Lett.* **16**, 183–188 (1994*b*).

AL ASHEH S., DUVNJAK Z.: Characteristics of phytase produced by *Aspergillus carbonarius* NRC 401121 in canola meal. *Acta Biotechnol.* **14**, 223–233 (1994*c*).

AL ASHEH S., DUVNJAK Z.: Phytase production and decrease of phytic acid content in canola meal by *Aspergillus carbonarius* in solid-state fermentation. *World J.Microbiol.Biotechnol.* **11**, 228–231 (1995*a*).

AL ASHEH S., DUVNJAK Z.: The effect of phosphate concentration on phytase production and the reduction of phytic acid content in canola meal by *Aspergillus carbonarius* during a solid-state fermentation process. *Appl.Microbiol.Biotechnol.* **43**, 25–30 (1995*b*).

Alko: Phytate-degrading enzyme, *e.g.* phytase and acid phosphatase over-production. *Pat. WO* 9 403 612 (1994).

Amano Pharmaceuticals: Novel phytase. *Japan Pat.* 07 067 635 (1995).

ANNO T., NAKANISHI K., MATSUNO R., KAMIKUBO T.: Enzymatic elimination of phytate in soybean milk. *J.Japan Soc.Food Sci. Technol.* **32**, 174–180 (1985).

ASADA K., TANAKA K., KASAI Z.: Formation of phytic acid in cereal grains. *Ann.N.Y.Acad.Sci.* **165**, 801–814 (1969).

Aveve: Synergistic enzyme composition for hydrolyzing phytic acid in feed. *Pat. EP* 619 369 (1994).

BALDI B.G., SCOTT J.J., EVERARD J.D., LOEWUS F.A.: Localization of constitutive phytases in lily pollen and properties of the pH 8 form. *Plant Sci.* **56**, 137–147 (1988).

BARRIENTOS L., SCOTT J.J., MURTHY P.P.: Specificity of hydrolysis of phytic acid by alkaline phytase from lily pollen. *Plant Physiol.* **106**, 1489–1495 (1994).

BERKA R.M., YODER W., TAKAGI S., BOOMINATHAN K.C. (*Novo-Nordisk-Biotech*): *Aspergillus foetidus* cells expressing heterologous enzyme. The fungal promotor is *Aspergillus niger* phytase. *Pat. WO* 9 515 390 (1995*a*).

BERKA R.M., YODER W., TAKAGI S., BOOMINATHAN K.C. (*Novo-Nordisk-Biotech*): *Aspergillus japonicus*-type cells expressing heterologous enzyme. The fungal promotor is *Aspergillus niger* phytase. *Pat. WO* 9 515 391 (1995*b*).

BERRIDGE M.J., IRVINE R.F.: Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature* **312**, 315–321 (1984).

BIEHL R.R., BAKER D.H.: Microbial phytase improves amino acid utilization in young chicks fed diets based on soybean meal but not diets based on peanut meal. *Poultry Sci.* **76**, 355–360 (1997).

BIEHL R.R., BAKER D.H., DELUCA H.F.: 1α-Hydroxylated cholecalciferol compounds act additively with microbial phytase to improve phosphorus, zinc and manganese utilization in chicks fed soy-based diets. *J.Nutr.* **125**, 2407–2416 (1995).

BILLINGTON D.C.: *The Inositol Phosphates. Chemical Synthesis and Biological Significance.* Verlag Chemie, Weinheim 1993.

BITAR K., REINHOLD J.G.: Phytase and alkaline phosphatase activities in intestinal mucosae of rat, chicken, calf, and man. *Biochim.Biophys.Acta* **268**, 442–452 (1972).

BLATNÝ P., KVASNIČKA F., KRINDLER E.: Time course of formation of inositol phosphates during enzymatic hydrolysis of phytic acid (*myo*-inositol hexaphosphoric acid) by phytase determined by capillary isotachophoresis. *J.Chromatogr. A* 679, 345–348 (1994).

Brocades: DNA sequence encoding phytase. *Pat. EP* 420 358 (1991*a*).

Brocades: Production of phytase in transgenic plant or plant organ. *Pat. EP* 449 375 (1991*b*).

Brocades: Use of seed containing heterologous recombinant enzyme, *e.g.* phytase, α-amylase from transgenic plant. *Pat. EP* 449 376 (1991*c*).

Brocades: A gene expression and protein secretion vector system for a fungus. *Pat. EP* 549 062 (1993).

BROZ J., OLDALE P., PERRIN-VOLTZ A.H., RYCHEN G., SCHULZE J., NUNES C.S.: Effect of supplemental phytase on performance and phosphorus utilization in broiler chickens fed a low phosphorus diet without addition of inorganic phosphates. *Brit.Poultry Sci.* 35, 273–280 (1994).

CASON J., ANDERSON R.J.: The chemistry of the lipids of tubercle bacilli. LVI. The wax of the bovine tubercle bacillus. *J.Biol. Chem.* 126, 527–541 (1938).

CHELIUS M.K., WODZINSKI R.J.: Strain improvement of *Aspergillus niger* for phytase production. *Appl.Microbiol.Biotechnol.* 41, 79–83 (1994).

CHEN L.H., PAN S.H.: cited in J.V. Erdman Jr.: Oilseed phytates: nutritional implications. *J.Am.Oil Chem.Soc.* 56, 739–741 (1979).

CONNEELY O.M.: From DNA to feed conversion: using biotechnology to improve enzyme yields and livestock performance. *Biotechnol.Feed Ind.* 57–66 (1992).

CORNELISSEN B.J.C., HOOFT VAN HUYSDUYNEN R.A.M., BOL J.F.: A tobacco mosaic virus-induced tobacco protein is homologous to the sweet-tasting protein thaumatin. *Nature* 321, 531–532 (1986).

COSGROVE D.J.: Ion-exchange chromatography of inositol polyphosphates. *Ann.N.Y.Acad.Sci.* 165, 677–686 (1969).

COSGROVE D.J.: Inositol phosphatases of microbial origin. Inositol phosphate intermediates in the dephosphorylation of the hexaphosphates of *myo*-inositol, *scyllo*-inositol, and D-*chiro*-inositol by a bacterial (*Pseudomonas* sp.) phytase. *Austral.J.Biol.Sci.* 23, 1207–1220 (1970).

COSGROVE D.J.: *Inositol Phosphates. Their Chemistry, Biochemistry and Physiology.* Elsevier, Amsterdam 1980.

CROMWELL G.L., COFFEY R.D., MONEGUE H.J., RANDOLPH J.H.: Efficacy of low-activity, microbial phytase in improving the bioavailability of phosphorus in corn–soybean meal diets for pigs. *J.Anim.Sci.* 73, 449–456 (1995*a*).

CROMWELL G.L., COFFEY R.D., PARKER G.R., MONEGUE H.J., RANDOLPH J.H.: Efficacy of a recombinant-derived phytase in improving the bioavailability of phosphorus in corn–soybean meal diets for pigs. *J.Animal Sci.* 73, 2000–2008 (1995*b*).

CROMWELL G.L., STAHLY T.S., COFFEY R.D., MONEGUE H.J., RANDOLPH J.H.: Efficacy of phytase in improving the bioavailability of phosphorus in soybean meal and corn–soybean meal diets for pigs. *J.Animal Sci.* 71, 1831–1840 (1993).

DASGUPTA S., DASGUPTA D., SEN M., BISWAS S., BISWAS B.B.: Interaction of *myo*-inositol trisphosphate phytase complex with the receptor for intracellular Ca^{2+} mobilization in plants. *Biochemistry* 35, 4994–5001 (1996).

DAY P.R.: Genetic modification of plants: significant issues and hurdles to success. *Am.J.Clin.Nutr.* 63, 651S–656S (1996).

DELUCCA A.J., DISCHINGER C., ULLAH A.H.J.: Identification of a phytase from *Citrobacter freundii*. *Abstr. Gen. Meet. Am. Soc. Microbiol.* 92 Meet. 385 (1992).

DISCHINGER H.C. Jr., ULLAH A.H.J.: Immobilization of *Aspergillus ficuum* phytase by carbohydrate moieties onto cross-linked agarose. *Ann.N.Y.Acad.Sci.* 672, 583–587 (1992).

DVOŘÁKOVÁ J., KOPECKÝ J., HAVLÍČEK V., KŘEN V.: *myo*-Inositol phosphates produced by microbial phytase. *15th Congr. Biochemistry*, Olomouc (Czech Republic) 1996; *Chem.listy* 90, 604–605 (1996).

DVOŘÁKOVÁ J., VOLFOVÁ O, KOPECKÝ J.: Characterization of phytase produced by *Aspergillus niger*. *Folia Microbiol.* 42, 349–352 (1997).

EASTWOOD D., LAIDMAN D.L.: The metabolization of macronutrient elements in the germinating wheat grain. *Phytochemistry* 10, 1275–1284 (1971).

EASTWOOD D., TAVENER R.J.A., LAIDMAN D.L.: Induction of lipase and phytase activities in the aleurone tissue of germinating wheat grains. *Biochem.J.* 113, Proc. Biochem. Soc., p. 32 (1969).

EBUNE A., AL ASHEH S., DUVNJAK Z.: Production of phytase during solid-state fermentation using *Aspergillus ficuum* NRRL 3135 in canola meal. *Bires.Technol.* 53, 7–12 (1995).

EDWARDS H.M. Jr.: Dietary 1,25-dihydroxycholecalciferol supplementation increases natural phytate phosphorus utilization in chickens. *J.Nutr.* 123, 567–577 (1993).

EHRLICH K.C., MONTALBANO B.G., MULLANEY E.J., DISCHINGER H.C. Jr., ULLAH A.H.: Identification and cloning of a second phytase gene (*phyB*) from *Aspergillus niger* (*ficuum*). *Biochem.Biophys.Res.Commun.* 195, 53–57 (1993).

ERDMAN J.W. Jr.: Oilseed phytates: nutritional implications. *J.Am.Oil Chem.Soc.* 56, 736–741 (1979).

Finnish National Public Health Institute: Gram-positive bacterial expression system for enhanced secretion of exoproteins. *Pat. WO* 9 419 471 (1994).

FREUND W.D., MAYR G.W., TIETZ C., SCHULTZ J.E.: Metabolism of inositol phosphates in the protozoan *Paramecium*. Characterization of a novel inositol hexakisphosphate-dephosphorylating enzyme. *Eur.J.Biochem.* 207, 359–367 (1992).

FROLICH W.: Chelating properties of dietary fiber and phytate. The role for mineral availability. *Adv.Exp.Med.Biol.* 270, 83–93 (1990).

GHAREIB M.: Biosynthesis, purification and some properties of extracellular phytase from *Aspergillus carneus*. *Acta Microbiol. Hung.* 37, 159–164 (1990).

GIBSON D.M.: Production of extracellular phytase from *Aspergillus ficuum* on starch media. *Biotechnol.Lett.* 9, 305–310 (1987).

GIBSON D.M., ULLAH A.H.: Purification and characterization of phytase from cotyledons of germinating soybean seeds. *Arch. Biochem.Biophys.* 260, 503–513 (1988).

GIBBINS M.P., NORRIS F.W.: Phytase and acid phosphatase in the dwarf bean, *Phaseolus vulgaris*. *Biochem.J.* 86, 67–71 (1963).

GREAVES M.P., ANDERSON G., WEBLEY D.M.: The hydrolysis of inositol phosphates by *Aerobacter aerogenes*. *Biochim.Biophys. Acta* 132, 412–418 (1967).

GREINER R., HALLER E., KONIETZNY U., JANY K.D.: Purification and characterization of a phytase from *Klebsiella terrigena*. *Arch.Biochem.Biophys.* 341, 201–206 (1997).

GREINER R., JANY K.D.: The reduction of phytate in animal feedstuffs by an *Escherichia coli* phytase. *Dechema-Biotechnol. Conf.* 5, Pt. B, 829–832 (1992).

GREINER R., KONIETZNY U., JANY K.D.: Purification and characterization of two phytases from *Escherichia coli*. *Arch.Biochem. Biophys.* 303, 107–113 (1993).

GREINER R., KONIETZNY U.: Construction of a bioreactor to produce special breakdown products of phytate. *J.Biotechnol.* 48, 153–159 (1996).

HAN Y.W., GALLAGHER D.J.: Phosphatase production by *Aspergillus ficuum*. *J.Ind.Microbiol.* 1, 295–301 (1987).
HAN Y.W., GALLAGHER D.J., WILFRED A.G.: Phytase production by *Aspergillus ficuum* on semisolid substrate. *J.Ind.Microbiol.* 2, 195–200 (1987).
HAN Y.W., YANG F., ZHOU A.G., MILLER E.R., KU P.K., HOOBERG M.G., LEI X.G.: Supplemental phytases of microbial and cereal sources improve dietary phytate phosphorus utilization by pigs from weaning through finishing. *J.Animal Sci.* 75, 1017–1025 (1997).
HARA A., EBINA S., KONDO A., FUNAGUMA T.: A new type of phytase from pollen of *Typha latifolia* L. *Agric.Biol.Chem.* 49, 3539–3544 (1985).
HOWSON S.J., DAVIS R.J.: Production of phytate-hydrolysing enzyme by fungi. *Enzyme Microbiol.Technol.* 5, 377–382 (1983).
Ichibiki: Phytase and its preparation. *Japan Pat.* 07 059 562 (1995).
IGBAL T.H., LEWIS K.O., COOPER B.T.: Phytase activity in the human and rat small intestine. *Gut* 35, 1233–1236 (1994).
IRVING G.C.J.: Phytase, in D.J. Cosgrove: *Inositol Phosphates. Their Chemistry, Biochemistry and Physiology*. Elsevier, Amsterdam 1980.
IUPAC–IUB Enzyme Nomenclature Recommendation Supplement 1: Corrections and additions (1975). *Biochim.Biophys.Acta* 429, 1 (1975).
JAREONKITMONGKOL S., OHYA M., WATANABE R., TAKAGI H., NAKAMORI S.: Partial purification of phytase from a soil isolate bacterium, *Klebsiella axytoca* MO-3. *J.Ferment.Bioeng.* 83, 393–394 (1997).
JOHNSON L.F., TATE M.E.: The structure of *myo*-inositol pentaphosphates. *Ann.A.N.Acad.Sci.* 165, 526–532 (1969).
JONGBLOED A.W., MROZ Z., KEMME P.A.: The effect of supplementary *Aspergillus niger* phytase in diets for pigs on concentration and apparent digestibility of dry matter, total phosphorus, and phytic acid in different sections of the alimentary tract. *J.Animal Sci.* 70, 1159–1168 (1992).
KETAREN P.P., BATTERHAM E.S., DETTMANN E.B., FARRELL D.J.: Phosphorus studies in pigs. 3. Effect of phytase supplementation on the digestibility and availability of phosphorus in soy-bean meal for grower pigs. *Brit.J.Nutr.* 70, 289–311 (1993).
KHARE S.K., JHA K., GUPTA M.N.: Entrapment of wheat phytase in polyacrylamide gel and its application in soy milk phytate hydrolysis. *Biotechnol.Appl.Biochem.* 19, 193–198 (1994).
KORNEGAY E.T., DENBOW D.M., YI Z., RAVINDRAN V.: Response of broilers to graded levels of microbial phytase added to maize–soybean-meal-based diets containing three levels of nonphytate phosphorus. *Brit.J.Nutr.* 75, 839–852 (1996).
KOSTREWA D., GRUNINGER LEITCH F., D'ARCY A., BROGER C., MITCHELL D., VAN LOON A.P.: Crystal structure of phytase from *Aspergillus ficuum* at 2.5 Å resolution. *Nat.Struct.Biol.* 4, 185–190 (1997).
KUJAWSKI M., ZYLA K.: Relationship between citric acid production and accumulation of phytate-degrading enzymes in *Aspergillus niger* mycelia. *Acta Microbiol.Polon.* 41, 187–191 (1992).
LABOURE A.M., GAGNON J., LESCURE A.M.: Purification and characterization of a phytase (*myo*-inositol hexakisphosphate phosphohydrolase) accumulated in maize (*Zea mays*) seedlings during germination. *Biochem.J.* 295, 413–419 (1993).
LAMBRECHTS C., BOZE H., MOULIN G., GALZY P.: Utilization of phytate by some yeasts. *Biotechnol.Lett.* 14, 61–66 (1992).
LAMBRECHTS C., BOZE H., SEGUEILHA L., MOULIN G., GALZY P.: Influence of culture conditions on the biosyntheses of *Schwanniomyces castelii* phytase. *Biotechnol.Lett.* 15, 399–404 (1993).
LARSEN T.: Dephytinization of a rat diet. Consequences for mineral and trace element absorption. *Biol.Trace Elem.Res.* 39, 55–71 (1993).
LAUMEN K., GHISALBA O.: Preparative-scale chemo-enzymatic synthesis of optically pure D-*myo*-inositol 1-phosphate. *Biosci. Biotech.Biochem.* 58, 2046–2049 (1994).
LEI X.G., KU P.K., MILLER E.R., YOKOYAMA M.T.: Supplementing corn–soybean meal diets with microbial phytase linearly improves phytate phosphorus utilization by weanling pigs. *J.Animal Sci.* 71, 3359–3367 (1993*a*).
LEI X.G., KU P.K., MILLER E.R., YOKOYAMA M.T., ULLREY D.E.: Supplementing corn–soybean meal diets with microbial phytase maximizes phytate phosphorus utilization by weanling pigs. *J.Animal Sci.* 71, 3368–3375 (1993*b*).
LEI X.G., KU P.K., MILLER E.R., ULLREY D.E., YOKOYAMA M.T.: Supplemental microbial phytase improves bioavailability of dietary zinc to weanling pigs. *J.Nutr.* 123, 1117–1123 (1993*c*).
LEI X.G., KU P.K., MILLER E.R., YOKOYAMA M.T., ULLREY D.E.: Calcium level affects the efficacy of supplemental microbial phytase in corn–soybean meal diets of weanling pigs. *J.Animal Sci.* 72, 139–143 (1994).
LI J., HEGEMAN C.E., HANLON R.W., LACY G.H., DENBOW M.D., GRABAU E.A.: Secretion of active recombinant phytase from soybean cell-suspension cultures. *Plant Physiol.* 114, 1103–1110 (1997).
LIM P.E., TATE M.E.: The phytase. II. Properties of phytase fractions F_1 and F_2 from wheat bran and the *myo*-inositol phosphates produced by fraction F_2. *Biochim.Biophys.Acta* 302, 316–328 (1973).
LOLAS M., MARKAKIS P.: Phytase of navy beans. *J.Food Sci.* 42, 1094–1097 (1977).
LYONS T.P.: Strategy for the future: the role of biotechnology in the feed industry. *Biotechnol.Feed Ind.* 1–22 (1992).
MADDAIAH V.T., KURNICK A.A., REID B.L.: Phytic acid studies. *Proc.Soc.Exp.Biol.Med.* 115, 391–393 (1964).
MANDAL N.C., BISWAS B.B.: Metabolism of inositol phosphates. I. Phytase synthesis during germination in cotyledons of mung beans, *Phaseolus aureus*. *Plant Physiol.* 45, 4–7 (1970).
MAUGENEST S., MARTINEZ I., LESCURE A.M.: Cloning and characterization of a cDNA encoding a maize seedling phytase. *Biochem.J.* 322, 511–517 (1997).
MCCOLLUM E.V., HART E.B.: On the occurrence of a phytin-splitting enzyme in animal tissue. *J.Biol.Chem.* 4, 497–500 (1908).
MEYER H., MAYER A.M., HAREL E.: Acid phosphatases in germinating lettuce – evidence for partial activation. *Physiol.Plant.* 24, 95–101 (1971).
MICHELL R.H.: Inositol phospholipids and cell surface receptor function. *Biochim.Biophys.Acta* 415, 81–147 (1975).
MITCHELL D.B., VOGEL K., WEIMANN B.J., PASAMONTES L., VAN LOON A.P.: The phytase subfamily of histidine acid phosphatase: isolation of genes for two novel phytases from the *Aspergillus terreus* and *Myceliophthora thermophila*. *Microbiology* 143, 245–252 (1997).
Mitsui Toatsu Chemicals: *myo*-Inositol preparation. *Japan Pat.* 4 365 489 (1992).
MOORE E., HELLY V.R., CONNEELY O.M., WARD P.P., POWER R.F., HEADON D.R.: Molecular cloning, expression and evaluation of phosphohydrolases for phytate-degrading activity. *J.Ind.Microbiol.* 14, 396–402 (1995).
MROZ Z., JONGBLOED A.W., KEMME P.A.: Apparent digestibility and retention of nutrients bound to phytate complexes as influenced by microbial phytase and feeding in pigs. *J.Animal Sci.* 72, 126–132 (1994).
MULLANEY E.J., GIBSON D.M., ULLAH A.H.J.: Positive identification of a λ gt11 clone containing a region of fungal phytase gene by immunoprobe and sequence verification. *Appl.Microbiol.Biotechnol.* 35, 611–614 (1991).
MURRY A.C., LEWIS R.D., AMOS H.E.: The effect of microbial phytase in a pearl millet–soybean meal diet on apparent digestibility and retention of nutrients, serum mineral concentration, and bone mineral density of nursery pigs. *J.Animal Sci.* 75, 1248–1291 (1997).

NAGAI Y., FUNAHASHI S.: Phytase (*myo*-inositol-hexakisphosphate phosphohydrolase) from wheat bran. *Agric.Biol.Chem.* **26**, 794–803 (1962).

NAGAI Y., FUNAHASHI S.: Phytase from wheat bran. II. Successive dephosphorylation of *myo*-inositol hexaphosphate by wheat bran phytase. *Agric.Biol.Chem.* **27**, 619–624 (1963).

NAIR V.C., DUVNJAK Z.: Reduction of phytic acid content in canola meal by *Aspergillus ficuum* in solid-state fermentation process. *Appl.Microbiol.Biotechnol.* **34**, 183–188 (1990).

NAIR V.C., LAPLAMME J., DUVNJAK Z.: Production of phytase by *Aspergillus ficuum* and reduction of phytic acid content in canola meal. *J.Sci.Food Agric.* **54**, 356–365 (1991).

NAYINI N.R., MARKAKIS P.: The phytase of yeast. *Lebensm.Wiss.Technol.* **17**, 24–26 (1984).

NAYINI N.R., MARKAKIS P.: Phytases, in *Phytic Acid: Chemistry and Applications.* Pilatus Press, Mineapolis 1986.

NEWMAN K.: Phytase: the enzyme, its origin and characteristics: impact and potential for increasing phosphorus availability, pp. 169–177 in *Biotechnology in the Feed Industry*, Proc. Alltech's 7th Ann. Symp. (T.P. Lyons, Ed.). Alltech Technical Publications, Nicholasville (Kentucky) 1991.

O'QUINN P.R., KNABE D.A., GREGG E.J.: Efficacy of Natuphos in sorghum-based diets of finishing swine. *J.Animal Sci.* **75**, 1299–1307 (1997).

OSTANIN K., HARMS E., STEVIS P.E., KUCIEL R., ZHOU M.M., VAN ETTEN R.L.: Overexpression, site-directed mutagenesis, and mechanism of *Escherichia coli* acid phosphatase. *J.Biol.Chem.* **267**, 22830–22836 (1992).

OTO Y.: cited in K. Bitar, J.G. Reinhold: Phytase and alkaline phosphatase activities in intestinal mucosae of rat, chicken, calf, and man. *Biochim.Biophys.Acta* **268**, 442–452 (1972).

PALLAUF J., RIMBACH G., PIPPIG S., SCHINDLER B., HOHLER D., MOST E.: Dietary effect of phytogenic phytase and an addition of microbial phytase to a diet based on field beans, wheat peas and barley on the utilization of phosphorus, calcium, magnesium, zinc and protein in piglets. *Z.Ernährungswiss.* **33**, 128–135 (1994).

Panlabs, Alko: Nucleic acid encoding phytase and acid 2.5 phosphatase. *Pat. WO* 9 403 072 (1994).

PASAMONTES L., HAIKER M., HENRIQUEZHUBCAS M., MITCHELL D.B., VAN LOON A.P.G.M.: Cloning of the phytases from *Emericella nidulans* and the thermophilic fungus *Talaromyces thermophilus*. *Biochim.Biophys.Acta* **1353**, 217–223 (1997).

PASAMONTES L., HAIKER M., WYSS M., TESSIER, VAN LOON A.P.: Gene cloning, purification, and characterization of a heat-stable phytase from the fungus *Aspergillus fumigatus*. *Appl.Environ.Microbiol.* **63**, 1696–1700 (1997).

PATWARDHAN V.N.: The occurrence of a phytin splitting enzyme in the intestines of albino rats. *Biochem.J.* **31**, 560–564 (1937).

PEN J., VERWOERD T.C., VAN PARIDON P.A., BEUDEKER R.F., VAN DEN ELZEN P.J.M.: Phytase-containing transgenic seeds as a novel feed additive for improved phosphorus utilization. *Bio/Technology* **11**, 811–814 (1993).

PEN J., VERWOERD T.C., VAN PARIDON P.A., VAN COYEN A.J.J., VAN DEN ELZEN P.J.M., HOEKEMA A.: Production of high-value proteins in plants. *J.Cell.Biochem.* **18A**, 77 (1994).

PERNEY K.M., CANTOR A.H., STRAW M.L., HERKELMAN K.L.: The effect of dietary phytase on growth performance and phosphorus utilization of broiler chicks. *Poultry Sci.* **72**, 2106–2114 (1993).

PFEFFER E.: cited in D.C. Billington: *The Inositol Phosphates. Chemical Synthesis and Biological Significance.* Verlag Chemie, Weinheim 1993.

PHILLIPPY B.Q., MULLANEY E.J.: Expression of an *Aspergillus niger* phytase (*phyA*) in *Escherichia coli*. *J.Agric.Food Chem.* **45**, 3337–3342 (1997).

PHILLIPPY B.Q., WHITE K.D., JOHNSTON M.R., TAO S.H., FOX M.R.S.: Preparation of inositol phosphates from sodium phytate by enzymatic and nonenzymatic hydrolysis. *Anal.Biochem.* **162**, 115–121 (1987).

PIDDINGTON C.S., HOUSTON C.S., PALOHEIMO M., CANTRELL M., MIETTINEN OINONEN A., NEVALAINEN H., RAMBOSEK J.: The cloning and sequencing of the genes encoding phytase (*phy*) and 2.5-optimum acid phosphatase (*aph*) from *Aspergillus niger* var. *awamori*. *Gene* **133**, 55–62 (1993).

PILEGGI V.J.: Distribution of phytase in rat. *Arch.Biochem.Biophys.* **80**, 1–8 (1959).

POSTERNAK T.: *The Cyclitols*. Hermann, Paris 1965.

POWAR V.K., JAGANNATHAN V.: Phytase from *Bacillus subtilis*. *Indian J.Biochem.* **4**, 184–185 (1967).

POWAR V.K., JAGANNATHAN V.: Purification and properties of phytate-specific phosphatase from *Bacillus subtilis*. *J.Bacteriol.* **151**, 1102–1108 (1982).

QIAN H., KORNEGAY E.T., CONNER D.E. Jr.: Adverse effects of wide calcium phosphorus ratios on supplemental phytase efficacy for weanling pigs fed two dietary phosphorus levels. *J.Animal Sci.* **74**, 1288–1297 (1996).

QIAN H., KORNEGAY E.T., DENBOW D.M.: Utilization of phytate phosphorus and calcium as influenced by microbial phytase, cholecalciferol, and the calcium: total phosphorus ratio in broiler diets. *Poultry Sci.* **76**, 37–46 (1997).

QIAN H., VIET H.P., KORNEGAY E.T., RAVINDRAN V., DENBOW D.M.: Effects of supplemental phytase and phosphorus on histological and other tibial bone characteristics and performances of broilers fed semi-purified diets. *Poultry Sci.* **75**, 618–626 (1996).

RAO R.K., RAMAKRISHNAN C.V.: Studies on inositol phosphatase in rat small intestine. *Enzyme* **33**, 205–215 (1985).

RAPOPORT S., LEVA E., GUEST G.M.: Phytase in plasma and erythrocytes of vertebrates. *J.Biol.Chem.* **139**, 621–632 (1941).

RICHTER G.: Use of microbial phytase at different phosphorus supply levels in broiler fatting. 1. Effect on fattening performance and tibia stability. *Arch.Tierernähr.* **45**, 235–244 (1993).

RIMBACH G., BRANDT K., MOST E., PALLAUF J.: Supplemental phytic acid and microbial phytase change zinc bioavailability and cadmium accumulation in growing rats. *J.Trace Elem.Med.Biol.* **9**, 117–122 (1995).

RIMBACH G., PALLAUF J.: The effect of a supplement of microbial phytase on zinc availability. *Z.Ernährungswiss.* **31**, 269–277 (1992).

RIMBACH G., PALLAUF J.: Enhancement of zinc utilization from phytate-rich soy protein isolate by microbial phytase. *Z.Ernährungswiss.* **32**, 308–315 (1993).

ROBERSON K.D., EDWARDS H.M. Jr.: Effects of 1,25-dihydroxycholecalciferol and phytase on zinc utilization in broiler chicks. *Poultry Sci.* **73**, 1312–1326 (1994).

RODEHUTSCORD M., BECKER A., PFEFFER E.: Effect of supplemental *Aspergillus niger* phytase on the utilization of plant phosphorus by rainbow trout (*Oncorhynchus mykiss*). *Arch.Tierernähr.* **48**, 211–219 (1995).

SAMANTA S., DALAL B., BISWAS S., BISWAS B.B.: *myo*-Inositol tris-phosphate–phytase complex as an elicitor in calcium mobilization in plants. *Biochem.Biophys.Res.Commun.* **191**, 427–434 (1993).

SANDBERG A.S., ANDERSSON H.: Effect of dietary phytase on the digestion of phytate in the stomach small intestine of humans. *J.Nutr.* **118**, 469–473 (1988).

SANDBERG A.S., HULTHEN L.R., TURK M.: Dietary *Aspergillus niger* phytase increases iron absorption in humans. *J.Nutr.* **126**, 476–480 (1996).

SANDBERG A.S., LARSEN T., SANDSTROM B.: High dietary calcium level decreases colonic phytate degradation in pigs fed a rapeseed diet. *J.Nutr.* **123**, 559–566 (1993).

SANDERS J.P.M.: Biotechnology closes natural product cycles. *Chem.Mag.* **10**, 475–777 (1993).
SATIRANA M.L., BIANCHETTI R.: The effects of phosphate on the development of phytase in the wheat embryo. *Physiol.Plant.* **20**, 1066–1075 (1967).
SCOTT J.J.: Alkaline phytase activity in nonionic detergent extracts of legume seeds. *Plant Physiol.* **95**, 1298–1301 (1991).
SCOTT J.J., LOEWUS F.A.: A calcium activated phytase from pollen of *Lilium longiflorum*. *Plant Physiol.* **82**, 333–335 (1986).
SEBASTIAN S., TOUCHBURN S.P., CHAVEZ E.R., LAGUE P.C.: The effect of supplemental phytase on the performance and utilization of dietary calcium, phosphorus, copper, and zinc in broiler chickens fed corn–soybean diets. *Poultry Sci.* **76**, 729–736 (1996).
SEGUEILHA L., LAMBRECHTS C., BOZE H., MOULIN G., GALZY P.: Purification and properties of the phytase from *Schwanniomyces castellii*. *J.Ferment.Bioeng.* **74**, 7–11 (1992).
SEGUEILHA L., MOULIN G., GALZY P.: Reduction of phytate content in wheat bran and glandless cotton flour by *Schwanniomyces castellii*. *J.Agric.Food Chem.* **41**, 2451–2454 (1993).
SHAH V., PAREKH L.J.: Phytase from *Klebsiella* sp. no. PG-2: purification and properties. *Indian J.Biochem.Biophys.* **27**, 98–102 (1990).
SHIEH T.R., WARE J.H.: Survey of microorganismus for the production of extracellular phytase. *Appl.Microbiol.* **16**, 1348–1351 (1968).
SHIEH T.R., WODZINSKI R.J., WARE J.H.: Regulation of the formation of acid phosphatase by inorganic phosphate in *Aspergillus ficuum*. *J.Bacteriol.* **100**, 1161–1165 (1969).
SHIMIZU M.: Purification and characterization of phytase from *Bacillus subtilis* (*natto*) N-77. *Biosci.Biotechnol.Biochem.* **56**, 1266–1269 (1992).
SHIMIZU M.: Purification and characterization of phytase and acid phosphatase produced by *Aspergillus oryzae* K1. *Biosci. Biotech.Biochem.* **57**, 1364–1365 (1993).
SHIRAI K., REVAH MOISEEV S., GARCIA-GARIBARY M., MARSHALL V.M.: Ability of some strains of lactic acid bacteria to degrade phytic acid. *Lett.Appl.Microbiol.* **19**, 366–369 (1994).
SIJMONS P.C., DEKKER B.M.M., SCHRAMMEYER B., VERWOERD T.C., VAN DEN ELZEN P.J.M., HOEKEMA A.: Production of correctly processed human serum albumin in transgenic plants. *Bio/Technology* **8**, 217–221 (1991).
SIMELL M., TURUNEN M., PIHONEN J., VAARA T.: Feed and food applications of phytase. Lecture at *3rd Meet. Industrial Applications of Enzymes*, Barcelona (Spain) 1989.
SIMONS P.C.M., VERSTEEGH H.A.J., JONGBLOED A.W., KEMME P.A., SLUMP P., BOS K.D., WOLTERS M.G.E., BEUDEKER R.F., VERSCHOOR G.J.: Improvement of phosphorus availability by microbial phytase in broilers and pigs. *Brit.J.Nutr.* **64**, 525–540 (1990).
SIREN M.: Stabilized pharmaceutical and biological material composition. *Pat. SE* 003 165 (1986*a*).
SIREN M.: New *myo*-inositol triphosphoric acid isomer. *Pat. SW* 052 950 (1986*b*).
SKOWRONSKI T.: Some properties of partially purified phytase from *Aspergillus niger*. *Acta Microbiol.Polon.* **27**, 41–48 (1978).
SOUPPE J.: Recent progress in the industrial application of enzymes. *C.R.Acad.Agric.Fr.* **81**, 19–26 (1995).
SPITZER R.S., PHILLIPS P.H.: cited in K. Bitar, J.G. Reinhold: Phytase and alkaline phosphatase activities in intestinal mucosae of rat, chicken, calf, and man. *Biochim.Biophys.Acta* **268**, 442–452 (1972).
SUTARDI M., BUCKLE K.A.: Characterization of extra- and intracellular phytase from *Rhizopus oligosporus* used in tempeh production. *Int.J.Food Microbiol.* **6**, 67–69 (1988).
SUZUKI U., YOSHIMURA K., TAKAISHI M.: cited in D.J. Cosgrove: *Inositol Phosphates. Their Chemistry, Biochemistry and Physiology*. Elsevier, Amsterdam 1980.
TAMBE S.M., KALU G.S., KELKAR S.M., PAREKH L.J.: Two distinct molecular forms of phytase from *Klebsiella aerogenes*: Evidence for unusually small active enzyme peptide. *J.Ferment.Bioeng.* **77**, 23–27 (1994).
TAMMINGA S.: Biotechnology and improvement of animal nutrition. *J.Meded.Fac. Landbouwwet Rijksuniv.Gent.* **55**, 1373–1382 (1990).
ULLAH A.H.J.: Production, rapid purification and catalytic characterization of extracellular phytase from *Aspergillus ficuum*. *Prep.Biochem.* **18**, 443–458 (1988*a*).
ULLAH A.H.J.: *Aspergillus ficuum* phytase: Partial primary structure, substrate selectivity, and kinetic characterization. *Prep. Biochem.* **18**, 459–471 (1988*b*).
ULLAH A.H.J., CUMMINS B.J.: *Aspergillus ficuum* extracellular phytase: Immobilization on glutaraldehyde-activated silicate. *Ann.N.Y.Acad.Sci.* **542**, 102–106 (1988).
ULLAH A.H.J., CUMMINS B.J.: Immobilization of *Aspergillus ficuum* extracellular phytase on fractogel. *Biotechnol.Appl.Biochem.* **9**, 380–388 (1987).
ULLAH A.H.J., CUMMINS B.J.: Purification, N-terminal amino acid sequence and characterization of pH 2.5 optimum acid phosphatase (EC 3.1.3.2) from *Aspergillus ficuum*. *Prep.Biochem.* **17**, 397–422 (1987).
ULLAH A.H.J., CUMMINS B.J., DISCHINGER H.C. Jr.: Cyclohexanedione modification of arginine at the active site of *Aspergillus ficuum* phytase. *Biochem.Biophys.Res.Commun.* **178**, 45–53 (1991).
ULLAH A.H.J., DISCHINGER H.C. Jr.: *Aspergillus ficuum* extracellular phytase: peptide mapping and purification. *Ann.N.Y.Acad. Sci.* **613**, 878–882 (1990).
ULLAH A.H.J., DISCHINGER H.C. Jr.: Identification of residues involved in active-site formation in *Aspergillus ficuum* phytase. *Ann.N.Y.Acad.Sci.* **672**, 45–51 (1992).
ULLAH A.H.J, DISCHINGER H.C. Jr.: *Aspergillus ficuum* phytase: Complete primary structure elucidation by chemical sequencing. *Biochem.Biophys.Res.Commun.* **192**, 475–453 (1993*a*).
ULLAH A.H.J., DISCHINGER H.C. Jr.: Identification of active site residues in *Aspergillus ficuum* extracellular pH 2.5 optimum acid phosphatase. *Biochem.Biophys.Res.Commun.* **192**, 754–759 (1993*b*).
ULLAH A.H.J., DISCHINGER H.C. Jr.: *Aspergillus ficuum* phytase active site: involvement of Arg and Trp residues. *Ann.N.Y. Acad.Sci.* **750**, 51–57 (1995).
ULLAH A.H.J., GIBSON D.M.: Extracellular phytase (E.C. 3.1.3.8) from *Aspergillus ficuum* NRRL 3135: purification and characterization. *Prep.Biochem.* **17**, 63–91 (1987).
ULLAH A.H.J., MULLANEY E.J.: Disulfide bonds are necessary for structure and activity in *Aspergillus ficuum* phytase. *Biochem. Biophys.Res.Commun.* **227**, 311–317 (1996).
ULLAH A.H.J., PHILLIPPY B.Q.: Substrate selectivity in *Aspergillus ficuum* phytase and acid phosphatase using *myo*-inositol phosphates. *J.Agric.Food Chem.* **42**, 423–425 (1994).
ULLAH A.H.J., PHILLIPPY B.Q.: Immobilization of *Aspergillus ficuum* phytase: product characterization of the reactor. *Prep. Biochem.* **18**, 483–489 (1988).

VAN ETTEN R.L., DAVIDSON R., STEVIS P.E., MACARTHUR H., MOORE D.L.: Covalent structure, disulfide binding, and identification of reactive surface and active site residues of human prostatic acid phosphatase. *J.Biol.Chem.* **266**, 2313–2319 (1991).

VAN HARTINGSVELDT W., VAN ZEIJL C.M., HARTEVELD G.M., GOUKA R.J., SUYKERBUYK M.E., LUITEN R.G., VAN PARIDON P.A., SELTEN G.C., VEENSTRA A.E., VAN GORCOM R.F.: Cloning, characterization and overexpression of the phytase-encoding gene (*phyA*) of *Aspergillus niger*. *Gene* **127**, 87–94 (1993).

VAN DEN HONDEL C.A.M.J.J., PUNT P.J., VAN GORCOM R.F.M.: Strategies for the over-production of fungal proteins in *Aspergillus*. *Prog.Biotechnol.* **9**, 287–290 (1994).

VAN DER KAAY J., VAN DER HAASTERT P.J.M.: Stereospecificity of inositol hexakisphosphate dephosphorylation by *Paramecium* phytase. *Biochem.J.* **312**, 907–910 (1995).

VERWOERD T.C., VAN PARIDON P.A., VAN OOYEN A.J., VAN LENT J.W., HOEKEMA A., PEN J.: Stable accumulation of *Aspergillus niger* phytase in transgenic tobacco leaves. *Plant Physiol.* **109**, 1199–1205 (1995).

VOLFOVÁ O., DVOŘÁKOVÁ J., HANZLÍKOVÁ A., JANDERA A.: Phytase from *Aspergillus niger*. *Folia Microbiol.* **39**, 481–484 (1994).

VOLFOVÁ O., DVOŘÁKOVÁ J., SOBOTKA M., ZOBAČ P., KUMPRECHT I.: Industrial production of enzymatic preparation phytase by fermentation. *Pat. CZ* 282 772 (1997).

Wakamoto Pharmaceuticals: Production of Ca immobilizing substance. *Japan Pat.* 192 839 (1988).

WHITAKER J.R.: New and future uses of enzymes in food processing. *Food Biotechnol.* **4**, 669–697 (1990).

WHITELAM G.C.: The production of recombinant proteins in plants. *J.Sci.Food Agric.* **68**, 1–9 (1995).

WILLIAMS S.A., CULP J.S., BUTLER L.G.: The relationship of alkaline phosphatase, CTPase and phytase. *Arch.Biochem.Biophys.* **241**, 10–13 (1985).

WILLIAMS P.J., TAYLOR T.G.: A comparative study of phytate hydrolysis in the gastrointestinal tract of the golden hamster (*Mesocricetus auratus*) and the laboratory rat. *Brit.J.Nutr.* **54**, 429–35 (1985).

YAMADA K., MINODA Y., YAMAMOTO S.: Phytase from *Aspergillus terreus*. I. Production, purification and some general properties of the enzyme. *Agric.Biol.Chem.* **32**, 1275–1282 (1968).

YAMAMOTO S., MINODA Y., YAMADA K.: Chemical and physicochemical properties of phytase from *Aspergillus terreus*. *Agric. Biol.Chem.* **36**, 2097–2103 (1972).

YANG W.J., MATSUDA Y., SANO S., MASUTANI H., NAKAGAVA H.: Purification and characterization of phytase from rat intestinal mucosa. *Biochim.Biophys.Acta* **1075**, 95–82 (1991*a*).

YANG W.J., MATSUDA Y., INOMATA M., NAKAGAVA H.: Developmental and dietary induction of 90 kDa subunits of rat intestinal phytase. *Biochim.Biophys.Acta* **1075**, 83–87 (1991*b*).

YI Z., KORNEGAY E.T., DENBOW D.M.: Supplemental microbial phytase improves zinc utilization in broilers. *Poultry Sci.* **75**, 540–546 (1996).

YI Z., KORNEGAY E.T., RAVINDRAN V., DENBOW D.M.: Improving phytate phosphorus availability in corn and soybean meal for broilers using microbial phytase and calculation of phosphorus equivalency values for phytase. *Poultry Sci.* **75**, 240–249 (1996).

YI Z., KORNEGAY E.T., RAVINDRAN V., LINDEMANN M.D., WILSON J.H.: Effectiveness of Natuphos phytase in improving the bioavailabilities of phosphorus and other nutrients in soybean meal-based semipurified diets for young pigs. *J.Animal Sci.* **74**, 1601–1611 (1996).

YOUNG L.G., LEUNISSEN M., ATKINSON J.L.: Addition of microbial phytase to diets of young pigs. *J.Animal Sci.* **71**, 2147–2150 (1993).

Zenkoku Nogyo Kyodo Ass.: A neutral phytase. *Japan Pat.* 06 038 745 (1994).

ZOBAČ P., KUMPRECHT L., VOLFOVÁ O., ŠIMEČEK K., DVOŘÁKOVÁ J.: The effect of microbial phytase applied in feed mixtures on phosphorus and calcium utilization in chicken broilers. *Živočišná výroba* **42**, 13–22 (1997).

ZYLA K.: Mould phytases and their application in the food industry. *World J.Microbiol.Biotechnol.* **8**, 4667-4672 (1992).

ZYLA K., KORELESKI J.: *In vitro* and *in vivo* dephosphorylation of rapeseed meal by means of phytate degrading enzymes derived from *Aspergillus niger*. *J.Sci.Food Agric.* **61**, 1–6 (1993).

ZYLA K., LEDOUX D.R., GARCIA A., VEUM T.L.: An *in vitro* procedure for studying enzymic dephosphorylation of phytate in maize–soybean feeds for turkey poults. *Brit.J.Nutr.* **74**, 3–17 (1995).

ELSEVIER

Animal Feed Science and Technology 47 (1994) 19–29

ANIMAL FEED SCIENCE AND TECHNOLOGY

Total phosphorus, phytate-phosphorus and phytase activity in plant feedstuffs

W. Eeckhout*, M. De Paepe

National Institute for Animal Nutrition, Agricultural Research Centre—Ghent, Scheldeweg 68, B-9090 Melle-Gontrode, Belgium

(Received 26 May 1993; accepted 2 November 1993)

Abstract

A total of 285 samples representing 51 feedstuffs used in Belgian feed-mills were quantitatively analysed for phytase activity, phytate-P and total P. It was concluded that the number of feedstuffs showing significant phytase activity (more than 100 units kg^{-1}) is rather limited ($n=13$). Of the cereals analysed, only rye (5130 units kg^{-1}), triticale (1688 units kg^{-1}), wheat (1193 units kg^{-1}) and barley (582 units kg^{-1}) were rich in phytase. Wheat by-products, such as fine bran meal (4601 units kg^{-1}) or pellets (2573 units kg^{-1})], middlings (4381 units kg^{-1}), feed flour (3350 units kg^{-1}) and bran (2957 units kg^{-1}) were very phytase rich. Pelleted wheat fine bran samples were, on average, only 56% as active as non-pelleted wheat fine bran. Malt sprouts pellets (877 units kg^{-1}) as well as corn distillers (385 units kg^{-1}) also showed a moderate phytase activity. All other feedstuffs analysed showed zero or extremely low phytase activity. Phytase activity was not related to total P content or phytate-P content.

A significant linear relationship was found between phytate-P and total P, for two feedstuff classes—wheat plus wheat by-products ($R^2=0.953$; RSD=0.060) and maize plus maize by-products ($R^2=0.928$; RSD=0.042)—but not for cereals or oil meals. Phytate-P content, as a percentage of total P content, was higher in cereals and in wheat by-products than in oil-seed meals and legume seeds. In malt sprouts, corn distillers and moist ensiled maize, phytate-P seems to be totally or partly hydrolysed as a result of processing or ensiling. In feedstuffs derived from roots and tubers, as well as in citrus pulp, cocoa shells, soyabean hulls, flax chaff, maize cob meal, dehydrated alfalfa and a dried mycelium sample, phytate-P was not detectable.

*Corresponding author.

SSDI 0377-8401(93)00577-I

1. Introduction

As a result of growing concern about the environment, intensification of animal production in many European countries is considered as a potential source of air pollution and a threat to soil and drinking water quality. In areas of intensive pig production (e.g. Belgium, Netherlands and France), pollution with nitrogen and phosphorus from manure is becoming a major problem. In some sandy regions of northern Belgium, pig manure production per unit of arable land greatly exceeds the requirements for reasonable crop production. In certain areas, phosphorus excretion by ruminants and pigs even exceeds 300 kg P_2O_5 ha^{-1} $year^{-1}$.

In such a situation, re-examination of nitrogen and phosphorus requirements, especially of pigs, is necessary to prevent the eventual deterioration of drinking water resources. Phosphorus pollution by monogastric animals is mainly due to the very low bio-availability of phytate-phosphorus, the most important P form in cereals and other seeds (Nelson, 1980; Jongbloed, 1987; Fourdin et al., 1988). To meet the animals' requirements for phosphorus, mixed feeds for pigs are supplemented with inorganic phosphates. This could at least partly be avoided if the absorbability of phytate-P could be increased by an appropriate use of phytases. Some plant feedstuffs (such as wheat or triticale) and some fungi (such as *Aspergillus* sp.) contain powerful phytases, capable of hydrolysing phytic acid in (bioavailable) inorganic P and inositol within the animals' digestive tract (Kemme and Jongbloed, 1989; Pointillart, 1991; Eeckhout and De Paepe, 1991, 1992). Consequently, the available phosphorus content of an all-plant pig meal depends on three factors: phytate-P content; non-phytate-P content; phytase activity of the diet. The possible destruction of phytase by pelleting or heat treatment must also be taken into account. A better understanding of the relative value of these factors would facilitate low-phosphorus diet formulation. However, until now, there has been no phosphorus evaluation system which takes into account vegetable phytase as a source of absorbable phosphorus.

Although the real significance of wheat phytase and triticale phytase has been clearly demonstrated with piglets and growing pigs (Eeckhout and De Paepe, 1991, 1992), little information has been published on the phytase activity for a variety of plant feedstuffs. Therefore, we collected 285 samples from 51 feedstuffs used in the Belgian animal feed industry and analysed them for total P, phytate-P and phytase activity.

2. Materials and methods

Clean, uncontaminated (not mouldy) samples were taken at various Belgian feed-mills and sent to our laboratory for analysis. The ground samples were analysed for total P by the EEC method for feedstuffs, and for phytate-P by the method of Haug and Lantzsch (1983). The choice of this method, which has been adopted

by the UK Agriculture and Advisory Service, was based on the encouraging results of a comparative study (Hopakins et al., 1989) involving two other methods.

Phytase activity was measured at pH 5.5 and 37°C by the following method. Finely ground samples (200 mg, or 100 mg for phytase-rich feedstuffs) were weighed in 50 ml volumetric flasks, and the flasks were filled to the mark with Na-phytate solution (1.722 g of Na-phytate (Sigma P 3168 from rice), 180 ml of H_2O and 820 ml of 0.25 M acetate buffer, pH 5.5). The flasks were shaken for 15 min and then placed in a water bath at 37°C. After 10 min incubation, a 2 ml portion of the incubate was transferred to a test tube containing 2 ml of 10% trichloro-acetic acid (TCA). After another 60 min, another 2 ml portion of incubate was transferred to a test tube containing 2 ml of 10% TCA. The contents of both tubes were filtered through an S&S (Schleicher & Schuell, Dassel) 589[3] filter paper and 1 ml of the filtrate was transferred to a cuvette, together with 1 ml of a freshly prepared colour reagent. The colour reagent was a mixture of four parts of Solution A (15 g of ammonium heptamolybdate.$4H_2O$, 55 ml of concentrated sulphuric acid and H_2O to 1 l) and one part of Solution B (27 g of $FeSO_4.7H_2O$, a few drops of concentrated sulphuric acid and H_2O to 250 ml). Colour was measured in a spectrophotometer at 700 nm and compared with a calibration series containing 1 ml of 10% TCA per cuvette, 1 ml of P standard solution (0, 10, 20, 30 or 40 μmol P ml^{-1}) and 2 ml of colour reagent. Phosphorus measured in the 10 min incubate was taken as a blank; therefore only the difference in optical density between the 70 min incubate and the 10 min incubate was attributed to phytase activity.

Phytase activity was calculated as follows: Phytase units $kg^{-1} = (P \times 1000)/(W \times 60)$, where P is micromoles of P liberated by phytase in 60 min, W is sample weight (g) and 60 is the incubation time taken into account (i.e. 70 min minus 10 min). From the method described, the phytase unit is defined as that amount of phytase activity which liberates inorganic phosphorus from a 0.0015 M Na-phytate solution, at a rate of 1 μmol min^{-1} at pH 5.5 and 37°C.

3. Results and discussion

The data were classified in the following manner: (1) feedstuffs with phytase activity (more than 100 units kg^{-1}); (2) feedstuffs with very low or no phytase activity (less than 100 units kg^{-1}); (3) feedstuffs without phytate-P; (4) relationships between phytate-P and total P and phytase activity.

3.1. Feedstuffs with phytase activity

Feedstuffs with an average phytase activity higher than 100 units kg^{-1} are presented in Table 1, which shows that, taking into account the borderline products (peas, corn distillers and rice bran), only a limited number of feedstuffs showed appreciable phytase activity. From a practical point of view, wheat and its by-products are by far the most important. Wheat by-products especially have powerful phytase activity, and the difference in activity between pelleted and non-

Table 1
Total P, phytate-P and phytase activity of feedstuffs with phytase activity of more than 100 units kg^{-1}

	n	Total P (%) mean ± SD (range)	Phytate-P (%) mean ± SD (range)	(Phytate-P/total P) × 100 mean ± SD (range)	Phytase (units kg^{-1}) mean ± SD (range)
Seeds					
Rye	2	0.36 (0.35–0.36)	0.22 (0.20–0.23)	61 (56–66)	5130 (4132–6127)
Triticale	6	0.37 ± 0.02 (0.35–0.40)	0.25 ± 0.02 (0.22–0.28)	67 ± 3.7 (61–70)	1688 ± 227 (1475–2039)
Wheat	13	0.33 ± 0.02 (0.31–0.38)	0.22 ± 0.02 (0.19–0.27)	67 ± 4.8 (61–78)	1193 ± 223 (915–1581)
Barley	9	0.37 ± 0.02 (0.34–0.39)	0.22 ± 0.01 (0.20–0.24)	60 ± 2.4 (55–62)	582 ± 178 (408–882)
Peas	11	0.38 ± 0.02 (0.36–0.40)	0.17 ± 0.03 (0.13–0.21)	45 ± 6.2 (36–53)	116 ± 54 (36–183)
By-products					
Wheat fine bran	6	0.95 ± 0.06 (0.88–1.03)	0.72 ± 0.08 (0.60–0.81)	76 ± 5.6 (68–84)	4601 ± 860 (3485–5345)
Wheat fine bran (pellets)	15	1.01 ± 0.08 (0.88–1.17)	0.78 ± 0.08 (0.62–0.88)	77 ± 5.6 (62–82)	2573 ± 0.59 (1206–4230)
Wheat middlings	5	0.80 ± 0.25 (0.53–1.20)	0.53 ± 0.14 (0.33–0.71)	66 ± 6.9 (59–76)	4381 ± 956 (2825–5042)
Wheat feed flour	11	0.56 ± 0.20 (0.26–0.91)	0.39 ± 0.16 (0.15–0.64)	70 ± 7.6 (56–76)	3350 ± 1244 (1007–4708)
Wheat bran	5	1.16 ± 0.14 (1.03–1.36)	0.97 ± 0.20 (0.77–1.27)	84 ± 7.3 (75–93)	2957 ± 1556 (1180–5208)
Malt sprouts (pellets)	4	0.60 ± 0.09 (0.52–0.73)	0.01 ± 0.03 (0–0.05)	2 ± 3.4 (0–7)	877 ± 242 (605–1174)
Corn distillers	3	0.90 (0.86–0.96)	0.19 (0.17–0.21)	21 (20–24)	385 (141–850)
Rice bran	2	1.71 (1.37–1.74)	1.10 (1.08–1.11)	64 (62–66)	122 (108–135)

pelleted wheat fine bran is very probably due to the partial destruction of phytase by heat developed during pelleting.

Of the cereals, rye has by far the highest phytase activity, followed by triticale, wheat and barley. Although variation in phytase activity within a single feedstuff is important, it should be noted that the average values for the four cereals are very different and that there is little or no overlapping. On the other hand, the higher variation and significant overlapping of wheat by-products is remarkable, and probably originates from different processing conditions rather than different cultivars. The higher variation in total P content of some wheat by-products supports this assumption.

The presence of an active phytase has been studied in germinating maize seeds (Chang, 1967); in ground dry maize, however, only very weak phytase activity can be demonstrated after a very long incubation time (Scheuermann et al., 1988).

The not negligible average phytase activity of the three corn distillers samples is mainly due to the high value of one sample (850 units kg^{-1}). Phytate-P, as measured by the Haug and Lantzsch (1983) method, is low in relation to the total P content (21%). The fermentation process that distiller's grains undergo seems to increase the P availability for pigs (Cromwell, 1992), presumably as a result of partial phytate hydrolysis. However, phytic acid seems to be totally hydrolysed during processing of malt sprouts, which have an average phytase activity higher than barley, notwithstanding pelleting.

The presence of phytase in legume seeds (Sutardi and Buckle, 1986), in aleurone particles of rice (Yoshida et al., 1975) and in rice (A. Pointillart and C. Colin, personal communication, 1993) has been demonstrated, but activity is weak. For 20 different pea cultivars, A. Pointillart (personal communication, 1993) found only 78 ± 15 units kg^{-1}, and concluded that phytase activity of peas is negligible.

Phytate-P content, as a percentage of total P content, for the seven cereals studied—four with phytase activity (Table 1) and three without (maize, oats and sorghum, Table 2)—was highest for sorghum (70%) and lowest for oats (59%), barley (60%) and rye (61%), whereas wheat (67%), triticale (67%) and maize (68%) were intermediate. These figures agree fairly well with those mentioned by Cromwell (1992). The corresponding value for peas is much lower (45%), in agreement with the values given by Sauveur (1989). All wheat by-products listed in Table 1 show a higher total P content than wheat, and also a higher phytate-P content, in relative as well as in absolute terms. This can be explained by the abundance of phytate-P in the aleurone layer of the grain (Reddy et al., 1989).

3.2. Feedstuffs without phytase activity

Table 2 presents 38 of the analysed feedstuffs with only very low (less than 100 units kg^{-1}) or no phytase activity. Among the cereals, there seems to be no naturally occurring phytase in maize, oats and grain sorghum capable of hydrolysing phytic acid in appreciable amounts under the test conditions used.

Although the bio-availability of phosphorus in high-moisture ensiled maize is three to four times higher than that of phosphorus in dry maize (Ross et al., 1983), practically no phytase activity was noted. However, there seems to be a considerable reduction in phytate-P content, which may explain the enhanced bio-availability of phosphorus.

Among the cereal by-products, oil meals, legume seeds, roots and tubers and the other products mentioned in Table 2, various feedstuffs have been heat-treated, and/or pelleted, so, theoretically, phytase that could potentially be present may have been destroyed. However, for most of these feedstuffs, these treatments are probably not responsible for the absence of noticeable phytase activity, with dehydrated wheat gluten feed perhaps the only exception.

Phytase activity of any significance was absent in all analysed maize by-products, in rice feed flour and extracted rice bran, as well as in oil meals and the legume seeds studied. Feedstuffs from roots and tubers, as well as hulls, shells

Table 2
Total P, phytate-P and phytase activity of feedstuffs with phytase activity of less than 100 units kg^{-1}

	n	Total P (%) mean ± SD (range)	Phytate-P (%) mean ± SD (range)	(Phytate-P/ total P) × 100 mean ± SD (range)	Phytase (units kg^{-1}) mean ± SD (range)
Cereals					
Maize	11	0.28 ± 0.03 (0.25–0.35)	0.19 ± 0.03 (0.16–0.26)	68 ± 5.9 (61–77)	15 ± 18 (0–46)
Oats	6	0.36 ± 0.03 (0.33–0.40)	0.21 ± 0.04 (0.16–0.28)	59 ± 11 (48–78)	42 ± 50 (0–108)
Sorghum	5	0.27 ± 0.05 (0.20–0.33)	0.19 ± 0.04 (0.14–0.24)	70 ± 6.2 (61–76)	24 ± 32 (0–76)
Maize (moist ensiled)[1]	7	0.30 ± 0.05 (0.24–0.38)	0.13 ± 0.02 (0.11–0.18)	43 ± 5.3 (35–49)	12 ± 11 (0–30)
Cereal by-products					
Maize gluten feed	9	0.87 ± 0.16 (0.63–1.10)	0.47 ± 0.06 (0.35–0.54)	54 ± 6.2 (44–62)	48 ± 68 (0–177)
Maize gluten feed (pellets)	5	0.89 ± 0.10 (0.75–0.99)	0.52 ± 0.08 (0.40–0.60)	58 ± 3.1 (53–61)	5 ± 7 (0–15)
Maize germs (extracted)	1	0.65	0.42	65	16
Maize feed flour	2	0.23 (0.22–0.24)	0.14 (0.12–0.16)	61 (55–67)	5 (3–6)
Maize feed flour (USA)	5	0.50 ± 0.04 (0.45–0.55)	0.27 ± 0.07 (0.20–0.36)	54 ± 9.7 (44–65)	37 ± 30 (0–78)
Rice feed flour	1	0.32	0.23	72	0
Rice bran (extracted)	4	1.89 ± 0.27 (1.57–2.21)	0.79 ± 0.19 (0.69–1.07)	42 ± 9.6 (31–54)	45 ± 67 (0–145)
Wheat gluten feed	6	0.78 ± 0.06 (0.71–0.87)	0.56 ± 0.10 (0.44–0.69)	71 ± 11.0 (39–90)	25 ± 61 (0–150)
Oil meals					
Peanut (extracted) (pellets)	3	0.68 (0.65–0.70)	0.32 (0.30–0.34)	47 (46–49)	3 (0–8)
Coconut (expeller)	4	0.53 ± 0.05 (0.47–0.58)	0.18 ± 0.03 (0.14–0.20)	34 ± 4.0 (30–39)	24 ± 37 (0–80)
Linseed (expeller)	4	0.75 ± 0.02 (0.73–0.78)	0.42 ± 0.02 (0.39–0.43)	55 ± 2.9 (52–58)	5 ± 6 (0–12)
Linseed (extracted)	1	0.82	0.47	57	41
Rapeseed (extracted)	5	1.12 ± 0.04 (1.07–1.17)	0.40 ± 0.05 (0.34–0.48)	36 ± 3.4 (32–41)	16 ± 16 (0–36)
Palm-kernel (expeller)	6	0.59 ± 0.03 (0.55–0.62)	0.39 ± 0.03 (0.33–0.41)	66 ± 3.9 (60–71)	37 ± 31 (0–91)
Sunflower (extracted) (pellets)	11	1.00 ± 0.11 (0.86–1.28)	0.44 ± 0.05 (0.32–0.51)	44 ± 3.9 (35–47)	62 ± 53 (0–185)
Soyabean 44 (extracted)	15	0.66 ± 0.03 (0.61–0.71)	0.35 ± 0.02 (0.33–0.39)	53 ± 2.5 (46–57)	40 ± 45 (0–120)
Soyabean 48 (extracted)	5	0.61 ± 0.01 (0.59–0.62)	0.32 ± 0.02 (0.28–0.33)	52 ± 3.7 (46–56)	8 ± 8 (0–20)
Soyabean 50 (extracted)	9	0.71 ± 0.02 (0.67–0.73)	0.38 ± 0.01 (0.37–0.40)	54 ± 2.2 (51–56)	31 ± 50 (0–149)

	n	Total P (%) mean ± SD (range)	Phytate-P (%) mean ± SD (range)	(Phytate-P/ total P) × 100 mean ± SD (range)	Phytase (units kg^{-1}) mean ± SD (range)
Legume seeds					
Soyabeans (heated)	4	0.57 ± 0.02 (0.55–0.59)	0.26 ± 0.02 (0.23–0.28)	46 ± 5.1 (39–51)	55 ± 89 (0–188)
Field beans (heated)	1	0.50	0.23	46	81
Lupins	1	0.25	0.05	20	0
Roots and tubers					
Beet pulp (pellets)	18	0.10 ± 0.01 (0.08–0.11)	0	0	3 ± 4 (0–13)
Potato (chips)	1	0.10	0	0	0
Potato starch	1	0.10	0	0	0
Cassava root (chips)	11	0.09 ± 0.01 (0.06–0.12)	0	0	6 ± 13 (0–40)
Cassava root (pellets)	7	0.08 ± 0.02 (0.06–0.12)	0	0	9 ± 8 (0–21)
Sweet potatoes	3	0.11 (0.10–0.13)	0	0	26 (0–73)
Other by-products (+ alfalfa)					
Citrus pulp (pellets)	4	0.10 ± 0.01 (0.09–0.11)	0	0	3 ± 6 (0–12)
Cocoa shells	1	0.40	0	0	65
Soyabean hulls	5	0.19 ± 0.02 (0.17–0.21)	0	0	99 ± 58 (0–150)
Flax chaff	1	0.10	0	0	58
Mycelium	2	0.14 (0.13–0.15)	0	0	77 (22–131)
Alfalfa (dehydrated) (pellets)	7	0.23 ± 0.07 (0.11–0.33)	0	0	60 ± 84 (15–250)
Maize cob	1	0.05	0	0	58

[1]On 88% dry matter basis. Lyophilized for phytase activity determination.

and chaff, citrus pulp pellets, dehydrated alfalfa pellets and a mycelium sample analysed, were not only free of phytase activity but no phytate-P was detectable with the method used. The absence of phytate-P in malt sprouts pellets, however, is attributed to hydrolysis of phytic acid during processing by a powerful phytase (877 units kg^{-1}). With this exception, it can be concluded that feedstuffs without phytate-P are free of phytase activity and show a rather low total P content.

However, it should be stressed that phytate-P was determined by the indirect method of Haug and Lantzsch (1983). Using other methods, Bos (1990) found traces of phytate-P in cassava, soya hulls and alfalfa, as did Simons et al. (1981) in cassava, grass meal and alfalfa. On the other hand, Simons et al. (1981) did not detect phytate-P in potato flakes, nor did Cromwell (1992) detect it in alfalfa

meal. These rather conflicting results are probably attributable to the difficulties of phytate-P determination at very low concentrations.

There is not always close agreement in the literature on the content of phytate-P as a percentage of total P, even when the same method for phytate-P determination is used. Lantzsch (1989) and Hopkins et al. (1989) used the Haug and Lantzsch method (1983), as we did. Nevertheless, widely different values were found, for example, for rapeseed meal—17% (Hopkins et al., 1989), 36% (this study) and 67% (Lantzsch, 1989). The corresponding values for sunflower meal were 32%, 44% and 75%. For soyabean oil meal, the respective values were 43%, 53% and 56%, and those for maize were 67%, 68% and 75%. As a rule, the values cited by Lantzsch are higher than those of Nelson et al. (1968), Hopkins et al. (1989) and from this study. In fact, there is better agreement between our results and those of Nelson et al. (1968), especially for cereals, although the latter used a different method for phytate-P determination. However, for palm-kernel meal, Lantzsch's value (66%) was similar to our results. The reasons for these conflicting results are not clear, although factors such as cultivars, processing conditions (e.g. cereal by-products) and number of observations per feedstuff and per researcher, as well as analytical methods used, may explain the observed variations.

3.3. Relationship between parameters studied

Simons et al. (1981) found good linear relationships between total P and phytate-P for several classes of vegetable feedstuffs, notwithstanding the fact that phytate-P data were from three different laboratories involving three different methods. We analysed linear relationship between phytate-P (y) and total P (x) for the following feedstuff classes (data from Tables 1 and 2): (1) cereals, $n=52$ (moist ensiled maize not included); (2) wheat and wheat by-products, $n=55$ (wheat gluten feed not included); (3) maize and maize by-products, $n=33$ (corn distillers, cob meal and moist ensiled maize not included); (4) oil meals, $n=63$; (5) legume seeds, $n=17$ (including peas).

Table 3 shows that, for each feedstuff class studied, total P content is positively

Table 3
Linear relationship between phytate-P (%) (y) and total P (%) (x)

Feedstuffs	n	Regression	R^2	RSD
Cereals	52	$0.49x+0.05$ (± 0.07)	0.518	0.022
Wheat + wheat by-products	55	$0.83x-0.06$ (± 0.02)	0.953	0.060
Maize + maize by-products	33	$0.50x+0.04$ (± 0.02)	0.928	0.042
Oil meals	63	$0.24x+0.18$ (± 0.04)	0.420	0.053
Legume seeds	17	$0.54x-0.04$ (± 0.07)	0.790	0.027

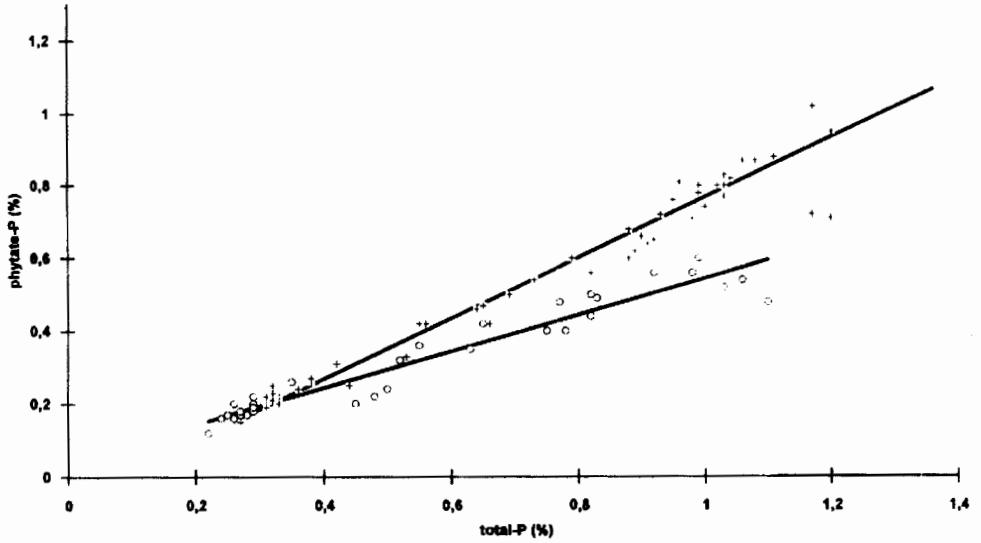


Fig. 1. Linear relationship between phytate-P (%) and total-P (%) for wheat and wheat by-product (+) and maize and maize by-products (O).

Table 4
Linear relationship between phytase activity (units kg^{-1}) (y) and total P (%) (x_1) or phytate-P (%) (x_2)

Feedstuffs	n	Regression	R^2	RSD
Barley	9	$3502x_1-725$ (± 3481)	0.126	177
		$7102x_2-996$ (± 3997)	0.311	158
Wheat	13	$-1207x_1+1594$ (± 2674)	0.018	230
		$-2818x_2+1837$ (± 2579)	0.102	220
Wheat by-products	27	$1606x_1+2462$ (± 841)	0.127	1236
		$1316x_2+2962$ (± 956)	0.070	1275

correlated with phytate-P content. However, from a practical standpoint, the predictability of phytate-P content from total P content is only justified for two feedstuff classes, wheat and wheat by-products ($R^2=0.953$; RSD=0.060) and maize and maize by-products ($R^2=0.928$; RSD=0.042). The regression coefficients of these two equations clearly show that a given increase in total P in wheat and wheat by-products results in a significantly higher increase in phytate-P com-

pared with maize and maize by-products (Fig. 1). The standard error of estimated phytate-P content, however, is relatively important.

Considering the relatively scarce information on phytase activity of feedstuffs, we considered it worth while to use the available phytase activity data for a linear regression analysis between the phytase activity of three feedstuff classes (barley, $n=9$; wheat, $n=13$; (non-pelleted) wheat by-products, $n=27$) on the one hand and total P or phytate-P content on the other. The results are summarized in Table 4. The results of this investigation suggest that it is not possible to predict phytase activity of barley, wheat or wheat by-products from total P or phytate-P content by linear regression analysis, and that no relationship can be established between phytase activity and total P or phytate-P contents.

References

Bos, K.D., 1990. Chemische achtergronden van fosforverbindingen en fytase in veevoeder. Mestproblematiek: aanpak via de voeding van varkens en pluimvee. Themadag: Veevoeding en Milieu, 19 April 1990, Lelystad, Netherlands, pp. 7–17.

Chang, C.W., 1967. Study of phytase and fluoride effects in germinating corn seeds. Cereal Chem., 44: 129–142.

Cromwell, G.L., 1992. The biological availability of phosphorus in feedstuffs for pigs. Pig News Inf., 13: 75N–78N.

Eeckhout, W. and De Paepe, M., 1991. The quantitative effects of an industrial microbial phytase and wheat phytase on the apparent phosphorus absorbability of a mixed feed by piglets. Meded. Fac. Landbouwwet. Rijksuniv. Gent, 56: 1643–1648.

Eeckhout, W. and De Paepe, M., 1992. Effect of phytase from wheat or triticale, and Ca level of a pig feed, on P digestibility with pigs. 4th Int. Conf. IMPHOS, Ghent, 8–11 September 1992. Institut Mondial du Phosphate, Casablanca, p. 66, abstract.

Fourdin, A., Camus, P., Cayron, B., Colin, C. and Pointillart, A., 1988. Amélioration de l'utilisation des phytates chez le porc par incorporation d'issues à haute activité phytasique: son de seigle, son de blé. Journ. Rech. Porc. Fr., 20: 327–332.

Haug, W. and Lantzsch, H.-J., 1983. Sensitive method for the rapid determination of phytate in cereals and cereal products. J. Sci. Food Agric., 34: 1423–1426.

Hopkins, J.R., Ballantyne, A.J. and Jones, J.L.O., 1989. Dietary phosphorus for laying hens. In: D.J.A. Cole and W. Haresign (Editors), Recent Developments in Poultry Nutrition. Butterworths, London, p. 231–238.

Jongbloed, A.W., 1987. Phosphorus in the feeding of pigs. Ph.D Thesis, Agricultural University, Wageningen, Netherlands.

Kemme, P.A. and Jongbloed, A.W., 1989. Effect van tarwe phytase op de Ca- en P-verteerbaarheid onder invloed van maalfijnheid, voorweken en pelleteren. Communication 202, IVVO, Lelystad, Netherlands, pp. 1–31.

Lantzsch, H.-J., 1989. Einführung und Stand der Diskussion zur intestinalen Verfügbarkeit des Phosphor beim Schwein. In: Mineralstoffempfehlungen beim Schwein unter besonderer Berücksichtigung der Phosphor-Verwertung. Referate der wissenschaftlichen Vortragstagung, Würzburg, 27 und 28 November 1989. Industrieverband Agrar e.V., Fachausschuss Futterphosphate, Frankfurt am Main, pp. 53–77.

Nelson, T.S., 1980. Phosphorus availability in plant origin feedstuffs for poultry and swine. Proc. 3rd Annual Int. Minerals Conf., 16 January, Orlando, FL. International Minerals & Chemical Corporation, Mundelein, IL, pp. 59–84.

Nelson, T.S., Ferrara, L.W. and Storer, N.L., 1968. Phytate phosphorus content of feed ingredients derived from plants. Poultry Sci., 47: 1372–1374.

Pointillart, A., 1991. Enhancement of phosphorus utilization in growing pigs, fed phytate-rich diets by using rye bran. J. Anim. Sci., 69: 1109–1115.

Reddy, N.R., Pierson, M.D., Sathe, S.K. and Salunkhe, D.K., 1989. In: Phytates in Cereals and Legumes. CRC Press, Boca Raton, FL, p. 7–14.

Ross, R.D., Cromwell, G.L. and Stahly, T.S., 1983. Biological availability of the phosphorus in high-moisture and pelleted corn. J. Anim. Sci., 57(Suppl. 7): 96.

Sauveur, B., 1989. Phosphore phytique et phytase dans l'alimentation des volailles. Prod. Anim., 2: 343–351.

Scheuermann, S.E., Lantzsch, H.-J. and Menke, K.H., 1988. In vitro und in vivo Untersuchungen zur Hydrolyse von Phytat. II. Aktivität pflanzlicher Phytase. J. Anim. Physiol. Anim. Nutr., 60: 64–75.

Simons, P.C.M., Terpstra, K., Teunis, G.R. and Zweers-Japing, H.M., 1981. Fytine fosfor en totaal fosfor in veevoeder-grondstoffen. Communication 335, Institute for Poultry Research, Beekbergen, Netherlands.

Sutardi and Buckle, K.A., 1986. The characteristics of soybean phytase. J. Food Biochem., 10: 197–201.

Yoshida, T., Tanaka, K. and Kasai, Z., 1975. Phytase activity associated with aleurone particles of rice grain. Agric. Biol. Chem., 39: 289–294.

Gibson, Rosalind
S.

1040

Proceedings of the Nutrition Society (2006), **65**, 160–168
DOI:10.1079/PNS2006489

Improving the bioavailability of nutrients in plant foods at the household level

Rosalind S. Gibson*, Leah Perlas† and Christine Hotz‡
Department of Human Nutrition, University of Otago, Dunedin, New Zealand

Plant foods are the major staples of diets in developing countries, in which the consumption of animal-source foods is often low because of economic and/or religious concerns. However, such plant-based diets are often associated with micronutrient deficits, exacerbated in part by poor micronutrient bioavailability. Diet-related factors in plant foods that affect bioavailability include: the chemical form of the nutrient in food and/or nature of the food matrix; interactions between nutrients and other organic components (e.g. phytate, polyphenols, dietary fibre, oxalic acid, protein, fat, ascorbic acid); pretreatment of food as a result of processing and/or preparation practices. Consequently, household strategies that reduce the content or counteract the inhibiting effects of these factors on micronutrient bioavailability are urgently needed in developing-country settings. Examples of such strategies include: germination, microbial fermentation or soaking to reduce the phytate and polyphenol content of unrefined cereal porridges used for young child feeding; addition of ascorbic acid-containing fruits to enhance non-haem-Fe absorption; heating to destroy heat-labile anti-nutritional factors (e.g. goitrogens, thiaminases) or disrupt carotenoid–protein complexes. Such strategies have been employed in both experimental isotope-absorption and community-based studies. Increases in Fe, Zn and Ca absorption have been reported in adults fed dephytinized cereals compared with cereals containing their native phytate. In community-based studies in rural Malawi improvements in dietary quality and arm-muscle area and reductions in the incidence of anaemia and common infections in young children have been observed.

Bioavailability: Plant foods: Household: Micronutrients: Phytate

In developing countries plant foods are the major staples of the diet and consumption of animal-source foods is often low because of economic and/or religious concerns. Such plant-based diets are, however, often associated with deficits in Ca, Fe, Zn and some vitamins. A major factor contributing to these deficits, particularly for diets based on unrefined cereals and legumes, is that bioavailability, which can be defined as the proportion of an ingested trace element in food that is absorbed and utilized for normal metabolic and physiological functions or storage (Jackson, 1997), is poor. Bioavailability is influenced by both dietary and host-related factors (Fairweather-Tait & Hurrell, 1996). The present review addresses the dietary factors and summarizes food preparation and processing practices that can be used in the household to enhance nutrient bioavailability. Examples of efficacy studies employing these strategies in developing countries are also given.

Diet-related factors in plant foods that affect bioavailability

Several dietary factors may affect the nutrient bioavailability of plant foods when they are consumed, including: (1) the chemical form of the nutrient in the food and the nature of the food matrix; (2) interactions occurring between nutrients and other organic components within the plant food; (3) pretreatment of the food during processing and/or preparation.

***Corresponding author:** Professor R. S. Gibson, fax +64 3 479 7958, email Rosalind.Gibson@stonebow.otago.ac.nz
†**Present address:** Food and Nutrition Research Institute, Department of Science and Technology, Bicutan, Taguig, Metro Manila, Philippines.
‡**Present address:** HarvestPlus, c/o International Food Policy Research Institute, 2003 K Street, NW, Washington DC 20006-1002, USA.

Table 1. Effects of non-competitive interactions involving organic substances on nutrient bioavailability in plant foods: inhibiting factors

Dietary component	Food sources	Main technical influences	Nutritional consequences
Phytate (*myo*-inositol hexaphosphate) plus magnesium, calcium or potassium phytate	Unrefined cereals, legumes, nuts, oil seeds	Binds certain cations to form insoluble complexes in gut	Zn, Fe, Ca and probably Mg are poorly absorbed (Heaney *et al.* 1991; Sandberg *et al.* 1999)
Soyabean protein	Some varieties of soyabeans, unfermented tofu, textured vegetable protein	Effect not explicable on basis of phytate content but instead depends on variety and processing method	Inhibits Fe and Zn absorption in some varieties. Some contain Fe as phytoferrin, which may be highly bioavailable (Murray-Kolb *et al.* 2003)
Polyphenols	Certain cereals (red sorghum), legumes (red kidney beans, black beans, black grams), spinach, betel leaves, oregano	Form insoluble complexes with Fe	Inhibit non-haem-Fe absorption
		Some polyphenols inactivate thiamin	Reduce thiamin absorption
		Bind certain salivary and digestive enzymes	Reduce digestibility of starch, protein and lipids
	Beverages: tea, coffee, cocoa, red wine	Enhance excretion of endogenous protein	Interfere with protein digestibility (Bravo, 1998)
Oxalic acid	Amaranth, spinach, rhubarb, yam, taro, sweet potato, sorrel, sesame seeds, black tea	Oxalates form insoluble complexes with Ca and possibly Fe	Reduce absorption of Ca and possibly Fe; increase urinary Ca (Savage, 2002)
Dietary fibre	Unrefined cereals, legumes, nuts, oil seeds, fruits and vegetables	Lignin and pectin bind bile acids	Reduces absorption of fats, fat-soluble vitamins and carotenoids; effects on folate bioavailability inconsistent
		Pectins, psyllium and gums retain water and form viscous solutions in gastrointestinal tract	Slows gastric emptying and digestion and absorption of nutrients (Gallagher & Schneeman, 2001)
		Dietary fibres are fermented in large intestine by microflora	SCFA produced that enhance Ca solubility (Demigne *et al.* 1995)

Sorghum, *Sorghum bicolor* (L.) Moench; red kidney beans, *Phaseolus vulgaris*; black beans, *Glycine max*; black gram, *Phaseolus mungo*; spinach, *Spinacia oleracea*; betel, *Piper betel*; oregano, *Origanum vulgare*; amaranth, *Amaranthus edulis*; rhubarb, *Rheum rhaponticum*; yam, *Dioscorea* spp.; taro, *Colocasia esculenta* var. *antiquorum*; sweet potato, *Ipomoea batatas*; sesame, *Sesamum orietale*.

In general, diet-related factors have a greater influence on the bioavailability of the micronutrients in plant foods, particularly Ca, Fe and Zn, than on the macronutrients. The absorption of Ca, Fe and Zn is particularly affected. The net effect on the nutrient bioavailability depends on the balance between factors that either inhibit or enhance nutrient absorption and/or utilization in the whole diet (Sandström, 2001). Increasingly, the influence of both synthetic micronutrient fortificants and intrinsic micronutrients on micronutrient bioavailability must be considered.

Chemical form of the nutrient and nature of the dietary matrix

The absorption and/or utilization of the trace elements Fe, Se and Zn, and of the vitamins niacin, provitamin A carotenoids and folate are most affected by their chemical form. Of these micronutrients, the bioavailability of the two forms of Fe in food (haem- and non-haem-Fe; Hallberg, 1981), certain isomeric forms of carotenoids (Yeum & Russell, 2002) and folate polyglutamates relative to monoglutamates (McNulty & Pentieva, 2004) have been reviewed in detail elsewhere.

The food matrix probably has the greatest effect on the absorption of provitamin A carotenoids and folate, both of which can be entrapped in the insoluble plant matrix, thus reducing their bioavailability. For example, β-carotene in raw carrots or lycopene in fresh tomato juice are poorly absorbed compared with pure β-carotene dissolved in oil (Yeum & Russell, 2002), whereas the bioavailability of folate from chopped spinach (*Spinacia oleracea*) is higher than that from whole spinach leaves (Castenmiller *et al.* 2000).

Interactions between nutrients themselves and with other organic components in the plant food

Direct competitive interactions between two (or more) inorganic nutrients with similar physico-chemical properties that share the same absorptive pathways are unlikely in plant foods because the intrinsic micronutrient levels are low. Even when plant staples serve as vehicles for fortification the risk of such interactions is small because of the presence of dietary ligands in food (Sandström *et al.* 1985).

In contrast, there are several organic components in plant foods that may form insoluble or soluble complexes with certain micronutrients in the acid pH of the stomach and gastrointestinal tract, thus inhibiting or facilitating their absorption. Re-absorption of endogenously-excreted Ca, Zn and Cu may also be affected (Sandström, 2001; Manary *et al.* 2002*a*). Examples of these non-competitive interactions are summarized in Tables 1 and 2; both inhibiting and enhancing factors are listed.

Table 2. Effects of non-competitive interactions involving organic substances on nutrient bioavailability in plant foods: enhancing factors

Dietary component	Food sources	Main technical influences	Nutritional consequences
Organic acids (citric, lactic, acetic, butyric, propionic and formic acids)	Fermented milk products (e.g. yoghurts), vegetables, sauerkraut, soya sauces, fermented cereals (e.g. Tobwa)	May form soluble ligands with some trace minerals in the gastrointestinal tract	Enhance absorption of Zn and possibly Fe (Sandström, 1997; Teucher *et al.* 2004)
Ascorbic acid	Citrus fruits and juices Other fruits: guavas, mango, papayas, kiwi, strawberries Vegetables: tomato, asparagus, Brussels sprouts, spinach etc.	Reduces Fe^{3+} to more soluble Fe^{2+}; forms Fe–ascorbate chelate	Enhances non-haem-Fe absorption (Teucher *et al.* 2004) Counteracts inhibitory effect of phytate
		May increase stability of folate during food processing and digestion	May enhance folate bioavailability (McNulty & Pentieva, 2004) May enhance or inhibit Se absorption, depending on the chemical form (Mutanen & Mykkanen, 1985; Levander, 1987). Ascorbic acid may also enhance Cr absorption (Offenbacher, 1994)
Protein		Amount and type (e.g. animal protein) form soluble ligands with Zn, Fe and Cu	Enhance absorption of Zn, Fe and Cu (Bjorn-Rasmussen & Hallberg, 1979; Turnlund *et al.*1983; Lönnerdal, 2000) Increase urinary Ca excretion (Heaney, 2000)
Fat	Oil seeds, nuts	Products of fat digestion + bile salts solubilize fat-soluble vitamins and carotenoids in intestinal milieu	Enhance absorption of fat-soluble vitamins and provitamin A carotenoids (Yeum & Russell, 2002)

Guava, *Psidium guajava* L.; mango, *Mangifera indica* L.; papaya, *Carica papaya*; kiwi, *Actinidia deliciosa*; strawberry, *Fragaria X ananassa*; asparagus, *Asparagus officinalis*; spinach, *Spinacia oleracea*.

Pretreatment of food in the household as a result of processing and/or preparation practices

The adverse effects of some of the organic components in plant foods on nutrient bioavailability can be reduced by household food processing and preparation practices; these practices are summarized in Table 3 and will be discussed.

Thermal processing. This treatment generally enhances the digestibility of proteins and carbohydrates, although if Maillard browning occurs in baked foods protein quality and digestibility may be reduced (Table 3). Thermal processing may also improve the bioavailability of certain vitamins and I, in some cases because of the destruction of heat-labile anti-nutritional factors (Erdman & Pneros-Schneier, 1994). For example, thiaminases in Brussels sprouts and red cabbage, which catalyse the cleavage of thiamin, are destroyed by cooking (Hilker & Somogyi, 1982). Cooking also destroys the goitrogens present in cabbage, Brussels sprouts, turnips, sweet potatoes (*Ipomoea batatas*), millet (hnatherum hymenoides), cassava (*Manihot esculenta* Crantz) and beans. Such goitrogens block the absorption or utilization of I and thus its uptake into the thyroid gland (Gaitan, 1990).

Thermal processing can also enhance the bioavailability of vitamins such as thiamin, vitamin B_6, niacin and carotenoids by releasing them from entrapment in the plant matrix. For example, greater increases in total serum β-carotene and serum lycopene have been reported after eating cooked carrots and spinach (Rock *et al.* 1998) and cooked tomatoes (van het Hof *et al.* 2000) compared with levels when they are consumed raw. This effect is attributed to softening or disruption of plant cell walls and the disruption of carotenoid–protein complexes so that the carotenoids are more available in the intestinal lumen for absorption (Yeum & Russell, 2002).

Reports on the effects of thermal processing on phytate degradation are inconsistent and depend on the plant species, temperature and/or pH. Hurrell *et al.* (2002) have reported that home thermal processing does not degrade phytate sufficiently to improve Fe absorption from home-prepared pancakes or chapattis. Other investigators (Kataria *et al.* 1989; Marfo *et al.* 1990; Khan *et al.* 1991) have suggested that conventional heat treatments such as boiling may induce moderate losses (i.e. 5–15%) of phytic acid in tubers (Marfo *et al.* 1990) and some legumes (Kataria *et al.* 1989) and cereals (Khan *et al.* 1991). Much higher losses have been reported after boiling white rice (i.e. 70%; Perlas & Gibson, 2002), attributed mainly to leaching of water-soluble sodium, potassium or magnesium phytate into the discarded cooking water.

Germination. This process, also termed malting, leads to an increase in phytase activity in certain cereals (e.g. maize, millet and sorghum (*Sorghum bicolor* (L.) Moench)), in most legumes and in oil seeds through *de novo* synthesis and/or activation of intrinsic phytase.

Table 3. Influence of household food processing and preparation methods on bioavailability of nutrients in plant foods

Processing method	Main technical influences	Nutritional consequences
Thermal processing	Releases some vitamins from poorly-digested complexes	Enhances bioavailability of vitamin B_6, niacin, folate and certain carotenoids
	Inactivates heat-labile anti-nutritional factors (e.g. protease inhibitors, α-amylase inhibitors, lectins, thiaminases, goitrogens)	Enhances digestibility of proteins and starch Enhances bioavailability of thiamine and I
	May degrade phytate, depending on temperature	May enhance Zn, Fe and Ca bioavailability
	Gelatinizes starch	Enhances digestibility
Baking	Induces Maillard browning in foods containing reducing sugars	Destroys basic essential amino acids lysine, arginine and methionine Reduces protein quality and protein digestibility
Boiling	Reduces oxalate content	Enhances Ca absorption
Germination and malting	Increases phytase activity via *de novo* synthesis or activation of endogenous phytase	Induces hydrolysis of phytate and hence increases Zn, Fe, Ca, and Mg absorption
	Reduces polyphenol content of some legumes (e.g. *Vicia faba*)	Enhances non-haem-Fe absorption
	Increases α-amylase content of cereals (e.g. sorghum and millet)	Facilitates starch digestion; may increase non-haem-Fe absorption through a change in consistency
Village-based milling or home pounding	Reduces phytate content of cereals with phytate localized in outer aleurone layer (rice, wheat, sorghum) or in germ (maize)	Enhances bioavailability of Zn, Fe, and Ca, although mineral content simultaneously reduced
Microbial fermentation	Induces hydrolysis of phytate by microbial phytase	Enhances bioavailability of Zn, Fe and Ca
	Increases content of organic acids	May form soluble ligands with non-haem-Fe and Zn, and enhance bioavailability
	Microbial enzymes may destroy protein inhibitors that interfere with N digestibility	May improve protein quality in maize, legumes, groundnuts and pumpkin and millet seeds

Sorghum, *Sorghum bicolor* (L.) Moench; millet, *Achnatherum hymenoides*; groundnut, *Apios americana* Medic.; pumpkin, *Cucurbita Pepo*.

Tropical cereals such as maize and sorghum have a lower endogenous phytase activity than do rye, wheat, triticale (*X Triticosecale* Wittmack), buckwheat (*Fagopyrum esculentum*) or barley (Egli *et al.* 2002). Phytases (*myo*-inositol hexakisphosphate 3-phosphohydrolase) hydrolyse phytic acid (*myo*-inositol hexaphosphate) as well as the salts, magnesium, calcium or potassium phytate, to yield inorganic orthophosphate and *myo*-inositol via intermediate *myo*-inositol phosphates (pentaphosphates to monophosphates). The rate of phytate hydrolysis varies with the species and variety, as well as the stage of germination, pH, moisture content, temperature (optimal range 45–57°C), solubility of phytate and the presence of certain inhibitors (Cheryan, 1980; Egli *et al.* 2003). Egli *et al.* (2002) have observed that during germination rice, millet and mungbean (*Vigna radiata* L.) have the largest reductions in phytate content, ranging from 50% (for mungbeans) to 64%.

Such reductions in the levels of higher inositol phosphates can have a major impact on mineral bioavailability because they form complexes with divalent and trivalent cations (particularly Zn, Fe, Ca and Mg) at the physiological pH conditions of the small intestine, making them unavailable for absorption (Oberleas & Harland, 1981; Hurrell, 2003; Egli *et al.* 2004; Hurrell *et al.* 2004); the higher inositol phosphates have no effect on Cu absorption (Egli *et al.* 2004). The hexa- and pentaphosphates may also complex endogenously-secreted minerals such as Zn (Sandström, 1997; Manary *et al.* 2000) and Ca (Morris & Ellis, 1985), making them unavailable for re-absorption into the body. In contrast, *myo*-inositol phosphates with less than five phosphate groups (i.e. monophosphates to tetraphosphates) do not have a negative effect on Zn absorption (Lönnerdal *et al.* 1989) and those with less than three phosphate groups do not inhibit non-haem-Fe absorption (Sandberg *et al.* 1999). There appears to be no adaptation to the inhibitory effect of a high-phytate diet on absorption of Fe (Brune *et al.* 1989) or exogenous Zn, although endogenous excretion of faecal Zn may be decreased in healthy subjects (Sandström *et al.* 1993).

Certain tannins and other polyphenols in legumes (e.g. *Vicia faba*) and red sorghum may also be reduced during germination as a result of the formation of polyphenol complexes with proteins and the gradual degradation of oligosaccharides (Camacho *et al.* 1992). Naturally-occurring polyphenol oxidase extracted from banana (*Musa X paridasiaca* L.) or avocado (*Persea americana* Mill.) and subsequently reduced by dialysis has also been used to reduce the polyphenol content of high-tannin sorghum (Matuschek & Svanberg, 2004).

α-Amylase activity is also increased during germination of cereals, particularly sorghum and millet. This enzyme hydrolyses amylase and amylopectin to dextrins and maltose, thus reducing the viscosity of thick cereal porridges (Gibson & Ferguson, 1998). A threefold increase in Fe absorption has been reported in amylase-treated

roller-dried rice cereal compared with the untreated roller-dried cereal, which is associated with the viscosity changes induced by α-amylase (Hurrell *et al.* 2002).

Milling or household pounding. In developing countries this process is used to remove the bran and/or germ from cereals such as wheat, sorghum, rice and maize. These processes also reduce the phytate content if the phytate is localized in the outer aleurone layer (e.g. rice, sorghum and wheat) or in the germ (i.e. maize; O'Dell *et al.* 1972). Milling can thus enhance mineral bioavailability, although the content of minerals and some vitamins of the milled cereals are simultaneously reduced. As a result, in some countries milled cereal flours are enriched to compensate for the micronutrients lost.

Microbial fermentation. Higher inositol phosphates are hydrolysed to lower inositol phosphates through the action of microbial phytase enzymes (Sandberg, 1991). These micro-organisms may occur naturally on the surface of cereals and legumes or can be introduced via inoculation with a starter culture. The extent of the reduction in higher inositol phosphate levels depends on the type of fermentation; sometimes ≥90% phytate can be removed by fermentation of maize, soyabeans, sorghum, cassava, cocoyam (*Colocasia esculenta*), cowpeas (*Vigna unguiculata*) and lima beans (*Phaseolus limensis*; Marfo *et al.* 1990; Sandberg, 1991; Svanberg *et al.* 1993). Fermentation of bread dough with yeast also induces phytate hydrolysis, although if Ca is added as a fortificant phytase activity in yeast is inhibited (Hallberg *et al.* 1991).

Organic acids are also produced during fermentation and can potentially enhance Fe and Zn absorption via the formation of soluble ligands (Charlton, 1983; Hazell & Johnson, 1987; Walter *et al.* 1998; Porres *et al.* 2001). They may also complex some of the minerals bound to phytate molecules, rendering them more susceptible to hydrolysis via phytase enzymes (Maenz *et al.* 1999), while simultaneously generating a pH that optimizes the activity of intrinsic phytase from cereal or legume flours (Porres *et al.* 2001). In contrast, organic acids may have an inhibitory effect on the activity of the intestinal brush-border enzyme glutamate caroboxypeptidase II, attributed to a lowering of the pH (McNulty & Pentieva, 2004).

Improvements in protein quality have also been documented after fermenting blended mixtures of plant-based complementary foods based on maize and legumes (Nnam, 1999), groundnuts (*Apios americana* Medic), pumpkin (*Cucurbita Pepo*) and millet seeds (Ezeji & Ojimelukwe, 1993) and cereal and soyabean blends (Sanni *et al.* 1999). Such improvements may be associated with the destruction by microbial enzymes of protein inhibitors that interfere with N digestibility (Nnam, 1999), or from the ability of starter cultures to synthesize certain amino acids (Odunfa, 1985).

Soaking. The soaking of cereal and most legume flours in water results in the passive diffusion of water-soluble sodium, potassium or magnesium phytate, which can be removed by decanting the water (De Boland *et al.* 1975; Chang *et al.* 1977; Perlas & Gibson, 2002). Nevertheless, the extent of the removal depends on the species, pH and length and conditions of soaking. Reductions in the penta- and hexaphosphates of 47, 57 and 98% respectively have been reported for mungbean, maize and rice flours after soaking (Hotz & Gibson, 2001; Perlas & Gibson, 2002; Temple *et al.* 2002); however, no reductions are achieved after soaking whole mungbeans and maize kernels for 6 h (Perlas & Gibson, 2002; Temple *et al.* 2002). Reductions in the content of other anti-nutrients such as glycosides, alkaloids, oligosaccharides, saponins, polyphenols and oxalates may also occur (Chang *et al.* 1977).

Application of household processing and preparation strategies to enhance nutrient bioavailability of plant foods in developing countries

There is an urgent need to improve the nutritional quality of plant-based foods in developing countries, especially those used for feeding infants and young children. In the past the emphasis has been on enhancing their protein quality by blending cereals with legumes (usually in ratios of 70:30 (w/w) to provide the optimal mixture of essential amino acids), and problems associated with mineral bioavailability have often been ignored. This approach is unfortunate because many of these cereal–legume blends have a very high phytate content and high phytate:Zn and phytate:Fe molar ratios.

The inhibitory effect of phytate on Zn absorption follows a dose-dependent response (Navert *et al.* 1985) and the molar ratio for phytate:Zn in the diet is used to estimate the proportion of absorbable Zn (Oberleas & Harland, 1981). For Fe, phytic acid begins to lose its inhibitory effect on Fe absorption when ratios are <1·0:1·0 and it still inhibits Fe absorption at ratios as low as 0·2:1·0 (Hallberg *et al.* 1989; Hurrell *et al.* 1992).

Both *in vitro* and *in vivo* methods have been used to estimate the bioavailability of Fe, Zn and Ca in plant foods. Some *in vitro* methods are based on a two-stage simulated digestive process of the food or test meal, followed by determination of the dialysable Fe, Zn or Ca released. In general, the magnitude of the responses measured using these methods are not the same as those observed in human subjects, but some of these methods have been used to rank foods with respect to the effect of processing and preparation practices on mineral bioavailability (Latunde-Dada *et al.* 1998). For example, increases in dialysable Fe, Zn and Ca have been reported after processing porridges prepared from legumes such as chickpea (*Cicer arietinum*) and black gram (*Phaseolus mungo*) flours and/or cereal flours such as maize, sorghum and rice by fermentation with a starter culture (Svanberg *et al.* 1993; Jood & Kapoor, 1997; Sharma & Khetarpaul, 1998) and/or by soaking and germination (Svanberg *et al.* 1993; Mbithi-Mwikya *et al.* 2002).

More recently, cultured human intestinal cells (i.e. a Caco-2 cell *in vitro* model) have been developed for studying the characteristics of Fe, and in some cases Zn and Ca, transport by the intestinal absorptive epithelium (Han *et al.* 1994; Wortley *et al.* 2005). However, currently, there is no standardized Caco-2 cell method and the magnitude of the effects observed appears to depend on the procedures used, making inter-laboratory comparisons difficult. Studies have applied this technique to screen and

rank selected staple-food genotypes for bioavailable Fe (Van Campen & Glahn, 1999), but further development of the Caco-2 cell model is needed before it can be used to determine the bioavailability of Zn and provitamin A carotenoids in plant foods.

To date, *in vivo* isotope studies to measure the bioavailability of Fe or Zn in plant foods modified using household strategies to enhance Fe or Zn absorption are limited. Studies on adults have demonstrated increases in Fe and Zn absorption when they are fed porridges used for infant feeding that have been prepared from a variety of dephytinized cereals as compared with those containing their native phytate. In a study of Fe absorption (Hurrell *et al.* 2003) phytic acid was degraded by the addition of exogenous commercial phytase enzyme, whereas in a Zn-absorption study (Egli *et al.* 2004) dephytinization of a wheat–soyabean blend was achieved by the use of phytase naturally occurring in wholegrain cereals (Egli *et al.* 2003). In a hospital-based study in Malawi (Manary *et al.* 2000) the reduction of phytate in a maize–soyabean porridge by using a commercial phytase enzyme has been shown to increase fractional and total Zn absorption and reduce endogenous Zn losses in children recovering from tuberculosis but has no effect on Zn absorption in apparently-well children.

Only a few community-based efficacy trials have assessed the impact of food-based strategies in the household designed to enhance nutrient bioavailability. Early studies focused on improving the bioavailability of non-haem-Fe have been reviewed by Ruel (2001). More recent studies have measured various outcomes, including absorption *in vivo* using stable isotopes of Fe (Diaz *et al.* 2003), nutrient adequacy (Gibson *et al.* 2003; Hotz & Gibson, 2005) and biochemical and/or functional health outcomes (Dewey *et al.* 1997; Manary *et al.* 2002*b*; Yeudall *et al.* 2002; Garcia *et al.* 2003; Mamiro *et al.* 2004).

In a recent study in rural Mexico no improvement in biochemical Fe status was observed among Fe-deficient women receiving 25 mg ascorbic acid from fresh lime juice twice daily on 6 d/week for 8 months compared with those receiving a placebo (Garcia *et al.* 2003), despite a twofold increase in Fe absorption, based on earlier stable-isotope results (Diaz *et al.* 2003). Similarly, after withholding coffee for 5 months no positive effect on Fe status was observed among Fe-deficient Guatemalan toddlers, except among those taking Fe supplements (Dewey *et al.* 1997), attributed to the relatively small amount of coffee ingested. Furthermore, in a large community-based double-blind randomized controlled trial in Tanzania (Mamiro *et al.* 2004), in which a processed complementary food (based on soaked and germinated finger millet (*Eleusine coracana*) and kidney beans (*Phaseolus vulgaris*), with roasted peanuts (*Arachis hypogea*) and mango (*Mangifera indica* L.) puree) and an identical unprocessed blend were fed to 6-month-old infants (n 309) for 6 months, no significant differences were found between the two groups at the end of the study in either Fe status, as measured by Hb and zinc protoporphyrin, or growth, perhaps in part because there was only a 34% reduction in the phytate content of the processed complementary food.

These results emphasize that an integrated approach that combines a variety of the strategies discussed earlier, including the addition of even a small amount of animal-source foods, is probably the best strategy to improve the nutrient bioavailability in diets based on plant foods. Two such community-based efficacy trials have been undertaken among weanlings and young children in rural Malawi. Both trials employed a quasi-experimental design with non-equivalent control groups and used a participatory approach to implement a combination of food-based strategies in the household to enhance their awareness, feasibility and acceptability to caregivers in the local community. Details of the strategies and their implementation have been published (Gibson *et al.* 1998, 2003; Yeudall *et al.* 2002, 2005; Hotz & Gibson, 2005); their efficacy was evaluated by determining knowledge, trial and adoption of the new practices and comparing dietary quality and the adequacy of the energy and nutrient intakes of the intervention and control groups post-intervention (Gibson *et al.* 2003; Hotz & Gibson, 2005) and, for the children only, changes in growth and body composition, morbidity and Hb and hair Zn concentrations (Yeudall *et al.* 2002).

Results of the Malawian studies suggest that a combination of household food-based strategies, comparable with those outlined earlier, can be designed to be feasible and acceptable to caregivers of weanlings and children in subsistence farming settings, although on-going nutrition education and social marketing efforts are required to enhance their adoption and to empower the community to sustain them. Nevertheless, even when such a combination of strategies is used, they are probably not sufficient to overcome the deficits in Ca, Fe and Zn, and possibly other micronutrients that exist in complementary diets in low-resource settings. In such cases additional strategies to enhance the micronutrient adequacy of these complementary diets are urgently required. Possible strategies include fortifying cereal-based dephytinized complementary foods with a fortificant containing balanced and physiological levels of multi-micronutrients. In the long term, biofortification of staple cereals involving strategies to enhance both micronutrient density and bioavailability may become a feasible option for improving the micronutrient status of the entire household in poor-resource settings.

References

Bjorn-Rasmussen E & Hallberg L (1979) Effect of animal proteins on the absorption of food iron in man. *Nutrition and Metabolism* **23**, 192–202.

Bravo L (1998) Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition Reviews* **56**, 317–333.

Brune ML, Rossander L & Hallberg L (1989) No intestinal adaptation to a high-phytate diet. *American Journal of Clinical Nutrition* **49**, 542–545.

Camacho L, Sierra C, Campos R, Guzman E & Marcus D (1992) Nutritional changes caused by germination of legumes commonly eaten in Chile. *Archivos Latinoamericanos de Nutricion* **42**, 283–290.

Castenmiller JJM, van de Poll, West CE, Brouwer IA, Thomas CMG & van Dusseldorp M (2000) Bioavailability of folate from processed spinach in humans. *Annals of Nutrition and Metabolism* **44**, 163–169.

Chang R, Schwimmer S & Burr HK (1977) Phytate: removal from whole dry beans by enzymatic hydrolysis and diffusion. *Journal of Food Science* **42**, 1098–1101.

Charlton RW (1983) The effects of organic acids, phytates and polyphenols on the absorption of iron from vegetables. *British Journal of Nutrition* **49**, 331–342.

Cheryan M (1980) Phytic acid interactions in food systems. *CRC Critical Reviews in Food Science and Nutrition* **13**, 297–335.

De Boland AR, Garner GB & O'Dell BL (1975) Identification and properties of phytate in cereal grains and oilseed products. *Journal of Agricultural and Food Chemistry* **23**, 1186–1189.

Demigne C, Levrat MA, Younes H & Remesy C (1995) Interactions between large intestine fermentation and dietary calcium. *European Journal of Clinical Nutrition* Suppl **3**, S235–S238.

Dewey KG, Romero-Abal ME, Quan de Serrano J, Bulux J, Peerson JM, Engle P & Solomons N (1997) Effects of discontinuing coffee intake on iron status of iron-deficient Guatemalan toddlers: A randomized intervention study. *American Journal of Clinical Nutrition* **66**, 168–176.

Diaz M, Rosado JL, Allen LH, Abrams SA & Garcia OP (2003) The efficacy of a local ascorbic-acid rich food to improve iron absorption from Mexican diets: a field study using stable isotopes. *American Journal of Clinical Nutrition* **78**, 436–440.

Egli I, Davidsson L, Juillerat M-A, Barclay D & Hurrell RF (2002) The influence of soaking and germination on the phytase activity and phytic acid content of grains and seeds potentially useful for complementary feeding. *Journal of Food Science* **67**, 3484–3488.

Egli I, Davidsson L, Juillerat M-A, Barclay D & Hurrell RF (2003) Phytic acid degradation in complementary foods using phytases naturally occurring in whole grain cereals. *Journal of Food Science* **68**, 1855–1859.

Egli I, Davidsson L, Zeder C, Walczyk T & Hurrell R (2004) Dephytinization of a complementary food based on wheat and soy increases zinc, but not copper apparent absorption in adults. *Journal of Nutrition* **134**, 1077–1080.

Erdman JW & Pneros-Schneier AG (1994) Factors affecting nutritive value in processed foods. In *Nutrition in Health and Disease*, pp. 1569–1578 [ME Shils, JA Olson and M Shile, editors]. Philadelphia, PA: Lea & Febiger.

Ezeji C & Ojimelukwe PC (1993) Effect of fermentation on the nutritional quality and functional properties of infant food formulations prepared from bamarra-groundnut, fluted-pumpkin and millet seeds. *Plant Foods for Human Nutrition* **44**, 267–276.

Fairweather-Tait S & Hurrell RF (1996) Bioavailability of minerals and trace elements. *Nutrition Research* **9**, 295–324.

Gaitan E (1990) Goitrogens in food and water. *Annual Review of Nutrition* **10**, 21–39.

Gallagher DD & Schneeman BO (2001) Dietary fiber. In *Present Knowledge in Nutrition*, pp. 83–91 [BA Bowman and RM Russell, editors]. Washington, DC: International Life Sciences Institute.

Garcia OP, Diaz M, Rosado JL & Allen LH (2003) Ascorbic acid from lime juice does not improve the iron status of iron-deficient women in rural Mexico. *American Journal of Clinical Nutrition* **78**, 267–273.

Gibson RS & Ferguson EL (1998) Food processing methods for improving the zinc content and bioavailability of home-based and commercially available complementary foods. *Micronutrient Interactions: Impact on Child Health and Nutrition*, pp. 50–57. Washington, DC: International Life Sciences Institute Press.

Gibson RS, Yeudall F, Drost N, Mitimuni B & Cullinan T (1998) Dietary interventions to prevent zinc deficiency. *American Journal of Clinical Nutrition* **68**, 484S–487S.

Gibson RS, Yeudall F, Drost N, Mitimuni BM & Cullinan TR (2003) Experiences of a community-based dietary intervention to enhance micronutrient adequacy of diets low in animal source foods and high in phytate: a case study in rural Malawian children. *Journal of Nutrition* **133**, 3992S–3999S.

Hallberg L (1981) Bioavailability of dietary iron in man. *Annual Review of Nutrition* **1**, 123–147.

Hallberg L, Brune M, Erlandsson M, Sandberg AS & Rossander-Hulten L (1991) Calcium: effect of different amounts on non-heme and heme-iron absorption in humans. *American Journal of Clinical Nutrition* **53**, 112–119.

Hallberg L, Brune M & Rossander L (1989) Iron absorption in man: ascorbic acid and dose-dependent inhibition by phytate. *American Journal of Clinical Nutrition* **49**, 140–144.

Han O, Failla ML, Hill AD, Morris ER & Smith JC (1994) Inositol phosphates inhibit uptake and transport of iron and zinc by a human intestinal cell line. *Journal of Nutrition* **124**, 580–587.

Hazell T & Johnson IT (1987) In vitro estimation of iron availability from a range of plant foods: influence of phytate, ascorbate and citrate. *British Journal of Nutrition* **57**, 223–233.

Heaney RP (2000) Dietary protein and phosphorus do not affect calcium absorption. *American Journal of Clinical Nutrition* **72**, 758–761.

Heaney RP, Weaver CM & Fitzsimmons ML (1991) Soybean phytate content: effect on calcium absorption. *American Journal of Clinical Nutrition* **53**, 745–747.

Hilker DM & Somogyi JC (1982) Antithiamins of plant origin: their chemical nature and mode of action. *Annals of the New York Academy of Sciences* **378**, 137–145.

Hotz C & Gibson RS (2001) Assessment of home-based processing methods to reduce phytate content and phytate/zinc molar ratios of white maize (*Zea mays*). *Journal of Agricultural and Food Chemistry* **49**, 692–698.

Hotz C & Gibson RS (2005) A participatory nutrition education intervention improves the adequacy of complementary diets of rural Malawian children: a pilot study. *European Journal of Clinical Nutrition* **59**, 226–237.

Hurrell RF (2003) Influence of vegetable protein sources on trace element and mineral bioavailability. *Journal of Nutrition* **133**, 2973S–2977S.

Hurrell RF, Juillerat MA, Reddy MB, Lynch SR, Dassenko SA & Cook JD (1992) Soy protein, phytate, and iron absorption in humans. *American Journal of Clinical Nutrition* **56**, 573–578.

Hurrell RF, Lynch S, Bothwell T, Cori H, Glahn R, Hertrampf E *et al.* (2004) Enhancing the absorption of fortification iron. A SUSTAIN Task Force report. *International Journal of Vitamin and Nutrition Research* **74**, 387–401.

Hurrell RF, Reddy MB, Burri J & Cook JD (2002) Phytate degradation determines the effect of industrial processing and home cooking on iron absorption from cereal-based foods. *British Journal of Nutrition* **88**, 117–123.

Hurrell RF, Reddy MB, Juillerat M-A & Cook JD (2003) Degradation of phytic acid in cereal porridges improves iron absorption by human subjects. *American Journal of Clinical Nutrition* **77**, 1213–1219.

Jackson M (1997) The assessment of bioavailability of micronutrients: introduction. *European Journal of Clinical Nutrition* **51**, S1–S2.

Jood S & Kapoor AC (1997) Improvement in bioavailability of minerals of chickpea and blackgram cultivars through

processing and cooking methods. *International Journal of Food Sciences and Nutrition* **48**, 307–312.
Kataria A, Chauhan BM & Gandhi S (1989) Antinutrients and protein digestibility (in vitro) of mungbean as affected by domestic processing and cooking. *Food Chemistry* **32**, 9–17.
Khan N, Zaman R & Elahi M (1991) Effect of heat treatment on the phytic acid content of maize products. *Journal of the Science of Food and Agriculture* **54**, 153–156.
Latunde-Dada GO, Bianchi MLP & de Oliviera JED (1998) On the methods for studying the mechanisms and bioavailability of iron. *Nutrition Reviews* **56**, 76–80.
Levander OA (1987) A global view of human selenium nutrition. *Annual Review of Nutrition* **7**, 227–250.
Lönnerdal B (2000) Dietary factors influencing zinc absorption. *Journal of Nutrition* **130**, 1378S–1383S.
Lönnerdal B, Sandberg A-S, Sandström B & Kunz C (1989) Inhibitory effects of phytic acid and other inositol phosphates on zinc and calcium absorption in suckling rats. *Journal of Nutrition* **119**, 211–214.
McNulty H & Pentieva K (2004) Folate bioavailability. *Proceedings of the Nutrition Society* **63**, 529–536.
Maenz DD, Engele-Schaan CM, Newkirk RW & Classen HL (1999) The effect of mineral chelators on the formation of phytase-resistant and phytase-susceptible forms of phytic acid in solution and in slurry of canola meal. *Animal Feed Science and Technology* **81**, 177–192.
Mamiro PS, Kolsteren PW, van Camp JH, Roberfroid DA, Tatala S & Opsomer AS (2004) Processed complementary food does not improve growth or hemoglobin status of rural Tanzanian infants from 6–12 months of age in Kilosa District, Tanzania. *Journal of Nutrition* **134**, 1084–1090.
Manary MJ, Hotz C, Krebs NF, Gibson RS, Westcott JE, Arnold T, Broadhead RL & Hambidge KM (2000) Dietary phytate reduction improves zinc absorption in Malawian children recovering from tuberculosis but not in well children. *Journal of Nutrition* **130**, 2959–2964.
Manary MJ, Hotz C, Krebs NF, Gibson RS, Westcott JE, Broadhead RL & Hambidge KM (2002*a*) Zinc homeostasis in Malawian children consuming a high-phytate, maize-based diet. *American Journal of Clinical Nutrition* **75**, 1057–1061.
Manary MJ, Krebs NF, Gibson RS, Broadhead RL & Hambidge KM (2002*b*) Community-based dietary phytate reduction and its effect on iron status in Malawian children. *Annals of Tropical Paediatrics* **22**, 133–136.
Marfo EK, Simpson BK, Idowu JS & Oke OL (1990) Effect of local food processing on phytate levels in cassava, cocoyam, yam, maize, sorghum, rice, cowpea, and soybean. *Journal of Agricultural and Food Chemistry* **38**, 1580–1585.
Matuschek E & Svanberg U (2004) Enzymatic treatment of high-tannin sorghum increases the bioaccessibility of iron. Abstr Th37. *Report of the 2004 International Nutritional Anemia Consultative Group Symposium: Iron Deficiency in Early Life: Challenges and Progress, Lima, Peru*, p. 60. Washington, DC: INACG; available at http://inacg.ilsi.org/file/INACGPeru-FINALREPORT.pdf
Mbithi-Mwikya S, van Camp J, Mamiro PRS, Ooghe W, Kolsteren P & Huyghebaert A (2002) Evaluation of the nutritional characteristics of a finger millet based complementary food. *Journal of Agricultural and Food Chemistry* **50**, 3030–3036.
Morris ER & Ellis R (1985) Bioavailability of dietary calcium. In *Nutritional Bioavailability of Calcium*, pp. 63–72 [C Kies, editor]. Washington, DC: American Chemical Society.
Murray-Kolb LE, Welch R, Theil EC & Beard JL (2003). Women with low iron stores absorb iron from soybeans. *American Journal of Clinical Nutrition* **77**, 180–184.
Mutanen M & Mykkanen HM (1985) Effect of ascorbic acid supplementation on selenium bioavailability in humans. *Human Nutrition Clinical Nutrition* **39**, 221–226.
Navert B, Sandstrom B & Cederblad A (1985) Reduction of the phytate content of bran by leavening in bread and its effect on zinc absorption in man. *British Journal of Nutrition* **53**, 47–53.
Nnam NM (1999) Nitrogen and mineral utilization of young children fed blends of fermented or unfermented corn (Zea may. L), African yam bean (Spenostylis stenocarpa) and cowpea (Vigna unguiculata) *Ecology of Food and Nutrition* **38**, 21–34.
Oberleas D & Harland BF (1981) Phytate content of foods: effect on dietary zinc bioavailability. *Journal of the American Dietetic Association* **79**, 433–436.
O'Dell BL, de Bowland AR & Koirtyohann SR (1972) Distribution of phytate and nutritionally important elements among the morphological components of cereal grains. *Journal of Agricultural and Food Chemistry* **20**, 718–721.
Odunfa SA (1985) African fermented foods. In *Microbiology of Food Fermentations*, pp. 151–191 [BJB Wood, editor]. London: Applied Science Publishers.
Offenbacher E (1994) Promotion of chromium absorption by ascorbic acid. *Trace Elements and Electrolytes* **11**, 178–181.
Perlas L & Gibson RS (2002) Use of soaking to enhance the bioavailability of iron and zinc from rice-based complementary foods used in the Philippines. *Journal of the Science of Food and Agriculture* **82**, 1115–1124.
Porres JM, Etcheverry P & Miller DD (2001) Phytate and citric acid supplementation in whole-wheat bread improves phytate-phosphorus release and iron dialyzability. *Journal of Food Science* **66**, 614–619.
Rock CL, Lovalvo JL, Emenhiser C, Ruffin MT, Flatt SW & Schwartz SJ (1998) Bioavailability of β-carotene is lower in raw than in processed carrots and spinach in women. *Journal of Nutrition* **128**, 913–916.
Ruel MT (2001) *Can Food-based Strategies Help to Reduce Vitamin A and Iron Deficiencies? A Review of Recent Evidence. Food Policy Review* no. 5. Washington, DC: International Food Policy Research Institute.
Sandberg A-S (1991) The effect of food processing on phytate hydrolysis and availability of iron and zinc. In *Nutritional and Toxicological Consequences of Food Processing*, pp. 499–508 [M Friedman, editor]. New York: Plenum Press.
Sandberg A-S, Brune M, Carlsson N-G, Hallberg L, Skoglund E & Rossander-Hulthen L (1999) Inositol phosphates with different numbers of phosphate groups influence iron absorption in humans. *American Journal of Clinical Nutrition* **70**, 240–246.
Sandström B (1997) Bioavailability of zinc. *European Journal of Clinical Nutrition* **51**, S17–S19.
Sandström B (2001) Micronutrient interactions: effects on absorption and bioavailability. *British Journal of Nutrition* **85**, S181–S185.
Sandström B, Davidsson L, Cederblad A & Lönnerdal B (1985) Oral iron, dietary ligands and zinc absorption. *Journal of Nutrition* **115**, 411–414.
Sandström B, Madsen E & Cederblad A (1993) Rate of endogenous zinc excretion at high phytate intake. In *Proceedings of the 8th International Symposium on Trace Elements in Man and Animals*, pp. 620–624 [M Anke, D Meissner and CF Mills, editors]. Gersdorf, Germany: Verlag Media Touristik.
Sanni A, Onilude AA & Ibidapo OT (1999) Biochemical composition of infant weaning food fabricated from fermented blends of cereal and soybean. *Food Chemistry* **65**, 35–39.
Savage GP (2002) Oxalates in human foods. *Proceedings of the Nutrition Society of New Zealand* **27**, 4–24.
Sharma A & Khetarpaul N (1998) Development of products incorporating fermented rice-legume-whey blends: effect on

phytic acid content and availability (*in vitro*) of calcium and iron. *Ecology of Food and Nutrition* **36**, 491–500.

Svanberg U, Lorri W & Sandberg A-S (1993) Lactic fermentation of non-tannin and high-tannin cereals: effects on *in vitro* estimation of iron availability and phytate hydrolysis. *Journal of Food Science* **58**, 408–412.

Temple L, Gibson RS & Hotz C (2002) Use of soaking and enrichment for improving the content and bioavailability of calcium, iron and zinc in complementary foods and diets of rural Malawian weanlings. *Journal of Food Science* **67**, 1926–1932.

Teucher B, Olivares M & Cori H (2004) Enhancers of iron absorption: ascorbic acid and other organic acids. *International Journal for Vitamin and Nutrition Research* **74**, 403–419.

Turnlund JR, Swanson CA & King JC (1983) Copper absorption and retention in pregnant women fed diets based on animal and plant proteins. *Journal of Nutrition* **113**, 2346–2352.

Van Campen DR & Glahn RP (1999) Micronutrient bioavailability techniques: accuracy, problems and limitations. *Field Crops Research* **60**, 93–113.

van het Hof KH, de Boer BCJ, Tijburg LBM, Lucius BRHM, Zijp I, West CE, Hautvast JGAJ & Weststrate JA (2000) Carotenoid bioavailability in humans from tomatoes processed in different ways determined from the carotenoid response in the triglyceride-rich lipoprotein fraction of plasma after a single consumption and in plasma after four days of consumption. *Journal of Nutrition* **130**, 1189–1196.

Walter A, Rimbach G, Most E & Pallauf J (1998) Effect of citric acid supplements to a maize-soya diet on the in vitro availability of minerals, trace elements, and heavy metals. *Zentralblatt Veterinarmedizin* **45A**, 517–524.

Wortley G, Leusner S, Good C, Gugger E & Glahn R (2005) Iron availability of a fortified processed wheat cereal: a comparison of fourteen iron forms using an *in vitro* digestion/human colonic adenocarcinoma (CaCo-2) cell model. *British Journal of Nutrition* **93**, 65–71.

Yeudall F, Gibson RS, Cullinan TR & Mitimuni B (2005) Efficacy of a community-based dietary intervention to enhance micronutrient adequacy of high-phytate maize-based diets of rural Malawian children. *Public Health Nutrition* **8**, 826–836.

Yeudall F, Kayira C, Umar E & Gibson RS (2002) Impact of a community-based dietary intervention on selected biochemical and functional outcomes in rural Malawian children. *European Journal of Clinical Nutrition* **56**, 1176–1185.

Yeum K-J & Russell RM (2002) Carotenoid bioavailability and bioconversion. *Annual Review of Nutrition* **22**, 483–504.

IUPAC-IUB

1043

Biochem. J. (1978) 171, 1–19
Printed in Great Britain

Nomenclature of Phosphorus-Containing Compounds of Biochemical Importance

(Recommendations 1976)[a]

IUPAC-IUB Commission on Biochemical Nomenclature[b]

The IUPAC Commissions on the Nomenclature of Inorganic and of Organic chemistry (CNIC and CNOC) have recently provided, in the "D-Rules"[1], recommendations for naming a large number of organic compounds containing phosphorus. Many such compounds are extremely important in biochemistry and hence in nearly all branches of biology and medicine. Most of the biochemically important ones are esters and/or anhydrides of various phosphorus-containing acids with complex organic alcohols and organic acids. Strict application of the "D-Rules"[1] to such compounds would result, in many cases, in rather complicated names, and these would be inconvenient for most biochemists and biologists to use.

However, other systems of nomenclature, in use in the biochemical literature, are available[2-5]. It is the purpose of this document to define and recommend certain of these for naming organic phosphorus-containing compounds in biochemical, biological, and medical publications.

A general summary and explanation of the principles involved in the nomenclature of biochemically important organic phosphorus compounds is given below. Representative compounds and their recommended names, together with those derived from more systematic nomenclature[6,7], including names formed according to the "D-Rules"[1] where appropriate, are listed in the Tables.

1. **Phosphoric esters**, $RO\text{-}PO(OH)_2$, are named as *O*-substituted phosphoric acids or as substituted alcohols (Table I). Thus, choline *O*-(dihydrogen phosphate) and *O*-phosphonocholine are both appropriate names. The latter may be contracted to *phosphocholine*, but not changed to phosphorylcholine; "phosphoryl" is defined (ref.[1], Rule 5.66) as OP< and requires, if used, the naming of all three groups attached to the phosphorus atom. However, "phosphoryl" is retained in derived terms such as the names of enzymes (e.g., phosphorylase) or of processes (e.g., phosphorylation).

Comment. The form *O*-phosphono-R stems from two considerations,
(i) the definition (ref.[1], Rule 5.51) of phosphonic acid as $HPO(OH)_2$, and

[a] Document of the IUPAC-IUB Commission on Biochemical Nomenclature (CBN), approved by IUPAC and IUB in 1976 and published by permission of the International Union of Pure and Applied Chemistry (IUPAC) and the International Union of Biochemistry (IUB). Comments and suggestions for future revisions of this document may be sent to any member of CBN[b]. Reprints may be obtained from the NRC Office of Biochemical Nomenclature (W.E. Cohn, Director), Biology Division, Oak Ridge National Laboratory, P.O. Box Y, Oak Ridge, Tenn., U.S.A., 37830.

[b] Members of CBN are: O. Hoffmann-Ostenhof (Chairman), W.E. Cohn (Secretary), A.E. Braunstein, H.B.F. Dixon, B.L. Horecker, W.B. Jakoby, P. Karlson, W. Klyne, C. Liébecq, E.C. Webb.

(ii) the principle in organic nomenclature of substitution of another atom or group for a hydrogen atom of a parent molecule, which, in this case, involves the replacement of the H of an OH group by -$PO(OH)_2$, the phosphono group (ref.[1], Rule 5.52).

2. **Phosphate** may be used for "(dihydrogen phosphate)", "(disodium phosphate)", etc., (1) if the nature of the counter-ions is not known or is of no importance in the context, or (2) if a mixture of ionic forms (free acid and/or monoanion and/or dianion) is in question. Thus, in most biochemical or biological systems, where the pH is around 7, "glucose phosphate" may be used in place of "glucose dihydrogen phosphate", the proper name for the protonated form.

Comments

(i) Although glucose phosphate is an ester, the term "phosphate ester" should not be used: "phosphoric ester" is the appropriate generic term.

(ii) When "phosphoric" is followed by a generic term (e.g., ester, amide, group), the word "acid" need not intervene. Hence "phosphoric ester" is complete and sufficient, and the residue transferred to glucose to form *O*-phosphonoglucose (see 1 above) is a "phosphoric residue".

(iii) To distinguish choline phosphate (ester) from choline phosphate (salt), the former could be written "choline *O*-phosphate". However, "phosphocholine" is unambiguous. (For *N*-phosphono compounds, see Section 6).

3. **Phosphoric anhydrides** are of two types, (a) those in which two or more phosphoric residues are linked by oxygen atoms to yield diphosphates, triphosphates, etc. (e.g., ADP, ATP, etc; Table II) and (b) those in which phosphoric acid forms a "mixed anhydride" with a different type of acid (generally a carboxylic acid, e.g., acetic acid) (Table VI). The latter are named (ref.[1], Rule 5.64) as "R-ic phosphoric anhydrides" or as "R-yl phosphates" (e.g., acetic phosphoric anhydride or acetyl phosphate).

Comments

(i) "Pyro" should not be used for the substituted phosphoric anhydrides (ref.[6], Rule 4.12) (Table II), but may be retained in such terms as inorganic pyrophosphate (ref.[6], Rule 5.213), pyrophosphatase, pyrophosphate-glycerol transferase, and pyrophosphorolysis[3]. (cf. Section 1 above *re* "phosphoryl").

(ii) The prefixes di, tri, tetra, etc. should not be used to indicate two or more independent phosphoric residues substituted on different oxygen (or other) atoms in a single compound; the appropriate multiplying prefixes for such compounds are bis, tris, tetrakis, etc. (ref.[1], Rule 5.51; ref.[6], Rules 2.251 and 4.12). For example, "fructose 1,6-diphosphate" could indicate a diphosphoric residue bridging positions 1 and 6 of fructose; the common biochemical substance is correctly named fructose 1,6-bis(phosphate) (ref.[4], Rule 4.4).

4. **Phosphodiesters** (Tables III and IV), which involve the bridging group -PO(OH)-, could be named in terms of phosphinic acid, $H_2PO(OH)$, for which the prefix form is phosphinico[1]. However, the use of this prefix, as in ref.[3], presents complications in placing the locants for unsymmetrical diesters. Hence, phosphinico is contracted to phospho, which is used as an infix between the names of the two alcohols. Thus, **glycerophosphocholine** is recommended[5] for the well-known phospholipid component (previously[8], but incorrectly, called glycerophosphorylcholine; cf. Section 1 above). This recommendation also illustrates the convention by which glycerol phosphate is contracted to **glycerophosphate**[5,8], but this should not be done in a context where "glycero" may be confused with the residue of glyceric acid, as in glycerolactone, or with the prefix *glycero* used in Carbohydrate Nomenclature (ref.[2], Rules Carb-8 and Carb-9). (For the placement of locants, see examples in Tables I and IV.)

Comments

(i) The use of "phosphoryl" in this situation requires an indication in the name that there is one hydroxyl group remaining on the phosphoric residue, and would thus further lengthen the name (see Section 1 above).

(ii) The diacyl derivatives of glycerophosphocholine are commonly expressed as derivatives

of phosphatidic acid (Table IV), i.e., diacylglycerophosphocholine ≡ phosphatidylcholine[5,8].

(iii) The trivial names for the acid radicals of nucleotides (Table III) include the phosphoric residue, hence the latter is not specified in oligo- or polynucleotide names, e.g., adenylylcytidine suffices for Ado-*P*-Cyd (locants omitted for clarity; cf.[9]).

(iv) The so-called cyclic phosphates (Table III), of which adenosine 3′,5′-phosphate* (cyclic AMP or cAMP) is the best-known example, are named in this form rather than in an inverted form, which would yield 3′,5′-phosphoadenosine. The word "cyclic", often added before "phosphate", is unnecessary if the locants are given.

(v) The infix "phospho" gives precedent for "diphospho", "triphospho", "tetraphospho", etc., for the doubly esterified oligophosphoric acids (Table II), e.g., uridinediphosphoglucose, adenosinediphosphoribose.

5. **Nucleoside triphosphate analogues**, in which a methylene group (-CH_2-), an imido group (-NH-), or a sulfur atom replaces an oxygen atom bridging two phosphorus atoms, could be named by an extension of the convention of inorganic nomenclature (ref.[6], Rule 4.15) that employs μ to indicate a bridging group. Thus the compound symbolized as Ado(5′)*P*[CH_2]*PP* could be named adenosine 5′-(1,2-μ-methylene)triphosphate, and Ado(5′)*PP*[CH_2]*P* might be named adenosine 5′-(2,3-μ-methylene)triphosphate. However, for the "methylene" part of these names, [α,β-methylene] and [β,γ-methylene] are unambiguous, are consistent with the use of Greek letters as locants in other situations[7], and are therefore recommended (see Table VIII). (The latter compound can be termed 5′-adenylyl methylenediphosphonate, but this name does not contain the significant term "triphosphate".)

Comments

(i) The use of square brackets here is similar to their use in amino-acid replacement[10], indicating a replacement of the normal constituent.

(ii) Although the bridging methylene group in the Ado(5′)*PP*[CH_2]*P* example should receive priority for numbering, i.e., should be 1,2-μ-methylene to accord with inorganic nomenclature (ref.[6], Rule 4.15), this would require an additional term (as in Table VIII, column 2); it is therefore not suitable in this context, in which it is desirable to give adenosine first consideration (i.e., it is always considered to be linked to the α phosphorus atom).

(iii) A terminal substitution (e.g., sulfur replacing oxygen on P^3) might be named adenosine 5′-[3-thio]triphosphate, but adenosine 5′-[γ-thio]-triphosphate is recommended (see ii above).

(iv) The rules of inorganic nomenclature (ref.[6], Table II) specify "imido" as the ligand name for -NH-; it is, in this case, an imide of phosphoric acid, hence "imido" is recommended for biochemical use with "triphosphate" or "diphosphate" (see Table VIII).

(v) The symbol for the nucleoside does not include the 5′-oxygen atom when the rest of the formula is written out in extenso. Thus Ado(5′)-*P*[CH_2]*P* ≡ Ado(5′)-O-PO(OH)-CH_2-PO_3H_2. Such extended representation may be useful for analogs such as Ado(5′)-CH_2-PO_3H_2, a methylene analog of AMP, and Ado(5′)-O-PO(OH)-CH_2-AsO_3H_2.

6. **Phosphoric amides** (Table V) are named by changing "acid" in the original acid name to "amide" (ref.[1], Rule 5.62). However, when the nitrogenous group supplying the amide moiety is known by a trivial name and that name is to be retained, the phosphoric amide may be named in the same manner as the esters (see Section 1 above), but not in the form in which "phosphate" is used as a suffix (see Section 2 above); e.g., **phosphocreatine** (for *N*-phosphonocreatine), but not "creatine phosphate", because "phosphate" means that all atoms attached to the phosphorus atom are oxygen atoms.

Comment. The contraction "phosphoamide" for phosphoric amide is often seen, but becomes unwieldy when either the amide or the phosphoric residue is substituted. Such compounds should be named as derivatives of phosphoramidic acid (or of a phosphoramidate), or of amidophosphoric acid (aminophosphate) [ref.[1], Rules 5.53 (a, b), 5.61 (a, b)].

* According to IUPAC Rules[7] in analogous cases [e.g. hydrocarbon bridges (A-34.1), epoxy and other epi compounds (C-212.2, 514.4, 701.1, 815.2), lactones (C-472.1)] and common use by Chemical Abstracts, the comma between the locants is preferred to the colon recommended earlier by CBN[9].

7. **Fluorophosphoric acids**, when doubly esterified, become fluorophosphates or phosphorofluoridates [ref.[1], Rules 5.53 (a, b), 5.61 (a, b)]. Thus the well-known compound $(Pr^iO)_2PO\text{-}F$ or $iPr_2P\text{-}F$[11] may be called diisopropyl fluorophosphate or diisopropyl phosphorofluoridate (see Table VI).

References

1 IUPAC, "Nomenclature of Organic Chemistry, Section D", IUPAC Information Bulletin No. 31, Appendix on Tentative Nomenclature, August 1973, pp. 60 - 86.

2 IUPAC-IUB, "Tentative Rules for Carbohydrate Nomenclature, Part 1, 1969", *Biochem. J.* **125**, 673 - 695 (1971); *Biochemistry* **10**, 3983 - 4004 (1971); *Biochim. Biophys. Acta* **244**, 223 - 302 (1971); *Eur. J. Biochem.* **21**, 455 - 477 (1971); *J. Biol. Chem.* **247**, 613 - 635 (1972).

3 IUB, "Enzyme Nomenclature (1972)", Elsevier Publ. Co. Amsterdam–New York; Supplement 1: *Biochim. Biophys. Acta* **429**, 1 - 45 (1976).

4 IUPAC-IUB, "Abbreviations and Symbols for Chemical Names of Special Interest in Biological Chemistry (1965 Tentative Rules)", *J. Biol. Chem.* **241**, 527 - 533 (1966); *Biochem. J.* **101**, 1 - 7 (1966); *Biochemistry* **5**, 1445 - 1453 (1966); *Eur. J. Biochem.* **1**, 259 - 266 (1967); *Hoppe-Seyler's Z. Physiol. Chem.* **348**, 245 - 255 (1967); and elsewhere.

5 IUPAC-IUB, "Nomenclature of Lipids, Recommendations 1976", *Hoppe-Seyler's Z. Physiol. Chem.* **358**, 617 - 631 (1977).

6 IUPAC, "Nomenclature of Inorganic Chemistry, Definitive Rules 1970", 2nd edit., Butterworths, London 1971.

7 IUPAC, "Nomenclature of Organic Chemistry, Definitive Rules for Sections A, B, C", 2nd edit., Butterworths, London 1971.

8 IUPAC-IUB, "Nomenclature of Lipids, (1967)", *J. Biol. Chem.* **242**, 4845– 4849 (1967) and **245**, 1511 (1970); *Eur. J. Biochem.* **2**, 127 - 131 (1967) and **12**, 1 (1970); *Hoppe-Seyler's Z. Physiol. Chem.* **350**, 279 - 285 (1969); and elsewhere. Revision (1976) see ref.[5].

9 IUPAC-IUB, "Abbreviations and Symbols for Nucleic Acids, Polynucleotides and their Constituents, Recommendations 1970", *Arch. Biochem. Biophys.* **145**, 425 - 436 (1971); *Biochem. J.* **120**, 449 - 454 (1970); *Biochemistry* **9**, 4022 - 4027 (1970); *Biochim. Biophys. Acta* **247**, 1 - 12 (1971); *Eur. J. Biochem.* **15**, 203 - 208 (1970); *Hoppe Seyler's Z. Physiol. Chem.* **351**, 1055 - 1063 (1970); *J. Biol. Chem.* **245**, 5171 - 5176 (1970); *Pure Appl. Chem.* **40**, 277 - 290 (1974); *Mol. Biol. (Moscow)* **6**, 167 - 178 (1972); and elsewhere.

10 IUPAC-IUB, "Rules for Naming Synthetic Modifications of Natural Peptides, Tentative Rules 1966 (amended 1972)", *Biochemistry* **6**, 362 - 364 (1967); *Eur. J. Biochem.* **1**, 379 - 381 (1967); *Hoppe-Seyler's Z. Physiol. Chem.* **348**, 262 - 265 (1967); and elsewhere.

11 IUPAC-IUB, "Symbols for Amino-Acid Derivatives and Peptides, Recommendations 1971", *Arch. Biochem. Biophys.* **150**, 1- 8 (1972); *Biochem. J.* **126**, 773 - 780 (1972); *Biochemistry* **11**, 1726 - 1732 (1972); *Biochim. Biophys. Acta* **263**, 205 - 212 (1972); *Eur. J. Biochem.* **27**, 201 - 207 (1972); *J. Biol. Chem.* **247**, 977 - 983 (1972); *Pure Appl. Chem.* **40**, 315 - 331 (1974).

12 IUPAC-IUB, "Nomenclature of α-Amino Acids, Recommendations 1974", *Biochemistry* **14**, 449 - 462 (1975); *Biochem. J.* **149**, 1 - 16 (1975); *Eur. J. Biochem.* **53**, 1 - 14 (1975); and elsewhere.

Table I: Phosphoric esters (phosphates). Footnotes a - n see p. 8.

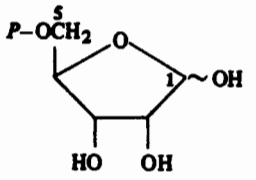
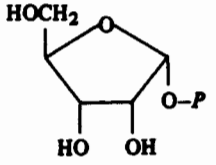
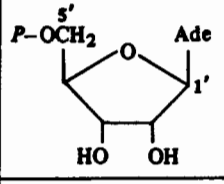
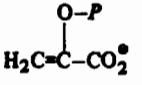
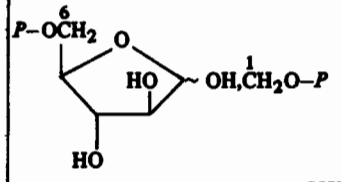
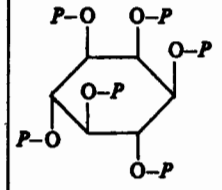
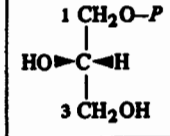
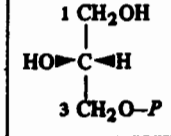
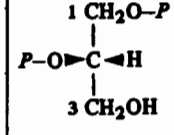
Names recommended for biochemical usage[a] *Phosphate names*	*O-Phosphono/phospho names*	*Systematic names*	*Abbreviations*[a, b]	*Structure*
1) D-Ribose 5-phosphate	5-*O*-Phosphono-D-ribose; 5-Phospho-D-ribose	D-Ribofuranose 5-(dihydrogen phosphate)	Ribose-5-*P*; Rib5*P*	
2) α-D-Ribose 1-phosphate; α-D-Ribosyl phosphate[c]	1-*O*-Phosphono-α-D-ribose; 1-Phospho-α-D-ribose	α-D-Ribofuranose 1-(dihydrogen phosphate); α-D-Ribofuranosyl dihydrogen phosphate[c]	Ribose-1-*P*; Rib1*P*	
3) Adenosine 5′-phosphate; 5′-Adenylic acid[d]	5′-*O*-Phosphonoadenosine; 5′-Phosphoadenosine	Adenosine 5′-(dihydrogen phosphate)	Adenosine-5′*P*; Ado5′*P*; *P*Ado; 5′AMP	
4) 2-Aminoethyl phosphate; 2-Aminoethanol *O*-phosphate; 2-Aminoethanol phosphate (ester)	2-Amino-*O*-phosphonoethanol; Phosphoethanolamine[e]	2-Aminoethyl dihydrogen phosphate; 2-Aminoethanol (dihydrogen phosphate) (ester); 2-Aminoethanol *O*-(dihydrogen phosphate)	*P*-Ethanolamine	
5) 2-Hydroxy-2-propenoate phosphate (ester)	*O*-Phosphono-*enol*-pyruvate; Phospho*enol*pyruvate[e]	2-(Phosphonooxy)-2-propenoate; 2-Hydroxy-2-propenoate (dihydrogen phosphate) (ester)	*P-enol*Pyruvate[f]	

Table I, continued.

Names recommended for biochemical usage[a] *Phosphate names*	*O-Phosphono/phospho names*	*Systematic names*	*Abbreviations*[a,b]	*Structure*
6) D-Fructose 1,6-bis(phosphate)	1,6-Di-*O*-phosphono-D-fructose 1,6-Bis-(phospho)-D-fructose	D-Fructofuranose 1,6-bis(dihydrogen phosphate)	Fructose-1,6-P_2; Fru(1,6)P_2	
7) *myo*-Inositol hexakis(phosphate)[g]	Hexa-*O*-phosphono-*myo*-inositol; Hexakis(phospho)-*myo*-inositol	*myo*-Inositol hexakis(dihydrogen phosphate)	P_6-Inositol	
8) *sn*-Glycerol 1-phosphate[h]; *sn*-Glycero-1-phosphate	1-*O*-Phosphono-*sn*-glycerol; 1-Phospho-*sn*-glycerol	(*S*)-[2,3-Dihydroxypropyl dihydrogen phosphate]; (*S*)-[Glycerol 1-(dihydrogen phosphate)]	*sn*-Glycerol-1-*P*	
9) *sn*-Glycerol 3-phosphate[i]; *sn*-Glycero-β-phosphate	3-*O*-Phosphono-*sn*-glycerol; 3-Phospho-*sn*-glycerol	(*R*)-[2,3-Dihydroxypropyl dihydrogen phosphate]; (*R*)-[Glycerol 1-(dihydrogen phosphate)]	*sn*-Glycerol-3-*P*	
10) *sn*-Glycerol 1,2-bis(phosphate)[j] *sn*-Glycero-1,2-bis(phosphate)	1,2-Di-*O*-phosphono-*sn*-glycerol; 1,2-Bis(phospho)-*sn*-glycerol	(*S*)-[(Hydroxymethyl)-ethylene bis(dihydrogen phosphate)]; (*S*)-[Glycerol 1,2-bis(dihydrogen phosphate)]; (*S*)-2,3-Bis(phosphonooxy)-1-propanol	*sn*-Glycerol-1,2-P_2	

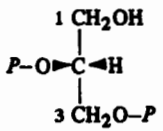
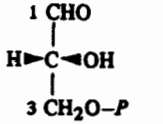
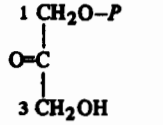
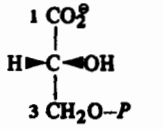
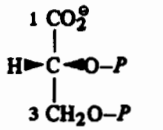
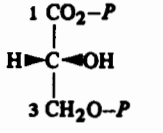
11) *sn*-Glycerol 2,3-bis(phosphate)[k]; *sn*-Glycero-2,3-bis(phosphate)	2,3-Di-*O*-phosphono-*sn*-glycerol; 2,3-Bis(phospho)-*sn*-glycerol	(*R*)-[(Hydroxymethyl)-ethylene bis(dihydrogen phosphate)]; (*R*)-[Glycerol 1,2-bis(dihydrogen phosphate)] (*R*)-2,3-Bis(phosphonooxy)-1-propanol	*sn*-Glycerol-2,3-P_2	
12) D-Glyceraldehyde 3-phosphate	3-*O*-Phosphono-D-glyceraldehyde; 3-Phospho-D-glyceraldehyde	2-Hydroxy-3-oxopropyl dihydrogen phosphate; 2-Formyl-2-hydroxyethyl dihydrogen phosphate; D-Glyceraldehyde 3-(dihydrogen phosphate)	D-Glyceraldehyde-3-*P*	
13) Glycerone phosphate[l]; 1,3-Dihydroxyacetone phosphate	1-*O*-Phosphonoglycerone; 1-Phosphoglycerone[l]	3-Hydroxy-2-oxopropyl dihydrogen phosphate; 1-Hydroxy-3-(phosphonooxy)-2-propanone	Glycerone-P[l]; Dihydroxyacetone-*P*	
14) D-Glycerate 3-phosphate	3-*O*-Phosphono-D-glycerate; 3-Phospho-D-glycerate	(*R*)-[2-Hydroxy-3-(phosphonooxy)propanoate]; (*R*)-[2,3-Dihydroxypropanoate 3-(dihydrogen phosphate)]	D-Glycerate-3-*P*	
15) D-Glycerate 2,3-bis(phosphate)[m]	2,3-Di-*O*-phosphono-D-glycerate; 2,3-Bis(phospho)-D-glycerate[m]	(*R*)-[2,3-Bis(phosphonooxy)propanoate]; (*R*)-[2,3-Dihydroxypropanoate 2,3-bis(dihydrogen phosphate)]	D-Glycerate-2,3-*P*	
16) (D-Glyceroyl phosphate) 3-phosphate[n]	3-*O*-Phosphono-D-glyceroyl phosphate; 3-Phospho-D-glyceroyl phosphate; 3-*O*-Phosphono-D-glyceric phosphoric monoanhydride; 3-Phospho-D-glyceric phosphoric monoanhydride	2-Hydroxy-3-(phosphonooxy)propionyl dihydrogen phosphate; 2,3-Dihydroxypropionyl dihydrogen phosphate 3-(dihydrogen phosphate); 2-Hydroxy-3-(phosphonooxy)propionic phosphoric monoanhydride	D-Glyceric-1,3-P_2	

Table I, continued.

[a] See Section 2 in the text. Stereospecific numbering denoted by *sn* is defined in ref.[5,8]. If fully defined in a paper, *sn* and D may be omitted from biochemical names and abbreviations.
[b] The symbol *P* represents a phosphoric residue and may precede or follow the organic residue. Approved symbols, such as Rib, Ado, Fru, etc. (see refs.[4,9,11]) are used to represent the organic residues. The symbols Ins, for inositol, and Gro, Gra, Grn, Gri, for glycerol, glyceraldehyde, glycerone (see footnote *l*, below), and glyceric acid respectively, are defined in ref.[5].
[c] Locants are not needed since the glycosyl radical, by definition, is formed at the hemiacetal position (ref.[2]).
[d] Nucleotide trivial name.
[e] Most commonly used name.
[f] "Pyruvenol" with the symbol *e*Prv has been suggested for "*enol*-pyruvate".
[g] Phytic acid.
[h] Originally named L-glycerol 1-phosphate *or* D-α-glycerophosphoric acid (D-glycerol 3-phosphate)[8].
[i] Originally named D-glycerol 1-phosphate *or* L-α-glycerophosphoric acid (L-glycerol-3-phosphate)[8].
[j] Previously named L-glycerol 1,2-bis(phosphate) *or* D-glycerol 2,3-bis(phosphate).
[k] Previously named D-glycerol 1,2-bis(phosphate) *or* L-glycerol 2,3-bis(phosphate).
[l] The name "glycerone" has been proposed for 1,3-dihydroxyacetone[5].
[m] Often called "diphosphoglycerate"; "bis" is correct (see Section 3).
[n] One phosphoric residue is an ester; the other is an anhydride. Glyceroyl is the acyl radical derived from glyceric acid (ref.[11], Rule C-411.1).

Table II. Representative oligophosphoric esters (oligophosphates).

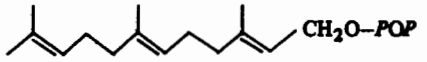
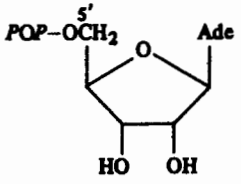
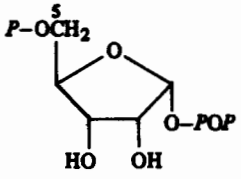
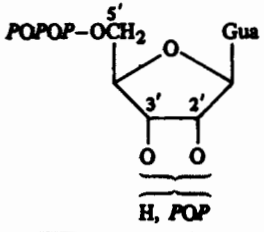
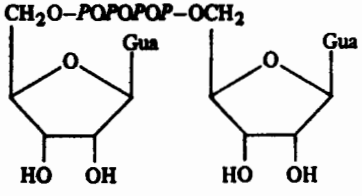
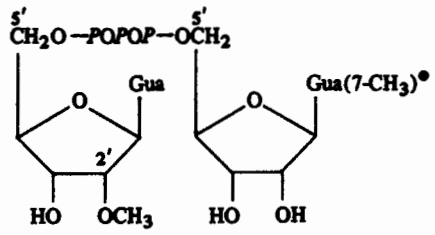
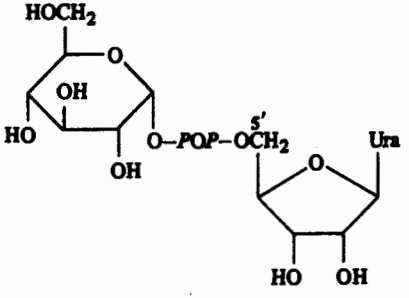
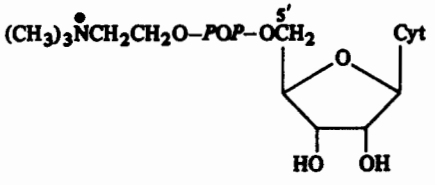
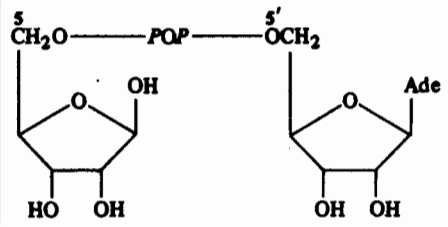
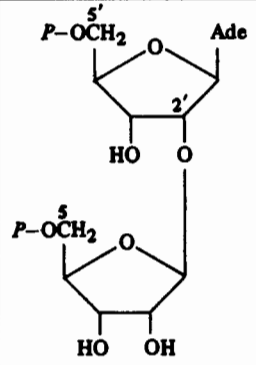
Recommended names	*Other names*	*Abbreviations*[a]	*Structure*
1) Farnesyl diphosphate; Farnesol diphosphate	Farnesyl trihydrogen diphosphate; Farnesol (trihydrogen diphosphate)		
2) Adenosine 5′-diphosphate[b]	5′-Diphosphoadenosine; 5′-Adenylyl phosphate	Adenosine-5′*PP*; Ado5′*PP*; PPAdo; ADP	
3) 5-Phospho-α-D-ribosyl diphosphate[c]; α-D-Ribosyl diphosphate 5-phosphate	5-*O*-Phosphono-α-D-ribosyl diphosphate; 5-Phospho-α-D-ribofuranosyl diphosphate; 1-Diphospho-5-phospho-α-D--ribofuranose; α-D-Ribose 1-diphosphate 5-phosphate	*PP*Rib*P*;[d] *P*Rib*PP*	
4) Guanosine 2′ (or 3′)-diphosphate 5′-triphosphate	2′(or 3′)-Diphospho-5′-triphosphoguanosine; 2′(or 3′)-Diphosphoguanylyl triphosphate	pppGpp; p_3Gp_2	
5) Guanosine(5′)tetraphospho(5′)-guanosine; Bis(guanylyl)diphosphate	P^1,P^4-Bis(5′-guanosyl) tetraphosphate; Bis(5′-guanylyl) diphosphate	G(5′)p_4(5′)G; $(ppG)_2$	

Table II, continued.

Recommended names	*Other names*	*Abbreviations*[a]	*Structure*
6) 7-Methylguanosine(5′)triphospho-(5′)-2′-*O*-methylguanosine[e]	P^1-(7-Methyl-5′-guanosinium-5′-yl) P^3-(2′-*O*-methyl-5′-guanosyl) triphosphate; 7-Methylguanosinium 5′-(2′-*O*-methylguanosine 5′-triphosphate)	$m^7G(5')ppp(5')Gm$	
7) Uridine(5′)diphospho(1)-α-D-glucose[c]	Uridine diphosphate glucose; Uridine 5′-(α-D-glucopyranosyl diphosphate); α-D-Glucopyranosyl 5′-uridylyl phosphate	UDP-Glc; U5′pp1Glc; UDPG[f]	
8) Cytidine(5′)diphosphocholine[c]	Cytidine diphosphate choline; Cytidine 5′-(choline diphosphate); Choline (5′-cytidylyl phosphate)	CDP-Choline; CDP-Cho	

9) Adenosine(5')diphospho(5)-β-D--ribose[c,g]	Adenosine diphosphate ribose; Adenosine 5'-(β-D-ribose 5-diphosphate)	ADP-Rib; A5'pp5Rib; (Rib5)ppA	
10) 2'-(5-Phospho-β-D-ribosyl)-adenosine 5'-phosphate[c,g]	2'-(5-Phospho-β-D-ribofuranosyl)-adenosine 5'-phosphate; 2'-(5-Phospho-β-D-ribofuranosyl)--5'-adenylic acid	AMP(*P*-Rib); *P*-Ado(*P*-Rib); pA2'(pRib); pRib(1-2')pA; iso(ADP-Rib); ψADP-Rib[h]	

[a] The symbol Rib for the ribose residue is defined in ref.[4]. Other symbols are defined in ref.[9], especially *P* or p = phosphoric residue.

[b] Triphosphates are treated similarly. Analogs of ATP are further delineated in Table VI. Other nucleoside terms may replace "adenosine", "adenylyl", and "Ado" (ref.[9]).

[c] Locants and descriptors are customarily omitted for the isomers shown, so that the terms may be written as, e.g., phosphoribosyl diphosphate, uridine-diphosphoglucose (compound 7).

[d] The abbreviation PPRP is seen often in the literature; it is not recommended.

[e] Bis(nucleotides) of this type, including the methylation as shown, appear to be the common 5'-terminus of many, if not all, of the RNAs of RNA-containing viruses. The use of 7-methylguanosine instead of the more correct 7-methyl-7-guanosinium is common.

[f] Common, but can be ambiguous in context with galactose. Glc for glucose is preferred.

[g] Cleavage of "poly(ADP-Rib)" at the2' linkage of the adenosine moieties gives compound 9; and between the phosphoric residues gives compound 10. Although compound 10 is not an oligophosphoric ester and does not really belong in this table, it is included because it is most often encountered in conjunction with the isomeric compound 9.

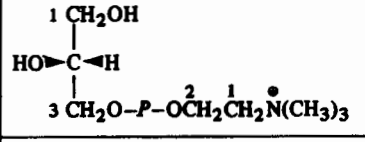
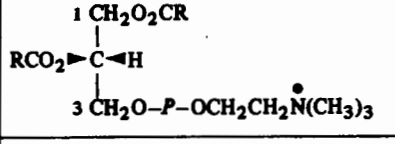
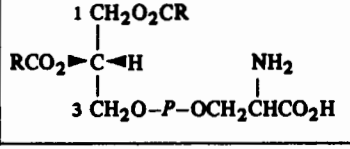
[h] Disfavored; given here because of occurrence in the literature (Ψ has other meanings in this context).

Table III. Representative bisnucleoside phosphates and cyclic phosphates[4,9].

Recommended name[a]	*Other names*[b]	*Abbreviation*
1) Adenylyl(3′-5′)cytidine	Cytidylyl(5′→3′)adenosine	A-C; Ado-3′*P*5′-Cyd
2) Adenylyl(3′-5′)cytidine 3′-phosphate	3′-Phosphocytidylyl(5′→3′)adenosine	A-Cp; Ado-3′*P*5′-Cyd*P*
3) 5′-Phosphoadenylyl(3′-5′)cytidine 3′-phosphate	3′-Phosphocytidylyl(5′→3′)adenosine 5′-phosphate	pA-Cp; *P*Ado-3′*P*5′-Cyd*P*
4) 5′-Phosphodeoxyadenylyl(3′-5′)thymidine[c]	Thymidylyl(5′→3′)deoxyadenosine 5′-phosphate	pdA-dT; d(pA-T); *P*-dAdo-3′*P*5′-dThd
5) 5′-Triphosphoguanylyl(3′-5′)cytidine	Cytidylyl(5′→3′)guanosine 5′-triphosphate	pppG-C
6) Cytidine 2′,3′-phosphate	Cytidine 2′,3′-(cyclic)phosphate[d]	Cyd>*P*; Cyd-2′,3′-*P*; C>p
7) Adenosine 3′,5′-phosphate	Adenosine 3′,5′-(cyclic)phosphate[d]	cyclic AMP; cAMP; Ado-3′,5′-*P*

[a] The infix (3′-5′) may be omitted if no ambiguity may arise.
[b] Many other names, including permutations of those given, are possible, e.g., adenosine(3′)phospho(5′)cytidine and 5′-(3′-adenylyl)cytidine for compound 1.
[c] The prefix "ribo" precedes "thymidine" when 5-methyluridine is meant. The symbols Thd and T indicate the latter; dThd and dT represent thymidine[4,9].
[d] "(Cyclic)" is redundant when locants are given (in compounds 5 and 7), but is helpful. It is necessary when, in repeated usage, the locants are omitted.

Table IV. Representative phospholipids (involving diesterified phosphoric acid).

Recommended names[a,b]	*Other names*	*Abbreviations*[c]	*Structure*
1) *sn*-Glycero(3)phosphocholine	Glycerol choline phosphate	Gro*P*Cho	
2)(3-*sn*-Phosphatidyl)choline	1,2-Diacyl-*sn*-glycero(3)phosphocholine; Lecithin[d]	PtdCho; acyl$_2$Gro*P*Cho	
3)(3-*sn*-Phosphatidyl)-L-serine	1,2-Diacyl-*sn*-glycero(3)phospho-L-serine; Cephalin[d]	PtdSer; acyl$_2$Gro*P*Ser	

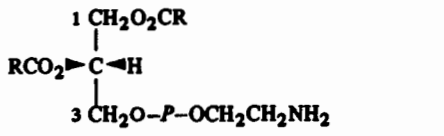
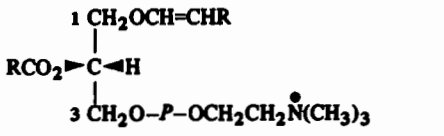
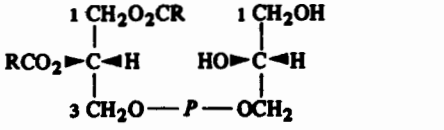
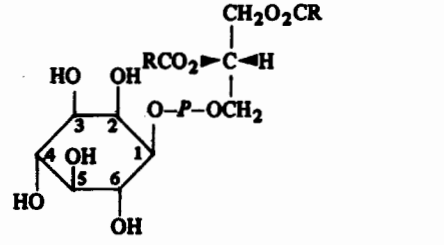
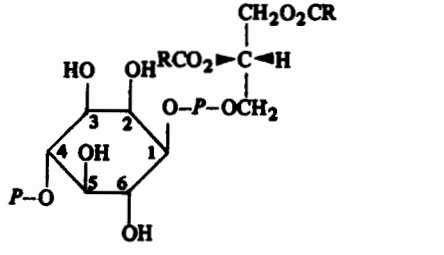
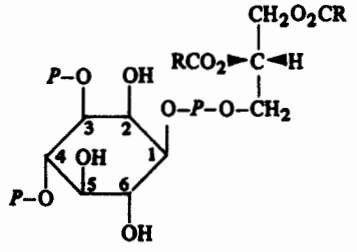
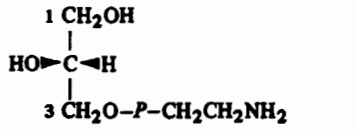
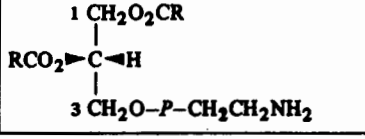
4) (3-*sn*-Phosphatidyl)ethanolamine	1,2-Diacyl-*sn*-glycero(3)phospho-ethanolamine; Cephalin[d]	PtdEtn; acyl$_2$Gro*P*Etn	
5)2-Acyl-1-(1-alkenyl)-*sn*-glycero-(3)-phosphocholine	Plasmenylcholine[e]; Plasmalogen		
6) 3-(3-*sn*-Phosphatidyl)-*sn*-glycerol	1,2-Diacyl-*sn*-glycero(3)phospho(3)-*sn*-glycerol	PtdGro	
7) 1-(3-*sn*-Phosphatidyl)-L-*myo*-inositol	1,2-Diacyl-*sn*-glycero(3)-phospho(1)-L-*myo*-inositol; Phosphoinositide[d]	PtdIns	
8) 1-(3-*sn*-Phosphatidyl)-L-*myo*-inositol 4-phosphate	1,2-Diacyl-*sn*-glycero(3)phospho(1)-L-*myo*-inositol 4-phosphate; 1,2-Diacyl-*sn*-glycero(3)phospho(1)-4-phospho-L-*myo*-inositol; Phosphonoinositide 4-phosphate[d]; Diphosphoinositide[d]	PtdIns4*P*	

Table IV, continued.

Recommended names[a,b]	*Other names*	*Abbreviations*[c]	*Structure*
9) 1-(3-*sn*-Phosphatidyl)-L-*myo*--inositol 3,4-bis(phosphate)	1,2-Diacyl-*sn*-glycero(3)phospho(1)--L-*myo*-inositol 3,4-bis(phosphate); 1,2-Diacyl-*sn*-glycero(3)phospho(1)--3,4-bis(phospho)-L-*myo*-inositol; Phosphoinositide 3,4-bis(phosphate)[d]; Triphosphoinositide[d]	PtdIns(3,4)P_2	
10) *sn*-Glycero(3)-2-phosphonoethylamine[f]	*sn*-Glycerol 3-[(2-aminoethyl)phosphonate		
11) (3-*sn*-Phosphatidyl)-ethylamine[f]	1,2-Diacyl-*sn*-glycero(3)-2-phosphonoethylamine		

[a] Stereospecific numbering denoted by *sn* is defined in ref.[5,8]. If fully defined in a paper, *sn* and various locants and descriptors may be omitted from the recommended names and abbreviations. The infix "phospho" replaces "phosphoryl" and "phosphinico", which have been used in the past[3,4] (see Sections 1 and 4 of the text).

[b] Phosphatidyl = 1,2-diacyl-*sn*-glycero(3)phospho, which may replace it when desired. For *O*-alkenyl- and *O*-alkyl-substitued glycero compounds ("lyso" compounds), see entry 5 and ref.[5].

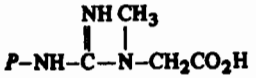
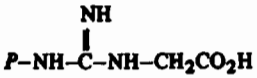
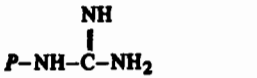
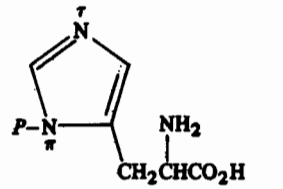
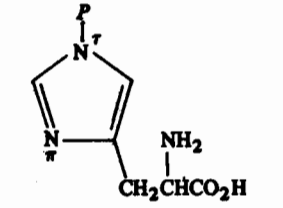
[c] The symbols Ptd for phosphatidyl, Gro for glycerol, Cho for choline, Ser for serine, Etn for ethanolamine, Ins for inositol and *P* for a phosphoric residue are defined in ref.[5].

[d] Trivial names occasionally used in the past; not recommended. Included here only for reference.

[e] Plasmenyl and plasmanyl are defined in ref.[5].

[f] Phosphonic derivatives, containing a P-C bond (compare entry 4, also Table VII).

Table V. Phosphoric amides (phosphoramidic acids or amidophosphoric acids).

Recommended names[a]	*Other names*[b,c]	*Structures*
1) Phosphocreatine	N^{ω}-Phosphonocreatine; *N*-(*N*-Phosphonoamidino)sarcosine	
2) Phosphoglycocyamine	N^{ω}-Phosphonoglycocyamine; N^{ω}-Phosphonoguanidinoacetic acid; *N*-(*N*-Phosphonoamidino)glycine	
3) Phosphoguanidine	*N*-Amidinophosphoramidate; *N*-Amidinophosphoramidic acid	
4) *pros*-Phosphohistidine[d]; π-Phosphohistidine	3(1)-Phosphonohistidine[c]	
5) *tele*-Phosphohistidine[d]; τ-Phosphohistidine	1(3)-Phosphonohistidine[c]	

[a] See Section 6 of the text for a discussion of the reasons for not using the "phosphate" form of name for the phosphoric amides. *P*-Creatine, creatine-*P* are valid for abbreviation purposes, on the assumption that the hyphen indicates a covalent bond; names such as creatine phosphate do not.

[b] The symbol ω is used to mean the NH_2 terminal group, not the NH group.

[c] The prefix "phosphonato" may be used to indicate an ionic form (ref.[1], Rule 5.52).

[d] For definition of *pros* (π) and *tele* (τ) locants, see refs.[11,12].

Table VI. Representative phosphoric anhydrides and fluorophosphates[a].

Recommended names	*Other names*	*Abbreviations*	*Structure*
1) Acetyl phosphate	Monoacetyl phosphate; Acetic phosphoric monoanhydride	Ac-*P*	CH_3CO_2-*P*
2) β-Aspartyl phosphate	Mono-β-aspartyl phosphate; β-Aspartic phosphoric mono-anhydride	Asp(β*P*)	$HO_2C\underset{\alpha}{C}H\underset{\beta}{C}H_2CO_2$–*P*, NH_2
3) Carbamoyl phosphate[b]	Monocarbamoyl phosphate; Carbamic phosphoric monoanhydride	Cbm-*P*	H_2NCO_2–*P*
4) Adenosine 5′-phosphosulfate	Adenosine 5′-*P*-phosphatosulfate[c]; 5′-Adenylyl sulfate; 5′-Adenylic sulfuric monoanhydride	APS[d]; Ado*PS*	HO_3SO–*P*–OCH_2(5′), Ade, HO, OH
5) Adenosine 3′-phosphate 5′-phosphosulfate; 3′-Phosphoadenosine 5′-phosphosulfate	Adenosine 3′-phosphate 5′-*P*-phosphatosulfate[c]; 3′-Phospho-5′-adenylyl sulfate; 3′-Phospho-5′-adenylic sulfuric monoanhydride	PAPS[d]; *P*Ado*PS*	HO_3SO–*P*–OCH_2(5′), Ade, 3′, *P*–O, OH
6) Seryl adenylate	1-*O*-(5′-Adenylyl)serine; Adenosine(5′)phospho(1)serine	AMP-Ser; Ser-*P*-Ado	$HOCH_2CHCO_2$–*P*–OCH_2(5′), NH_2, Ade, HO, OH

7) 3-Phosphoglyceroyl phosphate[e]	3-*O*-Phosphonoglyceric phosphoric monoanhydride; (Glyceroyl phosphate) 3-phosphate	Gri(1,3)P_2[f]	*P*–$OCH_2CH(OH)CO_2$–*P* 3 2 1
8) Diisopropyl fluorophosphate	Diisopropyl phosphorofluoridate	iPr_2P-F[g]	$[(CH_3)_2CHO]_2P(O)F$

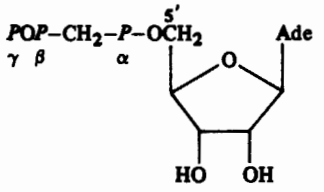
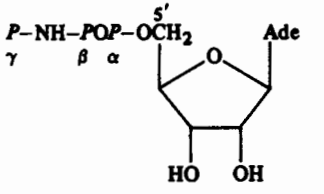
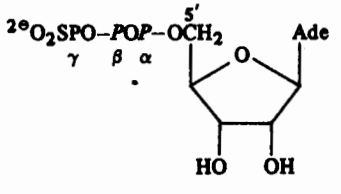
[a] See Sections 3 and 7 in text.
[b] Carbamyl, which is often used, is not in accord with the Organic Rules (ref.[7], Rule C-431.2).
[c] See Rules of Inorganic Nomenclature (ref.[6], Rule 4.211).
[d] Commonly used in the literature; Ado form is preferred for A and *P* and *S* for the acid residues.
[e] See 16 in Table I.
[f] Gri is defined in ref.[5].
[g] Equivalent to DIPF, FDIP, DFP, and Dip-F (ref.[11]).

Table VII. Representative *C*-phosphonates.

Recommended names	*Other names*[a]	*Structures*
1) (2-Aminoethyl)phosphonic acid[b]	2-Phosphonoethylamine; Ciliatine	*P*–$CH_2CH_2NH_2$ 2 1
2) (2-Oxoethyl)phosphonic acid	(Formylmethyl)phosphonic acid; Phosphonoacetaldehyde	*P*–CH_2CHO

[a] The prefix "phosphonato" may be used to indicate an ionic form (ref.[1], Rule D-5.52).
[b] See also entries 10 and 11 in Table IV.

Table VIII. Adenosine 5′-triphosphate analogs[a].

Recommended names	*Other names*[b]	*Abbreviations*	*Structure*
1)Adenosine 5′-[α,β-methylene]triphosphate	Adenosine (5′→O^1)-1,2-μ--methylenetriphosphate; Adenosine (5′→P^1)-1,2-μ--methylenetriphosphate	Ado*P*[CH_2]*PP*; pp[CH_2]pA	
2)Adenosine 5′-[β,γ-imido]triphosphate	Adenosine (5′→O^3)-1,2-μ--imidotriphosphate; Adenosine (5′→P^3)-1,2-μ--imidotriphosphate; 5′-Adenylyl imidodiphosphate; 5′-Adenylyl iminodiphosphonate[c]	Ado*PP*[NH]*P*; p[NH]ppA	
3) Adenosine 5′-[γ-thio]triphosphate	Adenosine (5′→O^3)-1-thiotriphosphate; Adenosine (5′→P^3)-1-thiotriphosphate	Ado*PPP*[S]; [S]pppA ATP[S]	

[a] See section 5 in text.
[b] Adaptation of principles of inorganic nomenclature for isopolyanions (ref.[6], Rule 4.15).
[c] "Iminodiphosphonate" is derived from organic nomenclature principles (ref.[7], Rules B-15.1 and C-815.1).

CORRECTIONS

(*a*) Table IV. The structures show the (naturally occurring) 1D isomers. Where appropriate, the names should have 1D in place of L.

(*b*) Table IV, entry 9). The naturally occurring bisphosphate is 4,5, not the 3,4 that is (correctly) depicted and named.

We are indebted to the Editors and Publishers of *Hoppe-Seyler's Zeitschrift für Physiologische Chemie* for permission to reproduce these Recommendations photographically.

Editorial note. These Recommendations are published as a service to the biochemical community. Publication does not imply endorsement of the Recommendations by the *Biochemical Journal*, but rather presents this information for consideration.

JECFA (1990)

This report contains the collective views of an international group of experts and does not necessarily represent the decisions or the stated policy of the World Health Organization or of the Food and Agriculture Organization of the United Nations

Evaluation of certain food additives and contaminants

Thirty-fifth Report of the
Joint FAO/WHO Expert Committee on
Food Additives

World Health Organization
Technical Report Series
789

World Health Organization, Geneva 1990

WHO Library Cataloguing in Publication Data

Joint FAO/WHO Expert Committee on Food Additives
Evaluation of certain food additives and contaminants: thirty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives.

(World Health Organization technical report series; 789)

1. Food additives – analysis 2. Food additives – toxicity 3. Food contamination I. Series

ISBN 92 4 120789 2 (NLM Classification: WA 712)
ISSN 0512-3054

PRINTED IN SWITZERLAND
89/8241 – Schüler SA – 6800

CONTENTS

JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES

Rome, 29 May–7 June 1989

Members invited by FAO

Mr J.F. Howlett, Head, Risk Assessment and Management Branch, Food Science Division, Ministry of Agriculture, Fisheries and Food, London, England

Mrs D.C. Kirkpatrick, Director, Bureau of Chemical Safety, Health Protection Branch, Health and Welfare Canada, Ottawa, Canada (*Joint Rapporteur*)

Professor K. Kojima, College of Environmental Health, Azabu University, Sagamihara-shi, Japan (*Chairman*)

Dr P.M. Kuznesof, Division of Food Chemistry and Technology, Center for Food Safety and Applied Nutrition, Food and Drug Administration, Washington, DC, USA

Mrs I. Meyland, Scientific Officer, National Food Agency, Ministry of Health, Søborg, Denmark

Members invited by WHO

Dr H. Blumenthal, Silver Spring, MD, USA

Professor K. Odusote, Associate Professor of Medicine, Department of Medicine, College of Medicine, University of Lagos, Lagos, Nigeria

Professor M.J. Rand, Department of Pharmacology, University of Melbourne, Melbourne, Victoria, Australia (*Vice-Chairman*)

Professor F.G. Reyes, Professor of Food Toxicology, Department of Food Science, State University of Campinas, Campinas, São Paulo, Brazil (*Joint Rapporteur*)

Professor A. Somogyi, Director, Max von Pettenkofer Institute of the Federal Health Office, Berlin (West)

Dr J.H. Steadman, Senior Principal Medical Officer, Division of Toxicology and Environmental Protection, Department of Health, London, England

Secretariat

Dr G. Burin, Toxicologist, Health Effects Division, Office of Pesticide Programs, Environmental Protection Agency, Washington, DC, USA (*WHO Consultant*)

Dr G.J. van Esch, Bilthoven, Netherlands (*WHO Temporary Adviser*)

Professor C.L. Galli, Professor of Toxicology, Institute of Pharmacological Sciences, University of Milan, Milan, Italy (*WHO Temporary Adviser*)

Mr T. Hallas-Moller, Commission of the European Communities, Brussels, Belgium (*WHO Temporary Adviser*)

Dr Y. Hayashi, Chief, Division of Pathology, National Institute of Hygienic Sciences, Tokyo, Japan (*WHO Temporary Adviser*)

Dr J.L. Herrman, Scientist, International Programme on Chemical Safety, Division of Environmental Health, WHO, Geneva, Switzerland (*Joint Secretary*)

Dr H. Irausquin, Division of Toxicological Review and Evaluation, Center for Food Safety and Applied Nutrition, Food and Drug Administration, Washington, DC, USA (*WHO Temporary Adviser*)

Dr T. Kemeny, Head, Food Chemicals Section, Toxicological Evaluation Division, Bureau of Chemical Safety, Health and Welfare Canada, Ottawa, Canada (*WHO Temporary Adviser*)

Dr N. Rao Maturu, Food Standards Officer, Joint FAO/WHO Food Standards Programme, FAO, Rome, Italy

Dr P. Shubik, Senior Research Fellow, Green College, Oxford, England (*WHO Temporary Adviser*)

Mr R. Top, Vice-Chairman, Codex Committee on Food Additives and Contaminants, Ministry of Agriculture and Fisheries, The Hague, Netherlands (*WHO Temporary Adviser*)

Professor R. Walker, Professor of Food Science, Department of Biochemistry, University of Surrey, Guildford, England (*WHO Temporary Adviser*)

Dr J. Weatherwax, Food Quality and Consumer Protection Group, Food Quality and Standards Service, Food Policy and Nutrition Division, FAO, Rome, Italy (*Joint Secretary*)

Monographs containing summaries of relevant data and toxicological evaluations are available from WHO under the title:

Toxicological evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 26, in press.

Specifications are issued separately by FAO under the title:

Specifications for the identity and purity of certain food additives. (To be published as an FAO Food and Nutrition Paper.)

INTERNATIONAL PROGRAMME ON CHEMICAL SAFETY

The preparatory work for toxicological evaluations of food additives and contaminants by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) is actively supported by certain of the Member States that contribute to the work of the International Programme on Chemical Safety (IPCS).

The International Programme on Chemical Safety (IPCS) is a joint venture of the United Nations Environment Programme, the International Labour Organisation, and the World Health Organization. One of the main objectives of the IPCS is to carry out and disseminate evaluations of the effects of chemicals on human health and the quality of the environment.

EVALUATION OF CERTAIN FOOD ADDITIVES AND CONTAMINANTS

Thirty-fifth Report of the Joint FAO/WHO Expert Committee on Food Additives

The Joint FAO/WHO Expert Committee on Food Additives met in Rome from 29 May to 7 June 1989. The meeting was opened by Dr P. Lunven, Director, Food Policy and Nutrition Division, FAO, on behalf of the Directors-General of the Food and Agriculture Organization of the United Nations and the World Health Organization. Dr Lunven noted that the work of the Joint FAO/WHO Committee on Food Additives in providing scientific assessments was invaluable to WHO, FAO, and Member States and, in particular, to the work of the Codex Alimentarius Commission. The Commission had been recognized as one of the key elements in removing barriers to trade and was expanding its work on food additives to provide uniform and comprehensive recommendations to governments. Dr Lunven also noted that a comprehensive compilation of specifications for the identity and purity of food additives was in preparation for publication.[1]

1. INTRODUCTION

As a result of the recommendations of the first Joint FAO/WHO Conference on Food Additives, held in September 1955 (*1*), there have been 34 previous meetings of the Expert Committee (Annex 1). The present meeting was convened on the recommendation made at the thirty-third meeting (Annex 1, reference *83*).

The tasks before the Committee were: (*a*) to prepare specifications for the identity and purity of certain food additives and to carry out toxicological evaluations of them; (*b*) to review specifications for selected food additives; and (*c*) to undertake toxicological evaluations of certain food additives and the contaminants polychlorinated biphenyls and patulin.

[1] *Specifications for the identity and purity of food additives* (being prepared by FAO).

2. GENERAL CONSIDERATIONS

2.1 Modification of the agenda

The issue of the safety of certain fungal enzyme preparations used in food was added to the agenda.

Four flavouring agents, dihydrocoumarin, ethyl vanillin, fumaric acid, and quinine hydrochloride, were placed on the agenda on the basis of application of the first three steps of the method for setting priorities for the safety review of food flavouring ingredients, which is summarized in the report of the thirty-third meeting (Annex 1, reference *83*).

2.2 Principles governing the toxicological evaluation of compounds on the agenda

In making recommendations on the safety of food additives and contaminants, the Committee took into consideration the principles established and contained in *Principles for the safety assessment of food additives and contaminants in food* (Annex 1, reference *76*). This publication, developed in response to repeated recommendations by the Committee, embraces the major observations, comments, and recommendations on the safety assessment of food additives and contaminants contained in the previous reports of the Committee and other associated bodies. The Committee noted that the document reaffirms the validity of recommendations that are still appropriate, and points out the problems associated with those that are no longer valid in the light of modern technical advances.

2.2.1 *Enzyme preparations*

In conjunction with the revision of general specifications for enzyme preparations (section 4.2), the Committee briefly reviewed the guidelines for evaluating enzyme preparations used in food processing that are given in Annex 3 of *Principles for the safety assessment of food additives and contaminants in food* (Annex 1, reference *76*).

It concluded that these guidelines provide a logical hierarchical procedure for determining the amount and kind of data required to establish the safety in use of enzyme preparations. The Committee stressed the advisory nature of the guidelines and recommended that

they and others given in Annex 3 of *Principles for the safety assessment of food additives and contaminants in food* (Annex 1, reference *76*) be reviewed at a future meeting.

2.2.2 *Flavouring agents*

Flavouring agents have been the subject of general comments in several previous reports of the Committee and in *Principles for the safety assessment of food additives and contaminants in food* (Annex 1, reference *76*). The view has repeatedly been expressed that, although flavouring agents should ideally be toxicologically evaluated in the same way as other food additives, special considerations dictate a degree of flexibility.

Although minimum requirements for the safety evaluation of flavouring agents have not been specified, any such evaluation should, in general, include at least a short-term feeding study, relevant metabolism studies, and mutagenicity studies.

The Committee had before it a number of flavouring agents for evaluation. In many instances, however, as is evident from section 3.1.3, the Committee had difficulty in carrying out an evaluation since data were lacking.

The Committee recognized the special problems involved in the safety evaluation of flavouring agents. However, it emphasized that a minimum amount of data was necessary to permit the development of a flexible procedure for evaluating these substances.

2.2.3 *Group ADIs for compounds that have a laxative effect*

In allocating a group acceptable daily intake (ADI) "not specified" to modified celluloses (section 3.1.5) and drawing attention to the laxative effect of an excessive intake of these substances, the Committee noted that similar considerations applied to polyols and that some gums and modified starches might also cause laxative effects at high intakes. At the Committee's twenty-seventh meeting (Annex 1, reference *62*), it was recommended that controls should be introduced to limit the consumption of polyols from all sources. At its present meeting, the Committee considered that other groups of thickeners and stabilizers that have laxative effects should also be subject to these controls since their effects are likely to be additive.

2.3 Principles governing the establishment and revision of specifications

2.3.1 *General*

The Committee reaffirmed the importance of specifications for identity and purity in the evaluation and safe use of food additives, as set out in *Principles for the safety assessment of food additives and contaminants in food* (Annex 1, reference *76*). Material subjected to toxicological testing should always be adequately defined. The Committee stressed that information on methods of manufacture, raw materials, and potential impurities should be assessed on a regular basis so that specifications can be drawn up that are both appropriate to the material used in food and consistent with the composition of the material toxicologically tested or evaluated.

In updating existing specifications, the Committee recognized the need, in certain instances, to change the terms "molecular weight" and "relative molecular mass" to "formula weight" in order to conform to accepted chemical principles (*2*). The Committee considered the term formula weight, which represents the mass corresponding to the simplest or empirical formula of a chemical compound, to be the correct term both for salts and for other chemicals that do not exist in nature as discrete molecules. Specifications reviewed at the present meeting were revised, where necessary, in accordance with this principle.

2.3.2 *Enzyme preparations*

Enzyme preparations were considered at the present meeting in response to a recommendation made at the Committee's thirty-first meeting. Questions had arisen from a discussion of the need to define the non-enzymic components of enzyme preparations and how information on such components might be taken into account from the point of view of the definition and safety of the products. The Committee concluded that a complete definition of all the components of an enzyme preparation can rarely, if ever, be achieved and that the identity and purity of preparations can therefore best be ensured by defining the processes by which they are produced and establishing criteria limiting the presence of contaminants and possible toxic metabolites derived from the source material or contaminating organisms.

The uncertainty as to the nature of the non-enzymic components mainly concerns the components that are derived in association with the active enzyme from the source material. In the case of enzymes derived from microbial sources, the potential for variability is related to both the identity of the organism concerned and the conditions under which it is cultured during the production of the enzyme. The Committee considered that it would be desirable, in specifications for microbial enzyme preparations, to define the source organism in terms not only of the species concerned but also of the strain or variant and to ensure that the culture conditions employed during the production of any particular preparation were the same as those under which the preparation subjected to toxicological testing was produced. Differences in either the strain of source organism or the conditions under which it was cultured would imply a change in the identity of the preparation and therefore require its re-evaluation. The Committee reiterated the principle already incorporated in the existing general specifications for enzyme preparations used in food processing (Annex 1, reference *69*) that non-enzymic components added for technological reasons (stabilizers, diluents, preservatives, etc.) and as immobilizing agents should be acceptable and appropriate for the intended uses of the enzyme preparations in food and food processing.

The Committee recognized that it may be inappropriate to impose limits on named mycotoxins in all microbial enzyme preparations regardless of source organism. It considered that, as individual specifications for enzymes are reviewed, the limits on named mycotoxins in the general specifications should be transferred, where relevant, to the individual source organisms. The Committee remained concerned, however, about the possibility of the production of as yet unidentified toxic metabolites. It considered that an appropriate battery of tests to screen for such potentially toxic metabolites should be developed for inclusion in the general specifications for enzyme preparations from microbial sources.

2.3.3 *Naturally occurring substances*

Substances of natural origin (e.g., spice oleoresins) may be introduced into commerce in forms that vary widely in composition. This variation is attributable to a number of factors, including the existence of different cultivars, the effects of climate and geography, the use of different extraction solvents and procedures, and the use

of diluents. Because of such compositional variation, specifications have tended to be broad and therefore not necessarily relevant to the substance for which a toxicological evaluation may be available. The Committee believed that specifications that simply state, for example, the content of the principal component (e.g., flavouring principle, colour principle) as "not less than declared on the label", while suitable for ensuring honesty in trade, could be inadequate for purposes of safety. It therefore recognized the need to continue exploring new principles for establishing adequate specifications for substances of natural origin that are both appropriate to the material used in food and consistent with the composition of the toxicologically evaluated material.

2.3.4 *Solvent residues*

During its deliberations on specifications for spice oleoresins, the Committee expressed the opinion that the use of dichloromethane and 1,2-dichloroethane as extraction solvents should be discouraged because of toxicological concerns. Because these and other solvents have not been recently evaluated and new data are now available, the Committee concluded that an overall review of solvents used in food processing would be appropriate.

In future reviews of existing specifications where provision has been made for the use of solvents, the Committee intends to request the user industry to provide justification for their use in addition to more specific data on typical levels of residues resulting from such use.

The Committee further stressed that levels of residues resulting from the use of any solvent should be the minimum technically achievable and toxicologically insignificant. Research leading to the development of new solvent systems of lower toxic potential is to be encouraged.

2.4 Methodology for analysing chemical contaminants in food

For assessing the health implications of dietary exposure to chemical contaminants, reliable information on the intake of such substances is needed. In particular, data are required on the actual levels of the substances of interest in various foods, and it is necessary to ensure that the analytical procedures employed to generate these data are both reliable and of adequate accuracy.

In connection with the chemical contaminants evaluated at its present meeting, the Committee was aware of the difficulties that could be encountered in the analysis of polychlorinated biphenyls (PCBs) in food and, in particular, in PCB isomer-specific analysis. It was informed of the ongoing activities of the WHO Regional Office for Europe related to PCBs (as well as other chlorinated hydrocarbons, including polychlorinated dibenzodioxins and polychlorinated dibenzofurans) and the assessment of health risks to infants associated with contamination of mothers' milk. Part of this project involves interlaboratory quality-control studies on levels of PCBs in human milk, and the results of the first round of such studies, which involved 12 laboratories, have been published (*3*). Planning of the second round of quality-control studies has already begun, and additional laboratories are expected to participate. The Committee expressed its support for studies of this type.

In the case of patulin, the results of numerous surveys of fruit products have been published over the last two decades. However, in many of the older surveys, the methods used were not sufficiently sensitive, and patulin was not positively identified. The Committee's evaluation of this mycotoxin took account of these facts.

3. COMMENTS ON SPECIFIC FOOD ADDITIVES AND CONTAMINANTS[1]

The Committee evaluated a number of food additives and contaminants for the first time and re-evaluated several substances considered at previous meetings. Information on the evaluations and on specifications is summarized in Annex 2. Details of further toxicological studies and of other information required or desired for certain substances are given in Annex 3.

3.1 Specific food additives

3.1.1 *Emulsifiers*

Polyglycerol esters of fatty acids

At the Committee's seventeenth meeting (see Annex 1, reference *32*), polyglycerol esters of fatty acids were evaluated and the

[1] Bibliographical references to toxicological studies are included in this section only for substances for which toxicological monographs (which would normally list such references) have not been prepared.

Committee agreed to convert the former conditional ADI to an ADI of 0–25 mg per kg of body weight.

At its thirty-first meeting (see Annex 1, reference *77*), the Committee revised the specifications but was unable to accept the request to increase the range of average polyglycerol chain lengths permitted from three to ten glycerol units without a review of the toxicological data on these substances. Since the data requested were not forthcoming, the Committee at its present meeting maintained the previous ADI of 0–25 mg per kg of body weight for polyglycerol esters of fatty acids having an average chain length of up to three glycerol units.

A toxicological monograph was not prepared.

The existing specifications for polyglycerol esters of fatty acids were maintained.

Sucrose esters of fatty acids and sucroglycerides

Sucrose esters of fatty acids are the mono-, di-, and triesters of sucrose with edible fatty acids. They may be prepared from sucrose and the methyl and ethyl esters of edible fatty acids, usually in the presence of a solvent. "Sucroglycerides" (a mixture of sucrose esters of fatty acids and mono- and diglycerides) are produced by reaction of edible fats or oils with sucrose; this reaction is also usually carried out in the presence of a solvent.

These substances were evaluated for the purpose of establishing an ADI at the Committee's thirteenth, seventeenth, twentieth, and twenty-fourth meetings (Annex 1, references *19, 32, 41*, and *53*). Separate toxicological monographs were prepared on each occasion for sucrose monoesters of individual fatty acids and for palm-oil sucrose esters and lard and tallow sucrose esters.

At its present meeting, the Committee was asked to consider the consequences of modifying the specifications for these substances when manufactured by a process in which dimethylsulfoxide, isobutanol, ethyl methyl ketone, or a combination of these is used as solvent. It was noted that an ADI (or provisional intake) had not been established for these solvents but that dimethylsulfoxide and isobutanol occur naturally in the diet and ethyl methyl ketone has been identified as a product of intermediary metabolism. The Committee concluded that, in foods as consumed, the levels of these solvents arising from residues in sucrose esters of fatty acids that comply with the specifications (as revised at the present meeting) are

insignificant relative to naturally occurring levels in the diet, and there is no reason to suppose that they present a hazard.

The Committee also reviewed new toxicological studies on a palm-oil sucroglyceride, including a long-term carcinogenicity study in rats and short-term studies in rats and dogs.

It was concluded that, both for sucrose esters of fatty acids manufactured by a process using dimethylsulfoxide, isobutanol, ethyl methyl ketone, or a combination of these as solvent, and for the palm-oil sucroglyceride, the previously established group ADI of 0–10 mg per kg of body weight for sucrose esters of fatty acids and sucroglycerides would apply.

An addendum to the toxicological monograph was prepared.

The specifications for sucrose esters of fatty acids were revised to include considerations on the use of the above-mentioned solvents.

The existing specifications for sucroglycerides were maintained.

3.1.2 *Enzyme preparations*

Enzyme preparations derived from Aspergillus niger

As a consequence of its review of general specifications for enzyme preparations, the Committee reconsidered the evaluation of enzymes derived from *Aspergillus niger* made at the thirty-first meeting (Annex 1, reference *77*). At that meeting, the Committee established a single ADI for several separate enzyme preparations derived from *Aspergillus niger* of 0–1 mg of total organic solids per kg of body weight. The enzyme preparations for which this ADI was established were carbohydrases, amyloglucosidases (EC 3.2.1.3), endo-1,3(4)-β-glucanase (EC 3.2.1.6), hemi-cellulase, pectinases (EC 3.1.1.11; 4.2.2.10; 3.2.1.15), and protease.

In view of the fact that *Aspergillus niger* is a common organism in food, that many strains have had a long history of use as an enzyme source, and that the numerous studies of various preparations from various strains have demonstrated no hazard to human health, the numerical ADI that was earlier established for each of the above-listed enzyme preparations from *Aspergillus niger* was changed to an ADI "not specified".

A toxicological monograph was not prepared.

None of the existing specifications for enzyme preparations derived from *Aspergillus niger* were reviewed.

3.1.3 *Flavouring agents*

Benzyl acetate

This compound was previously reviewed at the eleventh, twenty-seventh, twenty-ninth, and thirty-first meetings of the Committee (Annex 1, references *14, 62, 70,* and *77*).

At the thirty-first meeting, the Committee extended the temporary ADI of 0–5 mg per kg of body weight pending the evaluation of lifetime gavage studies with benzyl alcohol, a normal metabolite of benzyl acetate. These studies did not show an increased incidence of either hepatocellular or forestomach tumours in mice or pancreatic tumours in rats, although such effects had previously been observed in studies with benzyl acetate. However, there are difficulties in interpreting the results of the carcinogenicity studies with benzyl acetate since the compound was given by gavage. Since new long-term studies are under way with benzyl acetate incorporated into the diet of rats and mice, the Committee decided to extend the temporary ADI of 0–5 mg per kg of body weight until 1993 pending the evaluation of the results of these studies.

In view of a report of a positive result in an *in vitro* mutagenicity test on benzyl acetate, the Committee concluded that it would be desirable to ascertain whether the results of an existing *in vivo* study that demonstrated the absence of the induction of unscheduled DNA synthesis could be confirmed by an *in vivo* test for chromosome damage in bone marrow.

A toxicological monograph was prepared.

The existing specifications for benzyl acetate were maintained.

Cinnamaldehyde

Cinnamaldehyde was evaluated at the eleventh, twenty-third, twenty-fifth, and twenty-eighth meetings of the Committee (Annex 1, references *14, 50, 56,* and *66*).

At its twenty-third meeting, the Committee converted the previously established conditional ADI of 0–1.25 mg per kg of body weight into a temporary ADI of 0–0.07 mg per kg of body weight (Annex 1, reference *50*) because of inadequacies in the toxicity data. At the twenty-eighth meeting, the temporary ADI was extended and an extensive series of studies was requested.

Because the required data were not forthcoming, the Committee was unable to extend the temporary ADI at its present meeting. However, the Committee concluded that, of the information

requested at the twenty-eighth meeting, the results of the short-term feeding study in a non-rodent mammalian species and of adequate metabolic studies might be sufficient to make re-evaluation possible.

A toxicological monograph was not prepared.

The existing specifications for cinnamaldehyde were maintained.

Dihydrocoumarin

The safety of this substance was evaluated for the first time by the Committee at its present meeting.

Metabolites of dihydrocoumarin identified in rabbit urine include umbelliferone, 3-hydroxycoumarin, coumarin, *o*-coumaric acid, melilotic acid, melilotoylglycine, and *o*-coumaroylglycine (*4*). There is some evidence that the gut flora is responsible for the conversion to melilotic acid (*5*).

The toxicological information available was derived from acute toxicity tests in mice (*6*), rats (*7, 8*), and guinea-pigs (*7*), a 14-week study in rats in which loss of test compound in the diet mixture during storage precluded estimation of exact exposure levels (*9*), a short-term study in rats (90 days), in which a single dose level was used (*10*), and a study in which three dogs were treated at one of two dose levels of dihydrocoumarin for two years but for which there was no control group (*9*). Although no adverse effects were reported in these studies, the Committee considered the data inadequate for toxicological evaluation and was therefore unable to allocate an ADI for dihydrocoumarin. The Committee stated that, for a re-evaluation of this substance, the results of a short-term study in a rodent species and metabolic studies to investigate the extent of conversion to coumarin would be needed.

A toxicological monograph was not prepared.

New specifications were prepared for dihydrocoumarin and were designated as tentative. Further information is required (see Annex 3).

Ethyl vanillin

Ethyl vanillin was previously evaluated at the eleventh meeting of the Committee (Annex 1, reference *14*), when an ADI of 0–10 mg per kg of body weight was allocated to this compound on the basis of a long-term study in rats. Although the Committee had then considered it possible to allocate an ADI, it had noted that few metabolic studies were available and concluded that further studies of that type were desirable.

Ethyl vanillin was placed on the agenda of the present meeting on the basis of partial application of the method for setting priorities for the safety review of food flavouring ingredients (see section 2.1).

The Committee noted that none of the previously evaluated long-term or carcinogenicity studies met modern standards in that fewer animals per group had been used than would be the present norm.

Accordingly, it reduced the previous ADI to 0–5 mg per kg of body weight, and made it temporary. The Committee requested submission, by 1992, of the results of an adequate short-term study in rats and metabolic studies in rats.

A toxicological monograph was prepared.

The existing specifications for ethyl vanillin were revised.

Fumaric acid

Fumaric acid and sodium fumarate were evaluated for the purpose of establishing an ADI by the Committee at its tenth, eighteenth, and twenty-third meetings (Annex 1, references *13, 35,* and *50*). At the eighteenth meeting, the previous unconditional ADI for fumaric acid was confirmed as an ADI of 0–6 mg per kg of body weight. At the twenty-third meeting, the Committee decided to establish a group ADI for fumaric acid and its salts of 0–6 mg per kg of body weight.

Fumaric acid was placed on the agenda of the present meeting on the basis of partial application of the method for setting priorities for the safety review of food flavouring ingredients (see section 2.1).

Because fumaric acid is a normal constituent of tissues and is metabolized by the body, the Committee decided to change the previous group ADI to a group ADI "not specified" for fumaric acid and its salts, in agreement with the guidelines laid down in section 5.2.3 of *Principles for the safety assessment of food additives and contaminants in food* (Annex 1, reference *76*).

A toxicological monograph was not prepared.

The existing specifications for fumaric acid were revised.

Quinine hydrochloride

The safety of this substance was evaluated for the first time by the Committee at its present meeting. Specifications had been developed at the twenty-fourth and twenty-sixth meetings (Annex 1, references *53* and *59*).

Biochemical studies, short-term studies in rats, teratogenicity studies in rats, and mutagenicity studies were reviewed. In these

studies, no-effect levels ranged from 40 to 100 mg per kg of body weight per day. Mutagenicity studies gave negative results.

Varied complaints including headaches and transient visual problems were reported in human volunteers given doses of 100 mg of quinine hydrochloride per person per day. These findings were not confirmed in a second, controlled study using 120 mg per person per day. A third study showed electronystagmographic changes in stressed subjects for which a no-effect level of 52.5 mg of quinine per person per day was determined. The Committee concluded that an evaluation could be made on the basis of the human data. Since the toxic effects of concern were acute and reversible, and there is extensive experience of human consumption of quinine without reports of acute toxicity, except very rarely in hypersensitive individuals, the Committee saw no need to require a margin of safety. It established an acceptable intake of 52.5 mg of quinine per person per day, equivalent to an ADI of 0–0.9 mg of quinine per kg of body weight per day. However, the Committee considered that data from more extensive human studies should be submitted and therefore made the ADI temporary.

The Committee requested submission of the results of an adequate human study by 1992.

A toxicological monograph was prepared.

The existing specifications for quinine hydrochloride were revised.

3.1.4 *Food colours*

Canthaxanthin

This substance was last evaluated by the Committee at its thirty-first meeting (Annex 1, reference *77*), when it was noted that ingestion of canthaxanthin could, in some circumstances, lead to deposits of crystals in the human retina. At that time, the Committee reduced the previously allocated ADI to 0–0.5 mg per kg of body weight and made it temporary. In addition, it required: (*a*) further details of long-term studies in rats and mice; (*b*) clarification of the factors that influence deposition in the eye, including the establishment of the threshold dose, information on the influence of dose and duration of exposure and on the reversibility of pigment accumulation, and the investigation of potential animal models; and (*c*) clarification of whether pigment deposition is causally related to impaired visual function.

Canthaxanthin is used both as a direct food additive and as a feed additive for colouring salmonid fish and chicken egg yolks. Although there is some metabolic transformation to other carotenoids in egg yolks, the parent compound is present in the products derived from animals to which it has been given as a feed additive, so the present evaluation also covers its use for this purpose.

Since the previous evaluation, substantial amounts of new data have become available and these were reviewed by the Committee.

The Committee noted that the results of two long-term carcinogenicity studies in mice and rats did not provide evidence of carcinogenicity. However, at high dose levels, canthaxanthin produced liver damage in rats (with a non-dose-related increased incidence of benign nodules in female rats); mice appeared to be less sensitive to hepatic injury. It was concluded that, in addition to the eye, the liver was a target organ for canthaxanthin.

In the long-term studies in rats, it was not possible to establish a no-effect level. However, the Committee was informed that another long-term study in rats was in progress aimed at establishing a no-effect level in respect of pathological changes in the liver.

While distribution studies with radiolabelled canthaxanthin showed that relatively high concentrations accumulated in the eye in all the mammalian species studied, crystal deposition has, to date, been observed only in the human retina. The animal species studied have therefore not provided a suitable model for the study of the pathogenesis and reversibility of this phenomenon. However, the changes noted in electroretinograms in humans were reproduced in the electroretinograms of pigmented rabbits after canthaxanthin treatment.

The Committee concluded that the long-term toxicity of canthaxanthin in rats indicated potential hepatotoxicity in humans; this matter may be resolved by obtaining clinical data from human subjects showing retinal pigment deposition. However, the Committee considered that the primary problem associated with canthaxanthin was the deposition of crystals in the human retina.

In view of the irreversibility or very slow reversibility of the retinal crystal deposition, the significance of which is not known, the Committee was unable to establish an ADI for canthaxanthin when used as a food additive or animal feed additive. The previous temporary ADI was therefore not extended.

A toxicological monograph was prepared.

The existing specifications for canthaxanthin were maintained.

Carotene preparations from natural sources

These substances were reviewed at the eighteenth and thirty-first meetings of the Committee (Annex 1, references *35* and *77*). At the latter meeting, the Committee noted that, while there was a substantial toxicological data base relating to carotenes and an ADI had been established for synthetic β-carotene, the same ADI was not applicable to natural carotenes as they did not comply with the specifications for β-carotene.

At its present meeting, the Committee considered limited biochemical, acute, and short-term toxicological studies on material derived from three different algal species, namely *Dunaliella bardawil, D. salina,* and *D. kona*. Some of the preparations produced from these species were dried concentrates produced by lyophilization or spray-drying; another product was a vegetable oil extract.

The Committee concluded that there was insufficient evidence to indicate that data relating to one species of *Dunaliella* could be applied to others. It also decided that the specifications of the test materials were so different from one another that the results of the toxicity tests could not be generalized. There were insufficient data to evaluate any of these materials for the purpose of establishing an ADI.

The Committee considered that carotene isolated from algal sources would be acceptable for food additive use if it was of sufficient purity to meet the specifications for synthetic β-carotene. Acceptance of algal biomass or crude extracts of carotene from algal sources for use as food additives would be contingent on the provision of evidence of the safety of such materials.

A toxicological monograph was not prepared.

The existing tentative specifications for carotenes (algae) were revised. The Committee was aware that three different species of *Dunaliella* are used as sources of carotene, and recognized the need for further information regarding the differences between both the species and the resulting products, and the influence of the method of manufacture, such as spray-drying, on the quality. The Committee was also informed that carbon dioxide is not used for the extraction and it saw no need to test for urethane which might have resulted from such use. The existing tentative specifications for carotenes (vegetable) were also revised. The "tentative"

qualifications for both were maintained and certain further information is required (see Annex 3).

Curcumin and turmeric oleoresin

Turmeric and curcumin (the main colouring component of turmeric) were considered at the thirteenth, eighteenth, twenty-second, twenty-sixth, and thirtieth meetings of the Committee (Annex 1, references *19, 35, 47, 59,* and *73*). Toxicological monographs were prepared on each of these occasions (Annex 1, references *20, 36, 48, 60,* and *74*). At the thirtieth meeting, the Committee concluded that turmeric is often regarded as a food rather than as a food additive, and it is therefore not appropriate to allocate an ADI to this substance. The temporary ADI for curcumin was extended, and a temporary ADI was allocated to turmeric oleoresin at that meeting.

Curcumin. When the temporary ADI of 0–0.1 mg per kg of body weight was extended at the Committee's thirtieth meeting, the submission of the results of a carcinogenicity study and a reproduction/teratogenicity study was requested. The results of these studies were not made available at the present meeting, but the Committee was informed that the results of carcinogenicity studies, including fertility assessment phases, in B6C3F1 mice and F344 rats given turmeric oleoresin containing a high concentration of curcuminoids should be available in 1990. The Committee therefore extended the temporary ADI until 1992, with the requirement that the results of the above-mentioned studies should be made available for review at that time. Since a reproduction phase is included in the ongoing carcinogenicity studies, the Committee would wish to review these studies before deciding whether the reproduction/teratogenicity study requested earlier was still needed.

A toxicological monograph was not prepared.

The existing specifications for curcumin were maintained.

Turmeric oleoresin. At the thirtieth meeting, when the Committee established the temporary ADI of 0–0.3 mg per kg of body weight for turmeric oleoresin, it requested the results of an additional short-term study in pigs or another suitable non-rodent species in order to establish a clear no-effect level for effects on the thyroid gland, which had been observed in a study in pigs. The results of such a study were

not made available at the present meeting of the Committee and the temporary ADI was therefore not extended.

A toxicological monograph was not prepared.

The Committee reviewed the specifications for turmeric oleoresin and turmeric colour as recommended at the thirtieth meeting. The Committee, at that meeting, had suggested that the specifications for turmeric oleoresin be revised to bring them into line with those for turmeric colour. However, at its present meeting, the Committee concluded that specifications for turmeric colour were contained in those for the oleoresin, and therefore deleted the existing separate specifications for the former. The specifications for turmeric oleoresin were revised to emphasize the principal colouring components of the oleoresin and to incorporate the method of assay for content of total colouring matter that was previously part of the turmeric colour specifications.

The Committee pointed out that the revised specifications for turmeric oleoresin cover a range of products, some of which are used as colours and some as flavourings; components other than pure colouring principles (e.g., the volatile oils) should therefore be taken into account, as necessary, when such products are evaluated.

Paprika oleoresin

Paprika oleoresin was evaluated at the fourteenth meeting of the Committee (Annex 1, reference *22*), when no ADI was established because it was recognized that the use of this spice extract is self-limiting for technological and organoleptic reasons.

The Committee was informed that the extraction solvent 1,2-dichloroethane is being used to produce paprika oleoresin. At both the thirty-first meeting (Annex 1, reference *77*) and the present one, the Committee revised the specifications for paprika oleoresin but decided not to include 1,2-dichloroethane as an additional processing solvent. The toxicological data on 1,2-dichloroethane were therefore not reviewed, and a toxicological monograph was not prepared.

In addition to 1,2-dichloroethane, two other chlorinated hydrocarbons are among the solvents currently listed for use in paprika oleoresin production. The Committee expressed its general opinion that levels of residues resulting from the use of any solvent should be both the minimum technically feasible and of no toxicological concern.

In future reviews of the specifications for paprika oleoresin and other oleoresins, justification by the industry for the use of chlorinated hydrocarbon solvents will be sought, together with specific information on actual residues resulting from their use.

3.1.5 *Thickening agents*

Gum arabic

This substance was last evaluated at the twenty-sixth meeting of the Committee (Annex 1, reference *59*) and an ADI "not specified" was allocated.

At its present meeting the Committee reviewed further findings from teratogenicity and biochemical studies, and concluded that the results of these studies gave no reason to modify the previous evaluation. The Committee therefore confirmed the ADI "not specified". An addendum to the existing toxicological monograph was prepared.

The Committee's attention was drawn to the fact that products were being sold as gum arabic that were derived from species other than *Acacia senegal* (L) Willdenow and closely related species hitherto recognized as the source species of gum arabic. It was informed that all these gums would be covered by the existing specifications for gum arabic.

However, the Committee was also informed of extensive studies on the chemical composition of individual gums. These studies clearly showed that, while the composition of gums from *Acacia senegal* and closely related species originating from various geographical regions varied only slightly, there were significant differences in the composition of gums from other species, for example in the carbohydrate content and ratios of different amino acids.

The existing specifications were therefore revised to reflect more closely the gums that have been toxicologically evaluated.

Modified celluloses

Modified celluloses were reviewed at the fifth, seventh, tenth, thirteenth, seventeenth, twenty-sixth, twenty-seventh, and thirtieth meetings of the Committee (Annex 1, references *5, 7, 13, 19, 32, 59, 62*, and *73*). At the seventeenth meeting, a group ADI of 0–25 mg per kg of body weight was allocated for the five modified celluloses previously reviewed (methyl cellulose, methyl ethyl cellulose,

hydroxypropyl cellulose, hydroxypropyl methyl cellulose, and sodium carboxymethyl cellulose). A toxicological monograph on these five compounds was prepared (Annex 1, reference *33*).

At the twenty-sixth and twenty-seventh meetings of the Committee, ethyl cellulose and ethyl hydroxyethyl cellulose, respectively, were reviewed and it was decided that the group ADI of 0–25 mg per kg of body weight should also apply to them. A toxicological monograph on ethyl hydroxyethyl cellulose was prepared (Annex 1, reference *74*).

Since the previous evaluation, additional data have become available, including data from studies in rats on caecal enlargement and changes in caecal flora, teratogenicity, and development, and from *in vitro* mutagenicity studies on methyl cellulose and carboxymethyl cellulose. These studies confirmed the conclusion reached at the earlier meetings of the Committee that modified celluloses are of low toxicity.

In long-term/carcinogenicity studies on hydroxypropyl methyl cellulose, methyl cellulose, methyl ethyl cellulose, and sodium carboxymethyl cellulose in rats and mice, no evidence of mutagenicity or carcinogenicity was observed. In addition, in reproduction and teratogenicity studies in mice, rats, and rabbits, the consumption of hydroxypropyl cellulose, methyl cellulose, or sodium carboxymethyl cellulose did not interfere with the reproductive process, and no embryotoxic or developmental effects were observed.

A new substantial body of human data was available on the laxative effects of modified celluloses, which are seen in some subjects at levels as low as 5 g per person per day. At higher doses, diarrhoea was reported in some subjects, but in others constipation developed. The amounts ingested in studies in humans did not exceed 30 g per person per day, which has been recommended by the United States National Research Council as the upper safe level of dietary fibre in general (*11*).

The Committee allocated a group ADI "not specified" to these modified celluloses, and pointed out that their laxative properties should be taken into account when they are used as food additives (see section 2.2.3).

A toxicological monograph was prepared.

None of the existing specifications for modified celluloses were reviewed.

3.1.6 *Miscellaneous food additives*

Ferrous lactate

Ferrous lactate, a colour adjunct, was considered for the first time by the Committee.

At the seventeenth meeting, the Committee evaluated lactic acid and its ammonium, calcium, potassium, and sodium salts (Annex 1, reference *32*). Since lactic acid is a normal constituent of food and a normal intermediary metabolite in humans, the Committee decided at that meeting to establish a "not limited" ADI.

Iron was evaluated at the twenty-seventh meeting of the Committee and, on the basis of the data available, a provisional maximum tolerable daily intake (PMTDI) of 0.8 mg per kg of body weight was allocated (Annex 1, reference *62*). It was pointed out that the tolerable daily intake should not be used as a guideline in the fortifying of processed food (Annex 1, reference *60*, section 2.8).

At its present meeting, the Committee concluded that, because the iron in ferrous lactate is bioavailable, the amount of iron resulting from the use of ferrous lactate should be included with that from all other sources, and the total should not exceed the PMTDI for iron of 0.8 mg per kg of body weight.

A toxicological monograph was not prepared.

New specifications for ferrous lactate were prepared.

2-Nitropropane

2-Nitropropane was considered by the Committee at the twenty-third, twenty-fifth, and twenty-eighth meetings (Annex 1, references *50, 56*, and *66*). Toxicological monographs were prepared after each meeting (Annex 1, references *51, 57,* and *67*). At the twenty-eighth meeting, 2-nitropropane was considered to be temporarily acceptable for use as a fractionating solvent in the production of fats and oils, as long as its use continued to be limited and residue levels were kept to the lowest technically attainable.

The Committee noted that fractionated fats and oils have physical properties that limit their application and that present procedures for the processing of fats and oils with 2-nitropropane do not lead to detectable levels of this substance in the finished product. On the assumption that such treated fats and oils may contain 2-nitropropane at the limit of detection, namely, 10 µg/kg, and on the basis of a maximum projected intake of this substance in processed oils in the United States, the maximum intake of 2-nitropropane was

estimated to be 0.13 ng per kg of body weight per day. The Committee recognized that this was a worst-case intake estimate and that actual intakes of 2-nitropropane were probably lower.

The Committee reviewed a new inhalation study in which mice were exposed to 2-nitropropane; nodular hyperplasia of the liver was observed in females. A carcinogenic effect had previously been noted in rats after inhalational exposure to relatively high concentrations (100–800 mg/m^3) of 2-nitropropane (Annex 1, reference *66*). In addition, the Committee reviewed a new study in which all rats dosed by gavage with 2-nitropropane at a level of 89 mg per kg of body weight for 16 weeks (three days per week) developed hepatocarcinomas.

On the basis of these studies, 2-nitropropane was considered to be a potent liver carcinogen in rats, and the temporary acceptance of this substance for use as a fractionating solvent in the production of fats and oils was therefore not extended. However, if the technological need for this solvent could be demonstrated and if data were provided that could be used for establishing a safe level of intake of 2-nitropropane, the Committee would reconsider it at a future meeting.

A toxicological monograph was prepared.

The existing specifications for 2-nitropropane were revised, but maintained as tentative (see Annex 3).

Tannic acid

Tannic acid was reviewed at the fifth, tenth, fourteenth, and thirty-first meetings of the Committee (Annex 1, references *5, 13, 22,* and *77*).

At the thirty-first meeting (Annex 1, reference *77*), the previous temporary ADI was changed to a temporary ADI "not specified" for tannic acid used as a filtering aid. Further data were requested on the composition of tannic acid from different sources.

Information was provided to the Committee at its present meeting that permitted revision of the specifications so as to require a high degree of purity for tannic acid used as a filtering aid where the application of good manufacturing practice ensures that it is removed from food after use. An ADI "not specified" for this use was therefore established.

As detailed information on the composition of tannic acid from different botanical sources was not forthcoming and no new

toxicological data were available, it was not possible to consider its use as a direct food additive.

A toxicological monograph was not prepared.

The Committee received updated information on the manufacture of tannic acid and, as stated above, was in a position to revise the existing tentative specifications. It maintained the "tentative" qualification and renewed the request for data on the composition of tannic acid from different botanical sources. Data on actual uses and levels of use of tannic acid as a flavouring agent were also requested (see Annex 3).

Lactoperoxidase/thiocyanate/hydrogen peroxide system

At the twenty-ninth meeting (Annex 1, reference *70*), the Committee considered the practice of adding sodium thiocyanate and hydrogen peroxide (the lactoperoxidase/thiocyanate/hydrogen peroxide system) to raw milk to maintain its quality. Since then, the Joint FAO/WHO Committee of Government Experts on the Code of Principles concerning Milk and Milk Products has produced draft guidelines for the preservation of raw milk by use of the lactoperoxidase system in circumstances where refrigeration is virtually impossible. The underlying principles and application of the lactoperoxidase system have been further elaborated in the "Code of practice for the preservation of raw milk by the lactoperoxidase system" (*12*).

The lactoperoxidase system consists of three components: lactoperoxidase naturally present in bovine and buffalo milks; added sodium thiocyanate; and an added source of hydrogen peroxide.

The Committee reviewed the draft guidelines, and noted that milk preserved by this method would contain an amount of sodium thiocyanate greater by up to 14 mg/l than that naturally present in milk.

The Committee recognized that the potential major toxic effect of thiocyanate ion is interference with iodine uptake by the thyroid gland. Thiocyanate occurs normally in blood and urine as a result of ingestion of precursors in the diet. Epidemiological studies indicate that there is no significant toxicity from chronic dietary consumption of thiocyanate provided that iodine intake is adequate.

The Committee noted that the levels of hydrogen peroxide that would be introduced into milk through the use of sodium percarbonate, a hydrogen peroxide adduct of sodium carbonate ($2Na_2CO_3.3H_2O_2$), as the source of hydrogen peroxide in the

lactoperoxidase system were lower than those considered acceptable at the twenty-fourth meeting of the Committee (Annex 1, reference *53*) and therefore were not considered to be a cause for concern. Application of the lactoperoxidase system to raw milk requires a hydrogen peroxide concentration of approximately 10 mg per litre. This is significantly lower than the concentration of hydrogen peroxide required (300–800 mg/kg) when that substance is used alone for the preservation of raw milk.

The Committee recognized that the use of the lactoperoxidase system would increase total thiocyanate exposure but considered that that would not pose any toxicological problem provided that iodine intake was adequate. It concluded that, when used according to the draft guidelines, the lactoperoxidase system does not present a toxicological hazard and, furthermore, that the system should be used in preference to hydrogen peroxide alone for the preservation of raw milk, though only where absolutely necessary, i.e., in the absence of adequate refrigeration facilities.

A toxicological monograph was not prepared.

The existing specifications for sodium thiocyanate were maintained. New specifications for sodium percarbonate were prepared.

3.2 Contaminants

3.2.1 *Patulin*

Patulin has not been previously evaluated by the Committee. The Committee noted that fungi of several different genera, including *Penicillium, Aspergillus,* and *Byssochlamys*, are capable of producing patulin. The natural occurrence of this mycotoxin has been largely associated with *Penicillium expansum*, a common spoilage microorganism in apples.

The Committee reviewed studies on the biochemistry and toxicology of patulin as well as very limited information on observations in humans when patulin was tested as an antibiotic for treatment of the common cold.

In rats, most of the administered dose was eliminated within 48 hours, in faeces and urine, less than 2% being expired as carbon dioxide. No other metabolites have been identified. About 2% of the administered dose was still present after seven days, associated primarily with erythrocytes.

Patulin has a strong affinity for sulfhydryl groups, which explains why it inhibits the activity of many enzymes. Patulin adducts formed with cysteine were less toxic than the unmodified compound in acute toxicity, teratogenicity, and mutagenicity studies.

In acute and short-term studies, patulin caused gastrointestinal hyperaemia, distension, haemorrhage, and ulceration. Pigtail monkeys tolerated patulin consumption of up to 0.5 mg per kg of body weight per day for four weeks without adverse effects.

The results of two reproduction studies in rats were available. No reproductive or teratogenic effects were noted at levels of up to 1.5 mg per kg of body weight per day, but there was an increase in the frequency of fetal resorptions at that level.

The results of a carcinogenicity study in rats, with orally administered patulin, were negative. Short-term *in vitro* genotoxicity studies indicate that patulin is not mutagenic, but it has clastogenic activity in some test systems.

The Committee set a provisional tolerable weekly intake (PTWI) for patulin of 7 µg per kg of body weight based on a no-effect level of 0.1 mg per kg of body weight per day in a combined reproduction/long-term/carcinogenicity study in rats. An additional long-term/carcinogenicity study in a rodent species other than the rat is recommended for further evaluation of the toxicity of patulin.

The Committee had before it data on patulin levels in apple juice, which is often consumed by children. On the basis of surveys in limited areas of the world, a maximum intake by children of 0.26 µg per kg of body weight per day has been estimated. However, apple juice can occasionally be heavily contaminated and the Committee therefore considered that efforts should be made to avoid unnecessary exposure to this mycotoxin by adherence to good manufacturing practices whereby rotted or mouldy fruit is not used. This should reduce dietary exposure to levels below the PTWI. The Committee urged the application of such practices.

A toxicological monograph was prepared.

3.2.2 *Polychlorinated biphenyls (PCBs)*

Polychlorinated biphenyls (PCBs) have not been previously evaluated by the Committee.

PCBs are a class of stable chlorinated hydrocarbons which, prior to the 1970s, had been used extensively in a wide range of industrial

applications. About 50–60 different PCBs, with different degrees of chlorination and thus differing physical properties, are still found in industrial products. The higher the chlorine content of PCBs, the more resistant they are to biodegradation. When PCBs are ingested, the less highly chlorinated biphenyls are metabolized in the liver, primarily to hydroxylated compounds, which are rapidly excreted. The more highly chlorinated biphenyls are more metabolically stable and accumulate in body fat.

The Committee had before it data from a large number of toxicity studies, of varying merit, most of which were carried out with commercial mixtures of PCBs. The evaluation of these studies is both difficult and complicated since the PCB mixtures tested were ill-defined and many were contaminated with polychlorinated dibenzofurans and other related chlorinated compounds. The presence of these contaminants is thought to be at least partly responsible for a number of effects seen in the animal experiments. The Committee nevertheless concluded that sufficient data were available to enable some general conclusions regarding the effects of PCBs to be reached.

Some of the PCB mixtures were hepatocarcinogenic in rodent bioassays. However, human experience from long-term accidental exposures and from epidemiological studies of workers exposed occupationally is inconclusive in respect of an association between PCB exposure and increased cancer mortality.

From a comparison of data from animal studies and symptoms observed in accidentally exposed human populations, the monkey appears to be the most appropriate animal for use in studies on PCBs. The Committee concluded, on the basis of the available studies with monkeys, that 0.04 mg per kg of body weight per day was a no-effect level; only minor effects were observed at 0.1 mg per kg of body weight per day.

Because of the limitations of the available data and the ill-defined nature of the materials that were used in feeding studies, the Committee concluded that it was impossible to establish a precise numerical value for a tolerable intake for humans. In particular, the PCB mixtures used in the monkey studies cited above were not entirely the same as those to which humans are exposed in the diet. However, there is no reason to believe that humans would be more sensitive than monkeys to the effects of PCBs, and some indication of safe exposure levels can therefore be obtained from the no-effect level observed in the monkey studies.

The major foods in which contamination with PCBs is possible are fish, milk and other dairy products, and meat. Median levels in fish reported in various countries are of the order of 100 µg/kg compared with less than 20 µg/kg for other foods. An important exception is human milk, in which PCB median levels ranging from 15 to 100 µg/kg on a whole milk basis have been reported.

The dietary intake of PCBs by various populations has been estimated to range from 0.005 to 0.2 µg per kg of body weight per day. Such a wide variation in intakes can be explained not only by the type and amount of food consumed but also by the method used to estimate the dietary PCB intake. In the case of breast-fed infants, PCB intakes can be calculated to range from 2 to 12 µg per kg of body weight per day on the basis of the median levels noted previously and an average milk intake of 120 ml per kg of body weight.

In foods that contain higher levels of PCBs and/or contribute significantly to the total dietary PCB intake, preliminary studies have identified ten specific PCBs as predominant. In human breast milk, six of them account for approximately 70% of the total PCB content.

The Committee paid particular attention to the possible health consequences of the intake of PCBs by the suckling infant, but did not anticipate that adverse health effects would occur as a result of consuming breast milk. It should be kept in mind that the infant consumes breast milk for only a short period (1–2% of the total life span). In addition, the numerous benefits of breast milk, including its nutritional, immunological, and other properties, and the psychological advantages of breast-feeding should not be discounted; the disadvantages of breast milk substitutes, including potential contamination by infective agents and the consequences of incorrect preparation and inadequate hygiene, have been amply documented (*13, 14*). For these reasons, the Committee considered that the advantages to the infant of breast-feeding outweighed any potential hazards due to the PCB content of breast milk, and advised that there was absolutely no justification for discouraging this practice.

The Committee was reassured by the observation that the production of PCBs has largely ceased. It is expected, therefore, that levels of PCBs in the environment and food, and consequently in breast milk, will decrease with time.

The Committee suggested that further investigations be conducted to identify the PCBs most commonly present in foods and that safety studies be carried out on them, and particularly on the more highly chlorinated ones, in order to determine their toxicological potential. Furthermore, specific studies on the impact of these PCBs on the fetus and neonate were considered to be of great importance.

The Committee considered that the intake of PCBs should be kept as low as possible. In foods in which PCBs occur, and that are nutritionally essential, attempts should be made to set limits on PCBs, in particular for the most highly contaminated products. However, the Committee concluded that the consumption of PCBs at the dietary levels described above did not involve any long-term hazard. Finally, the Committee pointed out that good public health practices would require that a long-term goal should be the reduction of PCBs in the diet to a minimum.

An early draft of a document on PCBs that is being prepared for publication by WHO in the Environmental Health Criteria series was made available to the Committee. The working paper on which the Committee relied for its evaluation reproduced the discussion of the metabolism and toxicity studies contained in this draft document. In order to avoid duplication within WHO, a toxicological monograph was not prepared.

4. REVISION OF CERTAIN SPECIFICATIONS

4.1 General

Four substances were evaluated for specifications only (see Annex 2), and the specifications for all of them were revised.

The Committee revised the specification for carob bean gum so as to include the use of two solvents, ethanol and isopropanol, in a washing process used to purify the substance.

The previous specifications for citric and fatty acid esters of glycerol (Annex 1, reference *75*) had been designated as "tentative" because a suitable assay method and data on the amounts of individual components were lacking. The Committee revised the tentative specifications by including analytical methods and by specifying total citric acid, total fatty acids, and total glycerol. Consideration was also given to the sum of these components,

but the Committee decided not to specify it. The "tentative" qualification was deleted.

The existing specifications for iron oxides used as food colours were revised.

The specifications for modified starches (Annex 1, reference *79*) had been designated "tentative" as analytical methods were needed for carboxyl groups in oxidized starch and free adipic acid in acetylated distarch adipate. The Committee revised the specifications to include analytical methods for both and removed the "tentative" qualification.

4.2 General specifications for enzymes used in food processing

The existing general specifications for enzyme preparations used in food processing (Annex 1, reference *69*) were revised. The Committee recommended that, as and when enzyme preparations are reconsidered, or when new enzyme preparations are submitted for evaluation, their individual specifications should be reviewed to ensure they are consistent with the principles on which the new general specifications are based.

5. FUTURE WORK

1. The guidelines for the evaluation of various groups of food additives and contaminants that have been developed by the Committee and are given in Annex 3 of *Principles for the safety assessment of food additives and contaminants in food* (Annex 1, reference *76*) should be reviewed at a future meeting (see section 2.2.1).

2. Individual specifications for enzyme preparations used in food processing should be reviewed to take account of the principles set out in the revised general specifications (see section 4.2).

3. In view of the decision not to include the use of 1,2-dichloroethane as an extraction solvent in the specifications for paprika oleoresin despite its current listing for other spice oleoresins, the Committee should re-evaluate the toxicological basis for the listing of 1,2-dichloroethane in other specifications. Such a re-examination should be expanded to include a consideration of all solvent uses of chlorinated hydrocarbons.

4. A number of the specifications reviewed at the present meeting make reference to the general methods section of the *Guide to*

specifications (Annex 1, reference *65*). The Committee reiterated the need expressed at its thirtieth and thirty-third meetings to update the general methods and to include with them in a single publication the additional general methods adopted since the last revision of this compendium.

5. During its evaluation of specifications, the Committee noted that some of them had stood for a number of years without review or revision. All such long-standing specifications should be reviewed to ensure that they reflect current practices in the additive-manufacturing and food-processing industries, and that the methods of analysis remain appropriate in the light of modern developments in analytical techniques.

6. During the evaluation of specifications for several naturally occurring substances, the Committee recognized that many of them tended to be too broad to provide an effective basis for toxicological evaluation. It therefore recognized the need to develop new principles for establishing adequate specifications that would address this problem (see section 2.3.3).

7. In a number of specifications, gas chromatography using headspace sampling is given as the method of analysis. As this technique is not included in the general methods section of the *Guide to specifications* (Annex 1, reference *65*), the Committee should develop an appropriate general method.

8. General methods should be established for laying down microbiological criteria in specifications for food additives.

6. RECOMMENDATIONS

1. In view of the large number of food additives and contaminants requiring evaluation or re-evaluation, meetings of the Joint FAO/WHO Expert Committee on Food Additives should continue to be held regularly.

2. *Patulin*

(*a*) In many of the older studies on patulin levels in fruit and fruit products, methods were used that were of inadequate sensitivity and patulin was not positively identified. There is a need to expand the current limited data base on patulin levels in such products. In particular, to ensure that reliable data on levels of patulin in apple juice and other fruit products are available for

the assessment of dietary exposure, the Committee urged the application of appropriate analytical procedures that include confirmatory techniques.

(*b*) In view of the well established association between patulin occurrence and rotted fruit:

—the sorting out of rotted apples in accordance with good manufacturing practices should be emphasized in the industrial processing of apples; and

—educational programmes for consumers should highlight the need to remove visibly damaged parts of fruit prior to consumption and to avoid consuming visibly mouldy homogeneous products such as fruit jam.

3. *Polychlorinated biphenyls (PCBs)*

(*a*) There is a need to continue monitoring PCB levels in foods and, in particular, to determine the specific PCB isomers of individual congeners. To ensure the adequacy of analytical procedures and thus the availability of more reliable data on dietary exposure for risk assessment purposes, interlaboratory check-sample programmes are considered desirable. In the light of ongoing activities related to PCBs in human milk (see section 2.4), additional efforts should be made to determine the contribution of other important foods to the dietary intake of PCBs.

(*b*) Because of advances in the analytical determination of PCBs, it is now possible to ascertain which PCB isomers of individual congeners are most prevalent in the media being examined. Once the toxicity of these PCB isomers is known, a better assessment of their human health significance will be possible. In view of the foregoing:

—Future analytical studies should be aimed at identifying and quantifying the specific isomers that are major contributors to the overall dietary intake of PCBs.

—Safety studies should be carried out on the PCBs predominantly present in foods, and particularly on the more highly chlorinated ones, in order to determine their precise toxicological potential. Furthermore, specific studies on the impact of these PCBs on the fetus and neonate are considered to be of great importance.

4. To facilitate its review of solvent specifications, as suggested at its thirtieth meeting, the Committee recommends that the relevant

industries should be requested to provide justification for the use of solvents, together with data on typical levels of residues resulting from their use. Emphasis should be placed initially on chlorinated hydrocarbon solvents.

REFERENCES

1. *Joint FAO/WHO Conference on Food Additives.* FAO Nutrition Meetings Report Series, No. 11, 1956; WHO Technical Report Series, No. 107, 1956.
2. GOLD, V. ET AL., ed. *Compendium of chemical terminology: IUPAC recommendations.* Oxford, Blackwell, 1987.
3. YRJÄNHEIKKI, E., ed. *Levels of PCBs, PCDDs and PCDFs in breast milk.* Copenhagen, WHO Regional Office for Europe, 1989 (Environmental Health Series, No. 34).
4. FURUYA, T. Studies on the metabolism of naturally occurring coumarins. V. Urinary metabolites of coumarin and dihydrocoumarin. *Chemical and pharmaceutical bulletin,* **6**: 701–706 (1958).
5. SCHELINE, R.R. Studies on the role of the intestinal microflora in the metabolism of coumarin in rats. *Acta pharmacologica et toxicologica,* **26**: 325–331 (1968).
6. ITO, Y. ET AL. Coumarin derivatives for medicinal purposes. V. Sedative and hypnotic actions. *Journal of the Pharmaceutical Society of Japan,* **4**: 351–355 (1953).
7. JENNER, P.M. ET AL. Food flavourings and compounds of related structure. I. Acute oral toxicity. *Food and cosmetics toxicology,* **2**: 327–343 (1964).
8. OPDYKE, D.L.J. Monographs on fragrance raw materials. Dihydrocoumarin. *Food and cosmetics toxicology,* **12**: 521–522 (1974).
9. HAGAN, E.C. ET AL. Food flavourings and compounds of related structure. II. Subacute and chronic toxicity. *Food and cosmetics toxicology,* **5**: 141–157 (1967).
10. *Toxicological screening of components of food flavours. Class XI-B. Benzodihydropyrone.* Long Island, NY, Food and Research Laboratories, Inc., 1957 (unpublished report submitted to WHO by Trubeck Laboratories, Inc., East Rutherford, NJ, USA).
11. NATIONAL RESEARCH COUNCIL, COMMITTEE ON DIET AND HEALTH. Dietary fiber. In: *Diet and health. Implications for reducing chronic disease risk.* Washington, DC, National Academy Press, 1989, pp. 291–309.
12. Code of practice for the preservation of raw milk by the lactoperoxidase system. *Bulletin of the International Dairy Federation,* **234** (1988).
13. AKRÉ, J., ed. Infant feeding: the physiological basis. *Bulletin of the World Health Organization,* **67** (suppl) (1989).
14. *Principles for evaluating health risks from chemicals during infancy and early childhood: the need for a special approach.* Geneva, World Health Organization, 1986 (Environmental Health Criteria, No. 59).

Annex 1

REPORTS AND OTHER DOCUMENTS RESULTING FROM PREVIOUS MEETINGS OF THE JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES

1. *General principles governing the use of food additives* (First report of the Expert Committee). FAO Nutrition Meetings Report Series, No. 15, 1957; WHO Technical Report Series, No. 129, 1957 (out of print).
2. *Procedures for the testing of international food additives to establish their safety for use* (Second report of the Expert Committee). FAO Nutrition Meetings Report Series, No. 17, 1958; WHO Technical Report Series, No. 144, 1958 (out of print).
3. *Specifications for identity and purity of food additives (antimicrobial preservatives and antioxidants)* (Third report of the Expert Committee). These specifications were subsequently revised and published as *Specifications for identity and purity of food additives*, vol. I. *Antimicrobial preservatives and antioxidants*. Rome, Food and Agriculture Organization of the United Nations, 1962 (out of print).
4. *Specifications for identity and purity of food additives (food colours)* (Fourth report of the Expert Committee). These specifications were subsequently revised and published as *Specifications for identity and purity of food additives*, vol. II. *Food colours*. Rome, Food and Agriculture Organization of the United Nations, 1963 (out of print).
5. *Evaluation of the carcinogenic hazards of food additives* (Fifth report of the Expert Committee). FAO Nutrition Meetings Report Series, No. 29, 1961; WHO Technical Report Series, No. 220, 1961 (out of print).
6. *Evaluation of the toxicity of a number of antimicrobials and antioxidants* (Sixth report of the Expert Committee). FAO Nutrition Meetings Report Series, No. 31, 1962; WHO Technical Report Series, No. 228, 1962 (out of print).
7. *Specifications for the identity and purity of food additives and their toxicological evaluation: emulsifiers, stabilizers, bleaching and maturing agents* (Seventh report of the Expert Committee). FAO Nutrition Meetings Report Series, No. 35, 1964; WHO Technical Report Series, No. 281, 1964 (out of print).
8. *Specifications for the identity and purity of food additives and their toxicological evaluation: food colours and some antimicrobials and antioxidants* (Eighth report of the Expert Committee). FAO Nutrition Meetings Report Series, No. 38, 1965; WHO Technical Report Series, No. 309, 1965 (out of print).
9. *Specifications for identity and purity and toxicological evaluation of some antimicrobials and antioxidants*. FAO Nutrition Meetings Report Series, No. 38A, 1965; WHO/Food Add/24.65 (out of print).
10. *Specifications for identity and purity and toxicological evaluation of food colours*. FAO Nutrition Meetings Report Series, No. 38B, 1966; WHO/Food Add/66.25.
11. *Specifications for the identity and purity of food additives and their toxicological evaluation: some antimicrobials, antioxidants, emulsifiers, stabilizers, flour-treatment agents, acids, and bases* (Ninth report of the Expert Committee). FAO Nutrition Meetings Report Series, No. 40, 1966; WHO Technical Report Series, No. 339, 1966 (out of print).

12. *Toxicological evaluation of some antimicrobials, antioxidants, emulsifiers, stabilizers, flour-treatment agents, acids, and bases.* FAO Nutrition Meetings Report Series, No. 40A, B, C; WHO/Food Add/67.29, 1967.
13. *Specifications for the identity and purity of food additives and their toxicological evaluation: some emulsifiers and stabilizers and certain other substances* (Tenth report of the Expert Committee). FAO Nutrition Meetings Report Series, No. 43, 1967; WHO Technical Report Series, No. 373, 1967.
14. *Specifications for the identity and purity of food additives and their toxicological evaluation: some flavouring substances and non-nutritive sweetening agents* (Eleventh report of the Expert Committee). FAO Nutrition Meetings Report Series, No. 44, 1968; WHO Technical Report Series, No. 383, 1968.
15. *Toxicological evaluation of some flavouring substances and non-nutritive sweetening agents.* FAO Nutrition Meetings Report Series, No. 44A, 1968; WHO/Food Add/68.33.
16. *Specifications and criteria for identity and purity of some flavouring substances and non-nutritive sweetening agents.* FAO Nutrition Meetings Report Series, No. 44B, 1969; WHO/Food Add/69.31.
17. *Specifications for the identity and purity of food additives and their toxicological evaluation: some antibiotics* (Twelfth report of the Expert Committee). FAO Nutrition Meetings Report Series, No. 45, 1969; WHO Technical Report Series, No. 430, 1969.
18. *Specifications for the identity and purity of some antibiotics.* FAO Nutrition Meetings Report Series, No. 45A, 1969; WHO/Food Add/69.34.
19. *Specifications for the identity and purity of food additives and their toxicological evaluation: some food colours, emulsifiers, stabilizers, anticaking agents, and certain other substances* (Thirteenth report of the Expert Committee). FAO Nutrition Meetings Report Series, No. 46, 1970; WHO Technical Report Series, No. 445, 1970.
20. *Toxicological evaluation of some food colours, emulsifiers, stabilizers, anticaking agents, and certain other substances.* FAO Nutrition Meetings Report Series, No. 46A, 1970; WHO/Food Add/70.36.
21. *Specifications for the identity and purity of some food colours, emulsifiers, stabilizers, anticaking agents, and certain other food additives.* FAO Nutrition Meetings Report Series, No. 46B, 1970; WHO/Food Add/70.37.
22. *Evaluation of food additives: specifications for the identity and purity of food additives and their toxicological evaluation: some extraction solvents and certain other substances; and a review of the technological efficacy of some antimicrobial agents* (Fourteenth report of the Expert Committee). FAO Nutrition Meetings Report Series, No. 48, 1971; WHO Technical Report Series, No. 462, 1971.
23. *Toxicological evaluation of some extraction solvents and certain other substances.* FAO Nutrition Meetings Report Series, No. 48A, 1971; WHO/Food Add/70.39.
24. *Specifications for the identity and purity of some extraction solvents and certain other substances.* FAO Nutrition Meetings Report Series, No. 48B, 1971; WHO/Food Add/70.40.
25. *A review of the technological efficacy of some antimicrobial agents.* FAO Nutrition Meetings Report Series, No. 48C, 1971; WHO/Food Add/70.41.
26. *Evaluation of food additives: some enzymes. modified starches, and certain other substances: toxicological evaluations and specifications and a review of the technological efficacy of some antioxidants* (Fifteenth report of the Expert

Committee). FAO Nutrition Meetings Report Series, No. 50, 1972; WHO Technical Report Series, No. 488, 1972.

27. *Toxicological evaluation of some enzymes, modified starches, and certain other substances.* FAO Nutrition Meetings Report Series, No. 50A, 1972; WHO Food Additives Series, No. 1, 1972.
28. *Specifications for the identity and purity of some enzymes and certain other substances.* FAO Nutrition Meetings Report Series, No. 50B, 1972; WHO Food Additives Series, No. 2, 1972.
29. *A review of the technological efficacy of some antioxidants and synergists.* FAO Nutrition Meetings Report series, No. 50C, 1972; WHO Food Additives Series, No. 3, 1972.
30. *Evaluation of certain food additives and the contaminants mercury, lead, and cadmium* (Sixteenth report of the Expert Committee). FAO Nutrition Meetings Report Series, No. 51, 1972; WHO Technical Report Series, No. 505, 1972, and corrigendum.
31. *Evaluation of mercury, lead, cadmium, and the food additives amaranth, diethylpyrocarbonate, and octyl gallate.* FAO Nutrition Meetings Report Series, No. 51A, 1972; WHO Food Additives Series, No. 4, 1972.
32. *Toxicological evaluation of certain food additives with a review of general principles and of specifications* (Seventeenth report of the Expert Committee). FAO Nutrition Meetings Report Series, No. 53, 1974; WHO Technical Report Series, No. 539, 1974, and corrigendum (out of print).
33. *Toxicological evaluation of certain food additives including anticaking agents, antimicrobials, antioxidants, emulsifiers, and thickening agents.* FAO Nutrition Meetings Report Series, No. 53A, 1974; WHO Food Additives Series, No. 5. 1974.
34. *Specifications for identity and purity of thickening agents, anticaking agents, antimicrobials, antioxidants and emulsifiers.* FAO Food and Nutrition Paper, No. 4, 1978.
35. *Evaluation of certain food additives* (Eighteenth report of the Expert Committee). FAO Nutrition Meetings Report Series, No. 54, 1974; WHO Technical Report Series, No. 557, 1974, and corrigendum.
36. *Toxicological evaluation of some food colours, enzymes, flavour enhancers, thickening agents, and certain other food additives.* FAO Nutrition Meetings Report Series, No. 54A, 1975; WHO Food Additives Series, No. 6, 1975.
37. *Specifications for the identity and purity of some food colours, flavour enhancers, thickening agents, and certain food additives.* FAO Nutrition Meetings Report Series, No. 54B, 1975; WHO Food Additives Series, No. 7, 1975.
38. *Evaluation of certain food additives: some food colours, thickening agents, smoke condensates, and certain other substances* (Nineteenth report of the Expert Committee). FAO Nutrition Meetings Report Series, No. 55, 1975; WHO Technical Report Series, No. 576, 1975.
39. *Toxicological evaluation of some food colours, thickening agents, and certain other substances.* FAO Nutrition Meetings Report Series, No. 55A, 1975; WHO Food Additives Series, No. 8, 1975.
40. *Specifications for the identity and purity of certain food additives.* FAO Nutrition Meetings Report Series, No. 55B, 1976; WHO Food Additives Series, No. 9, 1976.

41. *Evaluation of certain food additives* (Twentieth report of the Expert Committee). FAO Food and Nutrition Series, No. 1, 1976; WHO Technical Report Series, No. 599, 1976.
42. *Toxicological evaluation of certain food additives.* WHO Food Additives Series, No. 10, 1976.
43. *Specifications for the identity and purity of some food additives.* FAO Food and Nutrition Series, No. 1B, 1977; WHO Food Additives Series, No. 11, 1977.
44. *Evaluation of certain food additives* (Twenty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 617, 1978.
45. *Summary of toxicological data of certain food additives.* WHO Food Additives Series, No. 12, 1977.
46. *Specifications for identity and purity of some food additives, including antioxidants, food colours, thickeners, and others.* FAO Nutrition Meetings Report Series, No. 57, 1977.
47. *Evaluation of certain food additives and contaminants* (Twenty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 631, 1978.
48. *Summary of toxicological data of certain food additives and contaminants.* WHO Food Additives Series, No. 13, 1978.
49. *Specifications for the identity and purity of certain food additives.* FAO Food and Nutrition Paper, No. 7, 1978.
50. *Evaluation of certain food additives* (Twenty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 648, 1980, and corrigenda.
51. *Toxicological evaluation of certain food additives.* WHO Food Additives Series, No. 14, 1980.
52. *Specifications for identity and purity of food colours, flavouring agents, and other food additives.* FAO Food and Nutrition Paper, No. 12, 1979.
53. *Evaluation of certain food additives* (Twenty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 653, 1980.
54. *Toxicological evaluation of certain food additives.* WHO Food Additives Series, No. 15, 1980.
55. *Specifications for identity and purity of food additives (sweetening agents, emulsifying agents, and other food additives).* FAO Food and Nutrition Paper, No. 17, 1980.
56. *Evaluation of certain food additives* (Twenty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 669, 1981.
57. *Toxicological evaluation of certain food additives.* WHO Food Additives Series, No. 16, 1981.
58. *Specifications for identity and purity of food additives (carrier solvents, emulsifiers and stabilizers, enzyme preparations, flavouring agents, food colours, sweetening agents, and other food additives).* FAO Food and Nutrition Paper, No. 19, 1981.
59. *Evaluation of certain food additives and contaminants* (Twenty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 683, 1982.
60. *Toxicological evaluation of food additives.* WHO Food Additives Series, No. 17, 1982.

61. *Specifications for the identity and purity of certain food additives.* FAO Food and Nutrition Paper, No. 25, 1982.
62. *Evaluation of certain food additives and contaminants* (Twenty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 696, 1983, and corrigenda.
63. *Toxicological evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 18, 1983.
64. *Specifications for the identity and purity of certain food additives.* FAO Food and Nutrition Paper, No. 28, 1983.
65. *Guide to specifications—General notices, general methods, identification tests, test solutions and other reference materials.* FAO Food and Nutrition Paper, No. 5, Rev. 1, 1983.
66. *Evaluation of certain food additives and contaminants* (Twenty-eighth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 710, 1984, and corrigendum.
67. *Toxicological evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 19, 1984.
68. *Specifications for the identity and purity of food colours.* FAO Food and Nutrition Paper, No. 31/1, 1984.
69. *Specifications for the identity and purity of food additives.* FAO Food and Nutrition Paper, No. 31/2, 1984.
70. *Evaluation of certain food additives and contaminants* (Twenty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 733, 1986, and corrigendum.
71. *Specifications for the identity and purity of certain food additives.* FAO Food and Nutrition Paper, No. 34, 1986.
72. *Toxicological evaluation of certain food additives and contaminants.* Cambridge, Cambridge University Press, 1987 (WHO Food Additives Series, No. 20).
73. *Evaluation of certain food additives and contaminants* (Thirtieth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 751, 1987.
74. *Toxicological evaluation of certain food additives and contaminants.* Cambridge, Cambridge University Press, 1987 (WHO Food Additives Series, No. 21).
75. *Specifications for the identity and purity of certain food additives.* FAO Food and Nutrition Paper, No. 37, 1986.
76. *Principles for the safety assessment of food additives and contaminants in food.* Geneva, World Health Organization, 1987 (WHO Environmental Health Criteria, No. 70).
77. *Evaluation of certain food additives and contaminants* (Thirty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 759, 1987, and corrigendum.
78. *Toxicological evaluation of certain food additives.* Cambridge, Cambridge University Press, 1988 (WHO Food Additives Series, No. 22).
79. *Specifications for the identity and purity of certain food additives.* FAO Food and Nutrition Paper, No. 38, 1988.
80. *Evaluation of certain veterinary drug residues in food.* (Thirty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 763, 1988.
81. *Toxicological evaluation of certain veterinary drug residues in food.* Cambridge, Cambridge University Press, 1988 (WHO Food Additives Series, No. 23).

82. *Residues of some veterinary drugs in animals and foods*. FAO Food and Nutrition Paper, No. 41, 1988.
83. *Evaluation of certain food additives and contaminants* (Thirty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 776, 1989.
84. *Toxicological evaluation of certain food additives and contaminants*. Cambridge, Cambridge University Press, 1989 (WHO Food Additives Series, No. 24).
85. *Evaluation of certain veterinary drug residues in food* (Thirty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 788, 1989.
86. *Toxicological evaluation of certain veterinary drug residues in food*. WHO Food Additives Series, No. 25, in press.
87. *Residues of some veterinary drugs in animals and foods*. FAO Food and Nutrition Paper, in press.

Annex 2

ACCEPTABLE DAILY INTAKES, OTHER TOXICOLOGICAL INFORMATION, AND INFORMATION ON SPECIFICATIONS

Substance	Specifications[1]	Acceptable daily intake (ADI) for humans and other toxicological recommendations
A. Food additives		
Emulsifiers		
Polyglycerol esters of fatty acids	S	0–25 mg/kg of body weight[2]
Sucrose esters of fatty acids	R	0–10 mg/kg of body weight[3]
Sucroglycerides	S	0–10 mg/kg of body weight[3]
Enzyme preparations		
Enzymes derived from *Aspergillus niger*	S	ADI "not specified"[4]
Flavouring agents		
Benzyl acetate	S	0–5 mg/kg of body weight[6]
Cinnamaldehyde	S	No ADI allocated[6]
Dihydrocoumarin	N, T	No ADI allocated[7]
Ethyl vanillin	R	0–5 mg/kg of body weight[8]
Fumaric acid	R	ADI "not specified"[4, 8]
Quinine	R	0–0.9 mg/kg of body weight[8]
Food colours		
Canthaxanthin	S	No ADI allocated[9]
Carotene preparations from natural sources	R, T[10]	No ADI allocated[11]
Curcumin	S	0–0.1 mg/kg of body weight[3]
Paprika oleoresin	R	No ADI allocated[12]
Turmeric oleoresin	R	No ADI allocated[9]
Thickening agents		
Gum arabic	R	ADI "not specified"[4]
Modified celluloses	S	ADI "not specified"[4, 13]
Miscellaneous food additives		
Ferrous lactate	N	[0.8 mg/kg of body weight[14]]
2-Nitropropane	R, T	No ADI allocated[15]
Tannic acid	R, T	ADI "not specified"[4, 16]
Lactoperoxidase/thiocyanate/hydrogen peroxide system for milk preservation	[17]	Acceptable[18]

Substance	Provisional tolerable weekly intake (PTWI) for humans
B. Contaminants	
Patulin	7 μg/kg of body weight
Polychlorinated biphenyls (PCBs)	PTWI not established[18]

Substance	Specifications[1]
C. Food additives (specifications only)	
Carob bean gum	R
Citric and fatty acid esters of glycerol	R
Iron oxides used as food colours	R
Modified starches	R

Notes to Annex 2

1. N, new specifications prepared; R, existing specifications revised; S, specifications exist, revision not considered or not required; and T, the existing, new, or revised specifications are tentative and comments are invited (see Annex 3).
2. Applies to polyglycerol esters of fatty acids having an average chain length of up to three glycerol units.
3. Group ADI for sucrose esters of fatty acids and sucroglycerides.
4. ADI "not specified" means that, on the basis of the available data (chemical, biochemical, toxicological, and other), the total daily intake of the substance, arising from its use at the levels necessary to achieve the desired effect and from its acceptable background in food, does not, in the opinion of the Committee, represent a hazard to health. For that reason, and for the reasons stated in the individual evaluations, the establishment of an ADI expressed in numerical form is not deemed necessary.
5. Temporary acceptance (see Annex 3).
6. The previous temporary ADI was not extended (see Annex 3).
7. See Annex 3.
8. Group ADI for fumaric acid and its salts.
9. The previous temporary ADI was not extended.
10. Specifications apply to carotenes from algal and vegetable sources (see Annex 3).
11. Insufficient information was available on toxicity and/or chemical composition to permit establishment of an ADI.
12. Self-limiting as a spice extract.
13. Group ADI for ethyl cellulose, ethyl hydroxyethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, methyl cellulose, methyl ethyl cellulose, and sodium carboxymethyl cellulose. The ability of modified celluloses to produce laxative effects should be taken into account when they are used as food additives.
14. Provisional maximum tolerable daily intake for iron from all sources.

15. The previous temporary acceptance of 2-nitropropane as a fractionating solvent in the production of fats and oils was not extended.
16. For use as a filtering aid where the application of good manufacturing practice ensures that it is removed from food after use.
17. Existing specifications for sodium thiocyanate were maintained. New specifications for sodium percarbonate were prepared.
18. When used according to the draft guidelines produced by the Joint FAO/WHO Committee of Government Experts on the Code of Principles concerning Milk and Milk Products, this system does not present a toxicological hazard.
19. The Committee concluded that the no-effect level in studies with monkeys was 40 µg per kg of body weight per day. Because of the limitations of the available data and the ill-defined nature of the materials that were used in the feeding studies, it was impossible to establish a precise numerical value for a tolerable intake for humans. In particular, the PCB mixtures that were used in the studies with monkeys were not entirely the same as those to which humans are exposed in the diet. However, there is no reason to believe that humans would be more sensitive than monkeys to the effects of PCBs, and some indication of safe exposure levels can therefore be obtained from the no-effect level observed in the studies with monkeys.

Annex 3

FURTHER TOXICOLOGICAL STUDIES AND OTHER INFORMATION REQUIRED OR DESIRED

Flavouring agents

Benzyl acetate

Submission is required by 1993 of the results of long-term studies, already in progress, in which benzyl acetate is incorporated into the diet of mice and rats.

An *in vivo* test for chromosome damage in bone marrow is desirable.

Cinnamaldehyde

Submission of the results of a short-term feeding study in a non-rodent mammalian species and of adequate metabolic studies might be sufficient to make re-evaluation of this substance possible.

Dihydrocoumarin

The results of a short-term study in a rodent species and metabolic studies to investigate the extent of conversion to coumarin would be needed for re-evaluation of this substance.

In addition, information is required on the method of manufacture, with respect to the possible presence of residues of catalysts in the final product, and on the refractive index measured at 25 °C.

Ethyl vanillin

Submission of the results of an adequate short-term study in rats and metabolic studies in rats is required by 1992.

Quinine hydrochloride

Submission of the results of an adequate human study is required by 1992.

Food colours

Carotenes (algae)

Information is required on the source algae (e.g., on the different species used and the differences in the composition of the resulting

products), the influence of the manufacturing process, such as spray-drying, on the quality of finished powder preparations, and the technological justification for the presence of ethanol residues of up to 10%.

Carotenes (vegetable)

Information is required on the composition of commercial products and method(s) of distinguishing between carotenes (vegetable) and synthetic colours.

Curcumin

Submission is required by 1992 of the results of carcinogenicity studies, already in progress, with mice and rats given turmeric oleoresin containing a high concentration of curcuminoids.

Miscellaneous

2-Nitropropane

Information is required on the range of refractive indices of the commercial product. Confirmation is also required of the adequacy of the method of assay.

Tannic acid

Information is required on the composition of tannic acid from different botanical sources, on a test to show that condensed tannins are absent, and on actual use and levels of use of tannic acid as a flavouring agent.

1045

INTERNATIONAL PROGRAMME ON CHEMICAL SAFETY

WORLD HEALTH ORGANIZATION

TOXICOLOGICAL EVALUATION OF SOME FOOD COLOURS, ENZYMES, FLAVOUR ENHANCERS, THICKENING AGENTS, AND CERTAIN FOOD ADDITIVES

WHO FOOD ADDITIVES SERIES 6

The evaluations contained in this publication were prepared by the Joint FAO/WHO Expert Committee on Food Additives which met in Rome, 4-13 June 1974[1]

World Health Organization Geneva 1975

[1] Eighteenth Report of the Joint FAO/WHO Expert Committee on Food Additives, Wld Hlth Org. techn. Rep. Ser., 1974, No. 557. FAO Nutrition Meetings Report Series, 1974, No. 54.

MICROBIAL GLUCOSE OXIDASE* (Aspergillus niger)

Explanation

This enzyme preparation has been evaluated for acceptable daily intake by the Joint FAO/WHO Expert Committee on Food Additives (see Annex 1, Ref. No. 27) in 1971.

Since the previous evaluation additional data have become available and are summarized and discussed in the following monograph. The previously published monograph has been expanded and is reproduced in its entirety below.

BIOLOGICAL DATA

BIOCHEMICAL ASPECTS

No data available.

TOXICOLOGICAL STUDIES

Acute toxicity

None available.

Short-term studies

Rat

Three groups of 10 male and 10 female Charles River rats were fed 0, 5 and 10% of Aspergillus niger mycelial preparation in their diet for 90 days. There were no overt signs of toxicity, and food consumption and growth were not affected. There was a drop in the efficiency of food utilization for all dosed groups, it was significant for the males in the high dose group. The haematological, the clinical and the ophthalmoscopic data revealed no significant differences between the groups. There were significant increases in the relative kidney weight of females in both dosage groups. The gross and microscopic findings were mostly related to chronic respiratory disease and kidney lesions including hydropelvis or hydronephrosis, which did not appear to be treatment-related (Swann & Cox, 1973).

* This enzyme preparation is prepared from some varieties of Aspergillus niger.

Duckling

Groups of five ducklings received in their diet either 0, 1, 5 or 10% of enzyme for 29 days. Growth, feed efficiency, behaviour and survival were similar in all groups except the 10% level which showed some growth retardation. No gross liver lesions or differences in mean liver weight were noted. Histopathology was normal. No toxic element was noted (FDRL., 1963).

Long-term studies

None available.

Comments:

Aspergillus niger is a common contaminant of food. The available information indicates that it is not pathogenic to man. A duckling study was done and a 90-day study in rats is now available showing no toxicological effects at 10% in the diet. This meets the requirements as laid down by the Committee.

EVALUATION

Acceptable daily intake not specified.*

REFERENCES

FDRL (1963) Unpublished report No. 84600d of the Food and Drug Research Laboratories submitted by Miles Chemical Co.

Swann, H. E. & Cox, G. E. (1973) Unpublished report la. No. 1223 submitted by Searle Biochemicals

* The statement "ADI not specified" means that, on the basis of the available data (toxicological, biochemical, and other), the total daily intake of the substance, arising from its use or uses at the levels necessary to achieve the desired effect and from its acceptable background in food, does not, in the opinion of the Committee, represent a hazard to health. For this reason, and for the reasons stated in individual evaluations, the establishment of an acceptable daily intake (ADI) in mg per kg of body weight is not deemed necessary.

See Also:
Toxicological Abbreviations

INTERNATIONAL PROGRAMME ON CHEMICAL SAFETY

WORLD HEALTH ORGANIZATION

TOXICOLOGICAL EVALUATION OF SOME FOOD COLOURS, ENZYMES, FLAVOUR ENHANCERS, THICKENING AGENTS, AND CERTAIN FOOD ADDITIVES

WHO FOOD ADDITIVES SERIES 6

The evaluations contained in this publication were prepared by the Joint FAO/WHO Expert Committee on Food Additives which met in Rome, 4-13 June 1974[1]

World Health Organization Geneva 1975

[1] Eighteenth Report of the Joint FAO/WHO Expert Committee on Food Additives, Wld Hlth Org. techn. Rep. Ser., 1974, No. 557. FAO Nutrition Meetings Report Series, 1974, No. 54.

MICROBIAL CARBOHYDRASE* (Aspergillus niger)

Explanation

This enzyme preparation has been evaluated for acceptable daily intake by the Joint FAO/WHO Expert Committee on Food Additives (see Annex 1, Ref. No. 27) in 1971.

Since the previous evaluation additional data have become available and are summarized and discussed in the following monograph. The previously published monograph has been expanded and is reproduced in its entirety below.

BIOLOGICAL DATA

BIOCHEMICAL ASPECTS

No information available.

TOXICOLOGICAL STUDIES

Special studies

Studies on sensitizing effect was tested on 20 albino guinea-pigs, 10 males and 10 females, of the Pirbright White strain by a challenge injection of 0.05 ml intracutaneous two weeks after 10 sensitizing injections of 1% Ultrazym 100 in a 2% carboxymethylcellulose solution. The challenge injection produced a more intense reaction than the sensitizing injections, indicating a skin sensitizing activity (Sachsse, 1971).

Acute toxicity

A 70% suspension of Ultrazym 100 in a 2% carboxymethyl-cellulose solution was dispersed over the shaved back of 12 RAC/f rats for 24 hours. Within 24 hours the rats of both dosage groups (1000 mg/kg and 2150 mg/kg) showed dyspnoea and lachrymation, but no skin irritation. Recovering after five days, no substance-related gross organ changes were observed at autopsy (Sachsse & Bathe, 1971b).

* This enzyme preparation is prepared from some varieties of Aspergillus niger.

Animal	Route	LD_{50} (mg/kg bw)	Reference
Mouse	Oral	>3 200	Hunt & Garvin, 1963
		>4 000	Hunt & Garvin, 1971
		>3 200	Willard & Garvin, 1963
		4 000	Garvin et al., 1966
Rat	Oral	10 000	Gray, 1960
		31 600	Kay & Calandra, 1962
		>3 200	Willard & Garvin, 1968
		>4 000	Garvin et al., 1966
		>10 000	Gray, 1960
Rabbit	Oral	>4 000	Garvin et al., 1966
Dog	Oral	>4 000	Garvin et al., 1966
Rat	Oral	7 750	Sachsse & Bathe (1971a)

Short-term studies

Rat

Four groups of 10 male rats received in their diet for 30 days enzyme at 0, 0.5 and 5%. There were no adverse effects related to treatment regarding growth, appearance, behaviour, survival, food consumption, haematology, organ weights and gross pathology (Garvin et al., 1966).

Two groups of 10 male and 10 female rats received daily for 91 days in their diet either 0 or 5% enzyme. There was no difference from controls regarding appearance, behaviour, survival, weight gain, haematology, organ weights and gross pathology (Garvin & Merubia, 1959).

One group of 20 male and 20 female and four groups of 10 male and 10 female ARS Sprague-Dawley rats were fed 0, 5 and 10% carbohydrase (Wallerstein Pectinase Evaporate) and 5 and 10% carbohydrase

(Wallerstein Amylo glycosidase Evaporate) in the diet for 90 days (roughly equivalent to 3,5 and 7 g preparation/kg bw/day). Appearance and behaviour were normal, no deaths. Growth rate and food consumption were similar in all groups. No changes were seen in haematology and blood chemistry as compared with the respective controls. The relative weight of thyroid, liver and spleen in males and liver and spleen in females fed pectinase showed a dose-related decrease. The relative weight of liver in males and heart in females fed amyloglucosidase showed a tendency for dose-related decrease. Gross pathology and the only partly performed histopathology showed chronic pneumonia and renal tubular obstruction with hyalin casts, which did not seem to be dose related (Garvin et al., 1972).

Three groups of 10 male and 10 female Charles River rats were fed 0, 5 and 10% of Aspergillus niger mycelial preparation in their diet for 13 weeks. There were no overt signs of toxicity. Food consumption and growth were not affected. There was a drop in the efficiency of food utilization for both dosage groups and both sexes, which was significant for the high dose males. The haematological, the clinical and the ophthalmoscopic data, revealed no significant differences among the groups. There were significant increases in the relative kidney weight of females in both dosage groups. The gross and microscopic findings were mostly related to chronic respiratory disease and kidney lesions including hydropelvis or hydronephrosis, which did not appear to be treatment related (Swann & Cox, 1973).

Duckling

Groups of five ducklings received in their diet either 0, 1, 5 or 10% of enzyme for 29 days. Growth, feed consumption, behaviour and development were comparable in all groups. No gross liver lesions were seen at autopsy and mean liver weights were similar to controls. Histopathology of the livers was normal. No toxic element was noted (FDRL., 1963).

Mouse, Rat, Cat

Enzyme preparations from Aspergillus niger, strain "Pectolytic" produced by different cultivation methods were in several different acute and short-term studies given to a total number of 300 mice, 123 rats and 17 cats. The conclusion of the author is, that surface and deep culture preparations on media rich in sugars (sugar beet pulp, wheat bran) gave effects in some experimental animals after 45-750 mg/kg bw/day, of the different preparations mainly given in the

drinking water. The main effects were decreased weight gain, increased leucocyte count, accelerated sedimentation of erythrocytes and abscesses different places in the body. A preparation isolated from the same mould, but cultivated on grain husk had no similar effects on the animals. The preparation methods are not described, especially is not revealed whether the preparations were sterile (Magnova, 1968).

Long-term studies

None available.

Comments:

Aspergillus niger is a common contaminant of food. The available information indicates that it is not pathogenic to man. Duckling studies were done on two preparations and an adequate 90 days' study in rats is now available showing no toxicological effects at 10% in the diet. This meets the requirements as laid down by the Committee.

EVALUATION

Acceptable daily intake not specified.*

REFERENCES

FDRL (1963) Unpublished report No. 84600f submitted by Miles Chem. Co

Garvin, P. & Merubia, J. (1959) Unpublished report submitted by Baxter Laboratories Inc.

Garvin, P. J. et al. (1966) Unpublished report submitted by Baxter Laboratories Inc.

Garvin, P. J. (1972) Unpublished report No. R.D. 6 - 106 submitted by R. M. Gesler

Gray, E. H. (1960) Unpublished report submitted by Miles Laboratories Inc.

Hunt, R. F. & Garvin, P. J. (1963) Unpublished report submitted by Baxter Laboratories Inc.

Hunt, R. F. & Garvin, P. J. (1971) Unpublished report submitted by Travenol Laboratories Ltd

* The statement "ADI not specified" means that, on the basis of the available data (toxicological, biochemical, and other), the total daily intake of the substance, arising from its use or uses at the levels necessary to achieve the desired effect and from its acceptable background in food, does not, in the opinion of the Committee, represent a hazard to health. For this reason, and for the reasons stated in individual evaluations, the establishment of an acceptable daily intake (ADI) in mg per kg of body weight is not deemed necessary.

Kay, J. H. & Calandra, J. C. (1962) Unpublished report submitted by Miles Chemical Co

Magonova, N. B. (1968) Voprosy pitania, 27, 67-72

Phillips, B. M., Hartnagel, R. E. & Thompson, D. (1967) Unpublished report submitted by Miles Chemical Co

Sachsse, K. (1971) Unpublished report No. Siss 826 submitted by Ciba Geigy

Sachsse, K. & Bathe, R. (1971) Unpublished report No. Siss 826 submitted by Ciba-Geigy

Swann, H. E. & Cox, G. E. (1973) Unpublished report laboratory No. 1223 submitted by Searle Biochemicals

Willard, R. L. & Garvin, P. J. (1968) Unpublished report submitted by Travenol Laboratories Ltd

See Also:
Toxicological Abbreviations

Jorquera, M.

0496

Microbes Environ. Vol. 23, No. 3, 182–191, 2008
http://wwwsoc.nii.ac.jp/jsme2/ doi:10.1264/jsme2.23.182

Minireview

Current and Future Biotechnological Applications of Bacterial Phytases and Phytase-Producing Bacteria

MILKO JORQUERA[1]*, OSCAR MARTÍNEZ[2], FUMITO MARUYAMA[3], PETRA MARSCHNER[4], and MARIA DE LA LUZ MORA[1]

[1]*Centro de Ciencias y Biotecnologia de Recursos Naturales, Universidad de La Frontera, Avenida Francisco Salazar 01145, 4811230 Temuco, Chile;* [2]*Programa de Doctorado en Ciencias de Recursos Naturales, Universidad de La Frontera, Avenida Francisco Salazar 01145, 4811230 Temuco, Chile;* [3]*Institute of Medical Science, Internacional Research Center for Infectious Diseases, University of Tokyo, 4–6–1 Shirokanedai, Minato-ku, Tokio 108–8639, Japan; and* [4]*Soil and Land Systems, School of Earth and Environmental Sciences, University of Adelaide, DP 636, Adelaide, SA 5005, Australia*

(Received May 12, 2008—Accepted June 20, 2008)

Phytases are a group of enzymes capable of releasing phosphate from phytate, one of the most abundant forms of organic phosphate in the natural environment. Phytases can be found in many organisms; in bacteria, they are particularly described in γ-proteobacteria. In recent years, bacterial phytases have been isolated, characterized and proposed as potential tools in biotechnology. Microbial phytases have been applied mainly to animal (swine and poultry) and human foodstuffs in order to improve mineral bioavailability and food processing. Here, we summarize the current knowledge of bacterial phytases and phytase-producing bacteria, as well as their potential biotechnological applications, including new fields poorly explored, such as fish nutrition, environmental protection and plant nutrition. Despite the recognized importance in biotechnology, information on bacterial phytases and phytase-producing bacteria is clearly limited and major efforts are required to improve the knowledge of phytases present in bacteria and their utilization.

Key words: bacterial phytase, biotechnological application, inositol phosphate, phosphorus, phytate

Introduction

Phytic acid (*myo*-inositol 1, 2, 3, 4, 5, 6-hexakis dihydrogen phosphate) and mixed cation salts of phytic acid, designated as phytates, are a group of organic phosphorus (P) compounds found widely in nature. In terrestrial ecosystems, they are synthesized by plants, accumulate in seeds during the ripening period and are regarded as the primary storage form of both phosphate and inositol in plant seed and grains[75,113]. They are strongly complexed in soils, representing an important class of organic P[8,114]. Phytic acid and phytates are of central interest as P sources in terms of bioavailability to plants[42], because P is considered an essential nutrient for plant growth and improvement in the ability of plants to use phytate as a source of P may help decrease the use of inorganic P fertilizers in agro-pastoral ecosystems. Moreover, phytic acid and phytates in soils are important due to their potential leaching into water bodies where they can contribute to eutrophication and algal bloom[6,102,104,112].

Phytase is a generic term used to describe phosphohydrolase enzymes, which catalyze the sequential release of inorganic orthophosphates from phytic acid and phytates[52,77]. Phytase enzymes are wide-spread in nature and can be derived from a number of sources including plants, animals and microorganisms[65,120]. Phytases have been classified as 3-phytases (EC 3.1.3.8), 6-phytases (EC 3.1.3.26) and 5-phytases (EC 3.1.3.72), based on the position of specificity of the initial hydrolysis of phytate. On the basis of pH optimum, phytases can be broadly divided in two major classes: acid and alkaline phytases. Phytases also exhibit variations in structure and catalytic mechanism, and consequently, have been categorized into cysteine phytases, histidine acid phosphatases, β-propeller phytases, and purple acid phosphatase[17,77,118].

During the last two decades, there has been an increase in the use of microbial phytases as feed additives in diets for swine and poultry to enhance the utilization of plant-based foods[33,99]. Apart from being commercially valuable in the feed and food industries, phytases have potential biotechnological applications in various other fields, such as environmental protection[69], aquaculture[11] and agriculture[48,88]. Nevertheless, many fundamental issues relating to biotechnological applications of phytates and phytases remain to be elucidated.

This review summarizes current information on bacterial phytases and phytase-producing bacteria and their applications in biotechnology, emphasizing the potential applications in less explored fields such as environmental protection and nutrition of swine, fish and plants.

Occurrence of phytase-producing bacteria

Phytases have a wide distribution in plants, animal tissues and microorganisms. Studies have shown that microbial phytases are most promising for biotechnological application[82,120]. Although the commercial production of phytases has focused on the fungus *Aspergillus*, studies have suggested bacterial phytases may be an alternative to the fungal enzymes because of their higher substrate specificity, greater

* Corresponding author. E-mail: mjorquera@ufro.cl; Tel: +56–45–325–450; Fax: +56–45–325–053.

resistance to proteolysis and better catalytic efficiency[62,89,122]. Bacteria able to degrade phytate have been isolated from a range of terrestrial and aquatic environments[45] and phytases have been detected in a wide variety of bacteria, such as bacilli, enterobacteria, anaerobic rumen bacteria and pseudomonads (Table 1).

Based on the microbial and environmental sequence database available at the National Center for Biotechnology Information (NCBI) and the Community Cyberinsfrastructure for Advanced Marine Microbial Ecology Research and Analysis (CAMERA[98]), Lim *et al.*[74] performed a study on the distribution and diversity of phytase-producing bacteria in various habitats. Representative genes from cysteine phytases (CPhy), histidine acid phosphatases (HAP) and β-propeller phytases (BPP) were obtained from public databases. CPhy-like sequences were mainly found in plant pathogenic, enteric and free-living bacteria, whereas HAP-like sequences were found in plant pathogenic and enteric bacteria. No CPhy and HAP-like sequences were found in aquatic bacteria. In contrast, BPP-like sequences were found in plants, soil and aquatic bacteria; suggesting that BPP phytases can play a major in the cycling of phytate in both soil and aquatic environments.

In relation to purple acid phosphatase (PAP), no bacterial enzyme has been purified, but a search of complete and partial genome sequences in the TIGR Microbial Database indicated that PAP-like sequences may be restricted to a small number of bacteria, such as myco- and cyanobacteria[96]. However, the current database is too small to provide adequate coverage of phytases present in the bacterial world[74]. This limitation can result in a inability to detect genes involved in phytase hydrolysis in environmental isolates, even when using modern techniques based on published phytase gene sequences as described by Hill *et al.*[44].

The NCBI and Swiss-Prot databases show that phytase-like proteins are found in a great diversity of bacterial groups (Table 2). It is worth noting that members of the genera *Pseudomonas* and *Xanthomonas* appear to contain genes for various types of phytases. *Pseudomonas syringae* and *Xanthomonas campestri* have been shown to produce all three classes of phytases (CPhy, HAP and BPP), whereas *X. axonopodis* and *X. oryzae* produce two classes of phytases (HAP and BPP). It is known that the genus *Pseudomonas* is a cosmopolitan opportunist *par excellence*. Members of the genus *Pseudomonas* are extremely metabolically versatile and omnivorous, some species being able to use more than 100 different sources of carbon and energy[110]. *Xanthomonas* constitutes a group of bacteria with diverse physiological traits and phytopathological specializations. This metabolic versatility may require the presence of different classes of phytases in the same species of pseudomonads and xanthomonads.

The highest frequency of phytase-like proteins has been reported in members of the γ-proteobacteria group (Table 2). This may simply be because γ-proteobacteria have the most fully sequenced genomes, including those species for which our knowledge of gene function is more complete than that for any other cellular organisms[9]. The γ-proteobacteria are commonly chosen in phylogenetic studies because this group represents a model of bacterial diversification and includes free-living and commensal species, intracellular symbionts, and plant and animal pathogens[71]. At the same time, γ-proteobacteria are sufficiently closely related to reduce the problem of lack of phylogenetic signals and to identify a large set of unambiguous orthologs. Figure 1, based on amino acid sequences taken from the NCBI and Swiss-Prot databases, shows the phylogenetic relations of representative phytase-like proteins in γ-proteobacteria. The phylogenetic tree shows a clustering of the tree classes of phytases (CPhy, HAP and BPP) reported. Due to the evident importance and the amount of information available about them, the following sections will focus on members of γ-proteobacteria as phytase-producing bacteria.

Table 1. Reported phytase-producing bacteria

Specie	Class of phytase	Reference
Bacillus amyloquefaciens	β-propeller phytase	36*, 38, 48, 60
Bacillus licheniformis		115
Bacillus subtilis	β-propeller phytase	36*, 57, 58, 61, 83, 103, 115
Bacillus sp.		15, 16, 30, 59
Citrobacter braakii		62
Escherichia coli	Histidine acid phosphatase	25, 26, 27, 29, 55, 67, 73*, 107
Enterobacter cloacae	Histidine acid phosphatase	43*
Enterobacter sp.		126
Klebsiella aerogenes		108
Klebsiella pneumoniae	Histidine acid phosphatase	121*
Klebsiella terrigena	Histidine acid phosphatase	28, 35*
Klebsiella oxytoca		54
Klebsiella sp.	3-phytase	92*, 100
Lactobacillus amylovorus		106
Megasphaera elsdenii		13, 124
Mitsuokella multiacidus		13, 124
Mitsuokella jalaludinii		68
Obesumbacterium proteus	Histidine acid phosphatase	129*
Pantoea agglomerans		31, 32
Prevotella sp.		13, 124
Pseudomonas syringae	Histidine acid phosphatase	14*
Pseudomonas fragi		50
Pseudomonas sp.		51, 86
Selenomonas ruminantium		13, 124, 125
Treponema sp.		13, 124
Yersinia intermedia	Histidine acid phosphatase	46*

* Asterisk denotes the reference describing the class of phytase.

Application of bacterial phytases in food

Monogastric animals (e.g. swine and poultry) are unable to degrade phytate in food, since they lack or have low levels of phytase activity in their digestive tracts[97]. Thus, inorganic phosphate is frequently added to feed to improve the growth of these animals. However, P uptake by monogastric animals is inefficient, 70% of the total P in feed is released in excreta[113]. Through leaching or surface run-off, the high levels of phytate and inorganic phosphate in the excretion of these animals can contribute to the eutrophication of surface water and algal blooms[6,19,102,104,112]. Furthermore, phytate acts as an anti-nutritional factor by chelating divalent metal cations such as Zn^{2+}, $Fe^{2+/3+}$, Ca^{2+}, Mg^{2+}, Mn^{2+} and Cu^{2+}, preventing the absorption of minerals in the digestive tracts of

Table 2. Bacteria containing phytase-like proteins reported in NCBI and Swiss-Prot databases

Classes of phytase	*Proteobacteria*				*Bacteroidetes*	*Firmicutes*	*Cyanobacteria*	*Actinobacteria*	*Acidobacteria*
	Alfa	Beta	Delta/Epsilon	Gamma					
Cysteine phytase (CPhy)		*Acidovorax*	*Bdellovibrio* *Stigmatella*	*Legionella* *Pseudomonas* *Xanthomonas*		*Clostridium* *Selenomonas*			
Histidine acid phosphatase (HAP)	*Gluconobacter* *Zymomonas*		*Lawsonia*	*Citrobacter* *Enterobacter* *Escherichia* *Erwinia* *Klebsiella* *Obesumbacterium* *Pseudomonas* *Salmonella* *Serratia* *Shigella* *Stenotrophomonas* *Xanthomonas* *Yersinia*					*Solibacter*
β-propeller phytase (BBP)	*Caulobacter* *Hyphomonas* *Maricaulis* *Oceanicaulis* *Parvularcula* *Sphingomonas*		*Desulfuromonas*	*Alteromonas* *Azotobacter* *Hahella* *Idiomarina* *Oceanobacter* *Pseudoalteromonas* *Pseudomonas* *Reinekea* *Saccharophagus* *Shewanella* *Stenotrophomonas* *Vibrio* *Xanthomonas*	*Chlorobium* *Flavobacterium* *Microscilla* *Polaribacter* *Prosthecochloris*	*Bacillus*	*Anabaena* *Cyanothece* *Gloeobacter* *Nostoc* *Nodularia*	*Kineococcus* *Streptomyces*	
Purple acid Phosphatase (PAP)		*Burkholderia*					*Synechocystis*	*Mycobacterium*	

Table 3. Summary of the potential applications of bacterial phytases and phytase-producing bacteria

	Main effect	Important properties	Limitations
Phytases as feed additives	Increased P utilization and metal bioavailability; decreased P concentration in excrements	Resistance to low pH and peptidase	Low effectiveness; cost
Inoculation of organic residues with phytase-producing bacteria	Increased P availability; decreased P pollution in water bodies	Resistance to physico-chemical changes during the stabilization process (changes in pH and temperature); capacity to utilize recalcitrant P forms	Low survival/activity during stabilization; rapid adsorption of P in soil after application
Inoculation of roots with phytase-producing bacteria	Increased uptake of P from organic forms by plants	P liberation should be greater than P requirement; rhizosphere competence	Low survival/activity in rhizosphere, sorption of phytases

animals[19,40,117]. The stability and solubility of metal cation-phytate complexes depends on the individual cation, pH, phytate: cation molar ratio, and the presence of other compounds in solution[80].

During the past 15 years, the inclusion of microbial phytase in poultry and swine diets has increased, mainly in response to heightened concerns over P pollution in the environment. The capacity of this enzyme to release P from phytate and reduce P excretion between 30% and 50% is now well documented[33,39,69,70,99]. Phytases used as feed additives should have certain properties for effective mineralization of phytate in the digestive tract of the animal. The main site for phytase activity is the stomach, thus an enzyme with an acidic pH optimum and high resistance to pepsin is desirable[66]. On the other hand, some enzymes, especially those of the genera *Enterobacter*, exhibit a pH optimum between 6.0 and 8.0[126]. Therefore, they are preferred as feed additives for poultry since this pH optimum is close to the physiological pH of the poultry crop[99]. Bacterial phytases could be of particular interest as feed additives as phytases of *E. coli* and *Citrobacter braakii* have been shown to be more resistant to pepsin and pancreatin than the commercial *Aspergillus* phytases[62,89], and the *E. coli* phytase is highly stable under acidic conditions, even at pH 2.0[27]. Several studies have described the effectiveness of supplemental *E. coli*-derived phytase in improving utilization of P by poultry and swine, resulting in greater feed intake and body weight gain[1,3,4]. Nevertheless, Rosen[91] argued that feed efficacy response to phytase has been declining recently, which can be attributed to concurrent improvements in broiler strains, feeds and management techniques.

With the aim of developing a sustainable and environmen-

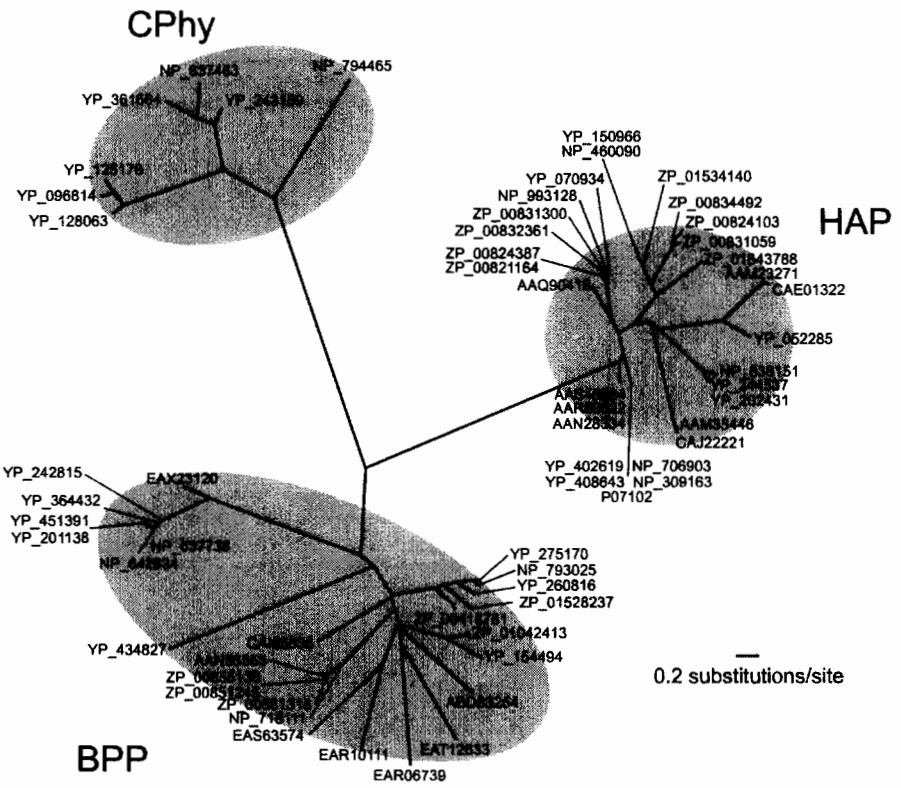


Fig. 1. Phylogenetic analysis of representative phytase-like proteins found in the γ-proteobacteria group. Amino acid sequences were aligned using the ClustalW program[109] and the neighbor-joining tree was constructed using Prodist and Drawtree in PHYLIP (*Phylogenetic Inference Package*, version 3.67, available from J. Felsenstein of the Department of Genome Sciences at the University of Washington, Seattle, WA, USA). The selected sequences analyzed were as follow: Cystein phytase-like sequences (**CPhy**): *Legionella pneumophila* str. Lens, YP_128063; *L. pneumophila* str. Philadephia, YP_096814; *L. pneumophila* str. Paris, YP_125176; *Xanthomonas campestris* str. 85-10, YP_361664; *X. campestris* ATCC 33913, NP_637463; *X. campestris* str. 8004, YP_243159; *Pseudomonas syringae* str. DC3000, NP_794465. Histidine acid phosphatase-like sequences (**HAP**): *Obesumbacterium proteus*, AAQ90419; *Yersinia bercovieri* ATCC 43970, ZP_00821164; *Y. mollaretii* ATCC 43969, ZP_00824387; *Y. intermedia* ATCC 29909, ZP_00832361; *Y. frederiksenii* ATCC 33641, ZP_00831300; *Y. pestis* str. 91001, NP_993128; *Y. pseudotuberculosis* IP 32953, YP_070934; *Salmonella enterica* ATCC9150, YP_150966; *Salmonella* Typhimurium LT2, NP_460090; *Serratia proteamaculans* 568, ZP_01534140; *Y. intermedia* ATCC 29909 (2), ZP_00834492; *Y. mollaretii* ATCC 43969 (2), ZP_00824103; *Y. frederiksenii* ATCC 33641 (2), ZP_00831059; *Stenotrophomonas maltophilia* R5513 ZP_01643788; *Klebsiella pneumoniae*, AAM23271; *K. terrigena*, CAE01322; *Erwinia carotovora* SCRI1043, YP_052285; *Xanthomonas campestris* ATCC 33913, NP_636151; *X. campestris* str. 8004, YP_244537; *X. oryzae* KACC10331, YP_202431; *X. axonopodis* str. 306, AAM35446; *X. campestris* str. 85-10, CAJ22221; *Shigella dysenteriae* Sd197, YP_402619; *S. boydii* Sb227, YP_408643; *S. flexneri* str. 301, NP_706903; *Escherichia coli* 0157:H7, NP_309163; *E. coli* K12 AppA, P07102; *E. coli* K12, AAN28334; *Citrobacter braakii*, AAS45884; *C. freundii*, AAR89622. β-propeller phytase-like sequences (**BBP**): *Pseudomonas syringae* 1448, YP_275170; *P. syringae* str. DC3000, NP_793025; *P. fluorescens* Pf-5, YP_260816; *P. mendocina* ymp, ZP_01528237; *Azotobacter vinelandii* AvOP, ZP_00418781; *Idiomarina baltica* OS145, ZP_01042413; *I. loihiensis* L2TR, YP_154494; *Saccharophagus degradans*, ABD83254; *Oceanobacter* sp. RED65, EAT12633; *Alteromonas macleodii* Deep ecotype, EAR06739; *Reinekea* sp. MED297, EAR10111; *Vibrio angustum* S14, EAS63574; *Shewanella oneidensis* MR-1, NP_718111; *Shewanella* sp. MR-4, ZP_00881318; *Shewanella* sp. ANA-3, ZP_00851245; *Shewanella* sp. MR-7, ZP_00855136; *S. oneidensis* PhyS, AAN55555; *Pseudoalteromonas haloplanktis* TAC125, CAI85536; *Hahella chejuensis* KCTC 2396, YP_434827; *Xanthomonas campestris* str. ATCC 33913, NP_637738; *X. axonopodis* str. 306, NP_642834; *X. oryzae* KACC10331, YP_201138; *X. oryzae* MAFF 311018, YP_451391; *X. campestris* str. 85-10, YP_364432; *X. campestris* str. 8004, YP_242815; *S. maltophilia* R551-3, EAX23120.

tal friendly aquaculture, several studies have been conducted on the use of plant protein-based foods, such as soybean meal and canola meal, in fish feed[11]. Similar to swine and poultry, monogastric and agastric aquatic animals lack intestinal phytases for efficient phytate hydrolysis during digestion[79], hence most phytate is excreted into the water which may cause algal growth[72]. Thus, phytase has been demonstrated to both increase the use of low-cost plant-based food in the aquaculture industry and maintain acceptable P levels in the water. Besides fungal phytase, 6-phytase from *E. coli* is a commonly used feed additive[11]. Studies have reported that between 20 and 80% of phytate-bound P can become available for intestinal absorption by adding phytase in fish fed plant-based diets[95,127]. Moreover, an increase in the weight of salmonids fed diets supplemented with phytases has been shown[93,119]. Microbial phytase supplementation also leads to improved availability of other minerals and trace elements in fish[123]. Nevertheless, there is still no consensus on whether phytase enhances protein and energy utilization or not[99], and further investigations about the application of microbial phytases in animal feed are needed.

Processing and manufacturing of human food is another potential area of application for phytases. Investigations in

this area are focused on better mineral absorption or technical improvement of food processing. The high phytate content in cereal and legume-based diets contributes to inhibition of mineral absorption[94]. In order to improve mineral absorption, the use of phytase has been suggested to improve phytate utilization in diets and reduce the risk for mineral deficiency in vulnerable groups such as child-bearing women, strict vegetarians and inhabitants of developing countries[34,39]. In relation to phytate degradation during food processing or preparation, phytases have been proposed for food fermentation to improve mineral bioavailability[33]. Hurrell *et al.*[47] reported that phytase fully degraded phytate during the manufacture of nine complementary foods based on flours from rice, wheat, maize, oat, sorghum, and wheat-soy blend. Hence, although currently phytases are used mainly as animal feed additives in diets of monogastric animals, there is a great potential for the use of this class of enzymes in processing and manufacturing of food for human consumption.

Environmental protection

Concern about the impact of P in the environment has greatly increased over the past decade. Over-application of P-containing materials, whether inorganic P-based fertilizers or organic residues such as crop residues, manures, composts, and municipal biosolids, can result in high levels of extractable soil inorganic phosphate[20,78]. If these soils are close to surface water (streams, lakes and rivers), this P can enter the water either as soluble P in runoff water or as P absorbed by eroded soil particles[75,78] thereby increasing P availability in these water bodies. This excess of P can potentially lead to eutrophication in water columns. Algal blooms (some of which may produce toxins), hypoxia and occasional anoxia can result with the excess of nutrients in aquatic habitats[6,102,104]. In this context, Knowlton[64] indicated that animal management has an important role in reducing P losses in the environment. This could be achieved by using technologies to increase the bioavailability of P in manures and excreta to produce an organic fertilizer with large amounts of available P[20]. A recent study on P dynamics in aerobic degradation of dairy cattle dung revealed the presence of phytate-utilizing bacteria during the entire degradation process (105 days)[21]. Two morphotypes with high P mineralizing capacity *in vitro*, genetically characterized as member of *Enterobacter* and *Rahnella*, were dominant as the phytate content decreased. Although the decreasing phytate content is probably mainly the result of the formation of recalcitrant P compounds during humification and polymerization, these bacteria may play a role in lowering the phytate concentration by mineralization. Similarly, inorganic phosphate could be released from organic P by bacterial mineralization during composting. For environmental protection it may become necessary to pre-treat organic wastes before their application in soils; phytase-producing bacteria could be useful tools to produce a fertilizer rich in available P.

Phytate-producing bacteria as plant growth-promoting agents

In many agricultural soils, a large proportion of P applied to soils as fertilizer (>90%) is not taken up by the crop because it rapidly becomes unavailable to plants due to its interaction with soil constituents by either adsorption or precipitation[101]. Total amounts of P in soils vary widely, depending on parent material and the extent of weathering. Phosphorus is incorporated into organic matter or relatively insoluble inorganic P minerals[78].

Plants and microorganisms increase P availability by solubilising inorganic P and mineralizing organic P. These processes are of particular importance in the rhizosphere where the activity of microorganisms is increased significantly because of the release of root exudates[88]. In the rhizosphere, a substantial number of bacteria may exert a beneficial effect on plant growth[81,128]; the so-called plant growth-promoting bacteria (PGPB)[5,63]. Among them, bacteria with the capacity to solubilize and/or mineralize P have been isolated and proposed as inoculants to improve yield[37,49,90]. Phosphate-solubilizing bacteria (PSB) may increase the transfer of P from poorly-available soil pools (e.g. tricalcium phosphates and rock phosphates) to plant-available forms[53]. Due to proximity of the plant root, this bacterial effect is particularly important in the rhizosphere; if PSB solubilize more soluble P than is required for their growth and metabolism, this surplus P is available for uptake by plants[76]. PSB have attracted the attention of microbiologists and agriculturists as biotechnological tools to improve plant growth and yield[2,10,12,18,85]. However, there have been few successful applications to the field. Clearly, microbial-plant interactions in soil are complex and have proven difficult to manipulate[49,88].

Organic P is generally estimated to contribute between 30 and 50% of total P in soil, being most abundant in soils with a high organic matter content[8,78]. Phytate is by far the most prevalent form of organic P in soil and typically represents between 10 and 50% of total organic P[78,114]. In terrestrial environments, phytates are synthesized in plants and the highest concentrations are found in the seeds, but it has also been reported in animal wastes, hay grass and in small amounts in bacteria, fungi and the roots of plants[111,113]. The amount of phytate in soils varies widely and depends on the properties, use and management of soils. Phytate also has a strong interaction with soil, either being adsorbed to clays, or precipitated as insoluble salts such oxides and hydroxides of Fe and Al in acidic soils and insoluble Ca salts in alkaline soils[113]. In British surface soils, the phytate content was estimated to be between 11 and 35% of total organic P[114]. In Chilean volcanic soils, Borie *et al.*[7] reported phytate contents of between 42 and 67% of total organic P and a possible accumulation in the organic fraction of soil matrix through Al and Fe bridges. On the other hand, it has been reported that phytate represents <5% of total organic P and <3% of total P in Australian soils[105]. The authors of the latter study suggest that phytate content may have been over-estimated in other studies due to mis-interpretation of the analytical results.

Phytases are actively excreted into soil by organisms and also accumulate in soil due to protection from sorption to organo-clay complexes[78]. These enzymes are important not only for the degradation of fresh organic residues, but also for the release of inorganic P from soil organic matter. Given the substantial amount of organic P, the hydrolysis of ortho-

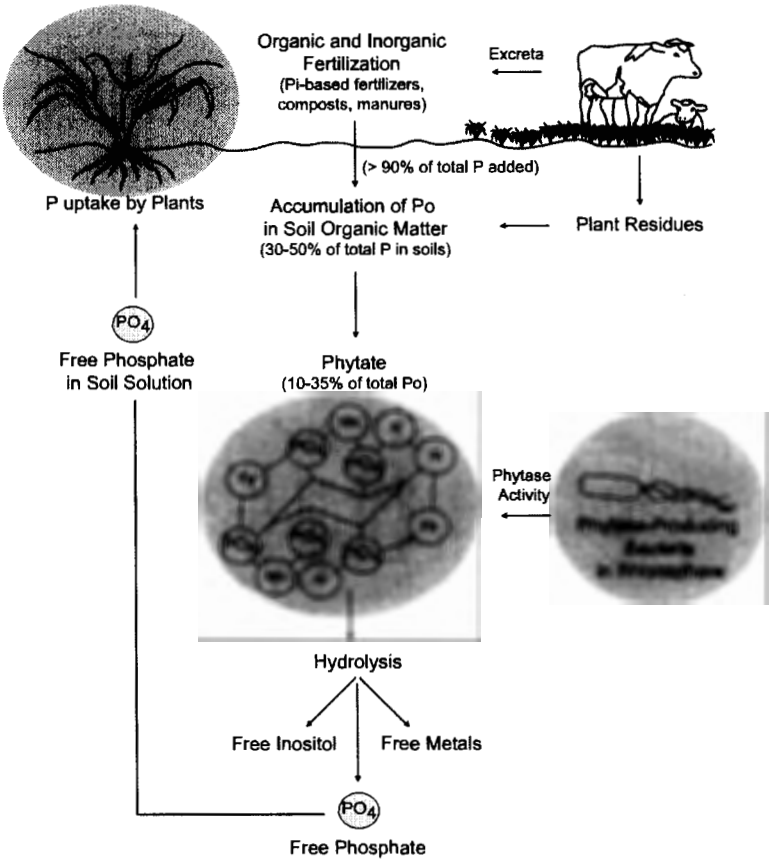


Fig. 2. Schematic illustration of free inorganic phosphate (PO_4) released by phytase-producing bacteria associated with rhizospheres of pasture plants. Pi: inorganic P; Po: organic P.

phosphate from these sources can be important for plant nutrition[78].

Plants producing phytases display only weak activity in roots and other plant organs[48]. Given the lack of extracellular phytase of plant origin and relative abundance of phytase-producing microorganisms, microbial phytases appear to be the key to organic P cycling in soils[41,118].

Despite that microbial phytases can play a fundamental role in the P cycle in soils and are of great agronomic and ecological value, the potential applications of phytase-producing bacteria in supporting plant growth under phosphate-limited conditions have been scarcely investigated. Rhizosphere bacteria that utilize phytate are being isolated and considered as potential PGPB to improve availability of P in the rhizosphere and uptake of P by plants[88,90]. Figure 2 illustrates the liberation of free inorganic phosphate (PO_4) from phytate in soils by the activity of phytase-producing bacteria associated with the rhizospheres of pasture plants. Phytate is represented as the salt of phytic acid (inositol hexakisphosphate) and divalent metals such as Al, Fe and Mn. Phytase released by bacteria hydrolyses phytate, producing free PO_4, metals, and inositol or its intermediate metabolites. The free PO_4 in soil solutions can be taken up by plants. Few studies have reported the isolation of phytase-producing bacteria from the rhizosphere and their effect on plant growth and P uptake. In 1996, Yoon *et al.*[126] isolated a bacterium characterized as *Enterobacter* from soil near the roots of leguminous plants which produced extracellular phytase. *Pseudomonas* spp. that utilize inositol hexaphosphate (IHP), isolated from diverse cropping soils in Australia, exhibited marked phytase activity and liberated up to 80% of the P from IHP[86]. Richardson[87] observed that the ability of pasture plants to acquire P from phytate was improved by the presence of soil microorganisms. In Japan, Unno *et al.*[116] isolated diverse bacteria with phytate-utilizing ability from the rhizosphere of white lupin (*Lupinus albus*). Almost all isolates were classified as members of the genus *Burkholderia* and some isolates significantly promoted plant growth. Recently, Jorquera *et al.*[56] isolated bacteria from the rhizospheres of plants most commonly cultivated in a volcanic soil in Chile. This study revealed bacteria with the capacity to solubilize phosphate and mineralize phytate. Moreover, the study also showed the capacity to release inorganic orthophosphate from IHP by some isolates, genetically characterized as members of the genera *Pseudomonas*, *Enterobacter* and *Pantoea*. The studies previously mentioned suggest that phytase-producing bacteria are ubiquitous in the rhizosphere of different plant species and members of γ-proteobacteria are most commonly reported. Thus, to circumvent the problem of P deficiency and the mobilization of the phytate

reserves present in agricultural soils, further investigations focused on the application of phytase-producing bacteria as inoculants for improving plant P uptake are required.

However, the effectiveness of phytases in soil may be reduced by sorption. For example, the rapid immobilization in soil of fungal-derived phytases released from roots of transgenic plants has been reported[23]. This immobilization may limit the capacity of phytases to interact with phytate and compromise the ability of transgenic plants to acquire P from endogenous soil phytate. A recent study revealed that two fungal phytases are rapidly absorbed to the soil solid phase, their physicochemical properties affecting their mobility, temporal stability and capacity to hydrolyze inositol phosphate in soil[24]. Absorption of microbial phytases may also reduce their affinity for inositol phosphate and thus reduce their effective activity[84]. Moreover, the effectiveness of phytases in increasing the availability of P to plants may be further limited by the concentration of organic P forms in the soil solution[22].

Concluding remarks and future trends

The importance of bacterial phytases as potential biotechnological tools has been recognized in various fields (Table 3). However, only a limited number of bacterial phytases have been reported and studied, and our knowledge of the mechanisms and factors regulating phytase activity is limited. Further research into developing new technologies and identifying the most efficient phytases for improving animal feed and food processing must continue. The use of phytase-producing bacteria for improving plant nutrition in the field also presents a promising potential application for agriculture, although major efforts will be required to refine and implement this technology prior to it becoming economically viable. With the collaborative efforts of scientists from different fields, effective solutions to the biotechnological development of phytases for mineral nutrition and environmental protection should be available in the near future.

Acknowledgements

This study was supported by Bicentenary Program in Science and Technology Grant PSD26, Fondecyt Grant No. 1061262, and International Cooperation Fondecyt Grant No. 7060093, CONICYT, Chile. Oscar Martinez acknowledges the Ph.D. Scholarship No. 21070354, CONICYT, Chile. This work was also partly supported by the JSPS Grant-in-Aid for Young Scientists (B) (20790111), Japan. The authors acknowledge the helpful suggestions made by the Editor and an anonymous reviewer to improve the quality of this Mini-Review.

References

1) Adedokun, S.A., J.S. Sands, and A. Adeola. 2004. Determining the equivalent phosphorus released by an *Escherichia coli*-derived phytase in broiler chicks. Can. J. Anim. Sci. **84**:437–444.
2) Anandham, R., K.H. Choi, P.I. Gandhi, W.J. Yim, S.J. Park, K.A. Kim, M. Madhaiyan, and T.M. Sa. 2007. Evaluation of shelf life and rock phosphate solubilization of *Burkholderia* sp. In nutrient-ammended clay, rice bran and rock phosphate-based granular formulation. World J. Microbiol. Biotechnol. **23**:1121–1129.
3) Applegate, T.J., D.M. Webel, and X.G. Lei. 2003. Efficacy of a phytase derived from *Escherichia coli* and expressed in yeast on phosphorus utilization and bone mineralization in turkey poults. Poult. Sci. **82**:1726–1732.
4) Augspurger, N.R., D.M. Webel, X.G. Lei, and D.H. Baker. 2003. Efficacy of an *E. coli* phytase expressed in yeast for releasing phytate-bound phosphorus in young chicks and pigs. J. Anim. Sci. **81**:474–483.
5) Bloemberg, G.V., and B.J.J. Lugtenberg. 2001. Molecular basis of plant growth promotion and biocontrol by rhizobacteria. Curr. Opin. Plant Biol. **4**:129–134.
6) Boesch, D.F., R.B. Brinsfield, and R.E. Magnien. 2001. Chesapeake bay eutrophication: scientific understanding, ecosystem restoration, and challenges for agriculture. J. Environ. Qual. **30**:303–320.
7) Borie, F., H. Zunino, and L. Martínez. 1989. Macromolecule P-associations and inositol phosphates in sole Chilean volcanic soils of temperate regions. Comm. Soil Sci. Plant Anal. **20**:1881–1894.
8) Borie, F., and R. Rubio. 2003. Total and organic phosphorus in Chilean volcanic soils. Gayana Bot. **60**:69–78.
9) Brown, J.R., and C. Volker. 2004. Phylogeny of γ-proteobacteria: resolution of one branch of the universal tree? BioEssays **26**:463–468.
10) Çakmakçi, R., F. Dönmez, A. Aydin, and F. Şahin. 2006. Growth promotion of plants by plant-promoting rhizobacteria under greenhouse and two different field soil conditions. Soil Biol. Biochem. **38**:1482–1487.
11) Cao, L., W. Wang, C. Yang, Y. Yang, J. Diana, A. Yakupitiyage, Z. Luo, and D. Li. 2007. Application of microbial phytase in fish feed. Enzyme Microb. Technol. **40**:497–507.
12) Chen, Y.P., P.D. Rekha, A.B. Arun, F.T. Shen, W.-A. Lai, and C.C. Young. 2006. Phosphate solubilizing bacteria from tropical soil and their tricalcium phosphate solubilizing abilities. Appl. Soil Ecol. **34**:33–41.
13) Cheng, K.J., L.B. Selinger, L.J. Yanke, H.D. Bae, L. Zhou, and C.W. Forsberg. 1999. Phytases of rumen micro-organisms, particularly of *Selenomonas ruminantium*, and uses thereof in feed additives and in transgenic plants. US patent 5, 985, 605, Nov. 16.
14) Cho, J., C. Lee, S. Kang, J. Lee, H. Lee, J. Bok, J. Woo, Y. Moon, and Y. Choi. 2005. Molecular cloning of a phytase gene (*phyM*) from *Pseudomonas syringae* MOK1. Curr. Microbiol. **51**:11–15.
15) Choi, Y.M., D.O. Noh, S.H. Cho, H.K. Lee, H.J. Suh, and S.H. Chung. 1999. Isolation of phytase-producing *Bacillus* sp. KHU-10 and its phytase production. J. Microbiol. Biotechnol. **9**:223–226.
16) Choi, Y.M., H.J. Suh, and J.M. Kim. 2001. Purification and properties of extracellular phytase from *Bacillus* sp. KHU-10. J. Prot. Chem. **20**:287–292.
17) Chu, H.M., R.T. Guo, T.W. Lin, C.C. Chou, H.L. Shr, H.L. Lai, T.Y. Tang, K.J. Cheng, B.L. Selinger, and A.H. Wang. 2004. Structures of *Selenomonas ruminantium* phytase in complex with persulfated phytate: DSP phytase fold and mechanism for sequential substrate hydrolysis. Structure **12**:2015–2024.
18) Chung, H., M. Park, M. Madhaiyan, S. Seshadri, J. Song, H. Cho, and T. Sa. 2005. Isolation and characterization of phosphate solubilizing bacteria from the rhizosphere of crop plants of Korea. Soil Biol. Biochem. **37**:1970–1974.
19) Feil, B. 2001. Phytic acid. J. New Seeds **3**:1–35.
20) Fuentes, B., N. Bolan, R. Naidu, and M.L. Mora. 2006. Phosphorus in organic waste-soil systems. J. Soil Sci. Plant Nutr. **6**:64–83.
21) Fuentes, B., M. Jorquera, and M.L. Mora. 2008. Dynamics of phosphorus and phytate-utilizing bacteria during aerobic degradation of dairy cattle dung. Chemosphere, In press.
22) George, T.S., P.J. Gregory, M. Wood, D. Read, and R.J. Buresh. 2002. Phosphatase activity and organic acids in the rhizosphere of potential agroforestry species and maize. Soil Biol. Biochem. **34**:1487–1494.
23) George, T.S., A.E. Richardson, and R.J. Simpson. 2005. Behaviour of plant-derived extracellular phytase upon addition to soil. Soil Biol. Biochem. **37**:977–988.
24) George, T.S., R.J. Simpson, P.J. Gregory, and A.E. Richardson. 2007. Differential interaction of *Aspergillus niger* and *Peniophora lycii* phytases with soil particles affects the hydrolysis of inositol phosphates. Soil Biol. Biochem. **39**:793–803.
25) Golovan, S., G.R. Wang, J. Zhang, and C.W. Forsberg. 2000. Characterization and overproduction of the *Escherichia coli* appA encoded bifunctional enzyme that exhibits both phytase and acid phosphatase activities. Can. J. Microbiol. **46**:59–71.

26) Greiner, R., and K.-D. Jany. 1991. Characterization of a phytase from *Escherichia coli*. Biol. Chem. Hopper-Seyler **372**:664–665.
27) Greiner, R., U. Konietzny, and K.-D. Jany. 1993. Purification and characterization of two phytases from *Escherichia coli*. Arch. Biochem. Biophys. **303**:107–113.
28) Greiner, R., E. Haller, U. Konietzny, and K.-D. Jany. 1997. Purification and characterization of a phytase from *Klebsiella terrigena*. Arch. Biochem. Biophys. **341**:201–206.
29) Greiner, R., N.-G. Carlsson, and M. Larsson Alminger. 2000. Stereospecificity of *myo*-inositol hexakisphosphate dephosphorylation by a phytate-degrading enzyme of *Escherichia coli*. J. Biotechnol. **84**:53–62.
30) Greiner, R., A. Farouk, M. Larsson Alminger, and N.-G. Carlsson. 2002. The pathway of desphosphorylation of *myo*-inositol hexakisphosphate by phytate-degrading enzymes of different *Bacillus* spp. Can. J. Microbiol. **84**:986–994.
31) Greiner, R. 2004. Purification and properties of a phytate-degrading enzyme from *Pantoea agglomerans*. Protein J. **23**:567–576.
32) Greiner, R. 2004. Degradation of *myo*-inositol hexakisphosphate by a phytate-degrading enzyme from *Pantoea agglomerans*. Protein J. **23**:577–585.
33) Greiner, R., and U. Konietzny. 2006. Phytase for food application. Food Technol. Biotechnol. **44**:125–140.
34) Greimer, R., U. Konietzny, and K.-D. Jany. 2006. Phytate—an undesirable constituent of plant-based foods? J. Ernährungsmedizin **8**:18–28.
35) Greiner, R., and N.-G. Carlsson. 2006. Myo-Inositol phosphate isomers generated by the action of a phytate-degrading enzyme from *Klebsiella terrigena* on phytate. Can. J. Microbiol. **52**:759–768.
36) Greiner, R., B.L. Lim, C. Cheng, and N.-G. Carlsson. 2007. Pathway of phytate dephosphorylation by β-propeller phytases of different origins. Can. J. Microbiol. **53**:488–495.
37) Gyaneshwar, P., G. Naresh Kumar, L.J. Parekh, and P.S. Poole. 2002. Role of soil microorganism in improving P nutrition of plant. Plant Soil **245**:83–93.
38) Ha, N.C., Y.O. Kim, T.K. Oh, and B.H. Oh. 1999. Preliminary X-ray crystallographic analysis of a novel phytase from a *Bacillus amyloliquefaciens* strain. Acta Crystallogr., Sect. D:Biol. Crystallogr. **55**:691–693.
39) Haefner, S., A. Knietsch, E. Scholten, J. Braun, M. Lohscheidt, and O. Zelder. 2005. Biotechnological production and applications of phytases. Appl. Microbiol. Biotechnol. **68**:588–597.
40) Harland, B.F., and D. Oberleas. 1999. Phytic acid complex in feed ingredient, p. 69–76. *In* M.B. Coelho, and E.T. Kornegay (ed.), Phytase in Animal Nutrition and Waste Management. BASF, Mexico.
41) Hayes, J.E., A.E. Richardson, and R.J. Simpson. 1999. Phytase and acid phosphatase activities in extracts from roots of temperate pasture grass and legume seedlings. Aust. J. Plant Physiol. **26**:801–809.
42) Hayes, J.E., R.J. Simpson, and A.E. Richardson. 2000. The growth and phosphorus utilization of plants in sterile media when supplied with inositol hexaphosphate, glucose 1-phosphate or inorganic phosphate. Plant Soil **220**:165–174.
43) Herter, T., O.V. Berezina, N.V. Zinin, G.A., Velikodvorskaya, R. Greiner, and R. Borriss. 2006. Glucose-1-phosphatase (*Agp*E) from *Enterobacter cloacae* displays enhanced phytase activity. Appl. Microbiol. Biotechnol. **70**:60–64.
44) Hill, J.E., D. Kysela, and M. Elimelech. 2007. Isolation and assessment of phytate-hydrolysing bacteria from the DelMarVa Peninsula. Environ. Microbiol. **9**:3100–3107.
45) Hill, J.E., and A.E. Richardson. 2007. Isolation and assessment of microorganisms that utilize phytate, p. 61–77. *In* B.L. Turner, A.E. Richardson, and E.J. Mullaney (ed.), Inositol Phosphates: Linking Agriculture and Environment. CABI Publishing, Wallington, UK.
46) Huang, H., H. Luo, P. Yang, K. Meng, Y. Wang, T. Yuan, Y. Bai, and B. Yao. 2006. A novel phytase with preferable characteristics from *Yersinia intermedia*. Biochem. Biophys. Res. Comm. **350**:884–889.
47) Hurrell, R.F., M.B. Reddy, M.A. Juillerat, and J.D. Cook. 2003. Degradation of phytic acid in cereal porridges improves iron absorption by human subject. Am. J. Clin. Nutr. **77**:1213–1219.
48) Idriss, E., O. Makarewicz, A. Farouk, K. Rosner, R. Greiner, H.T. Bochow, T. Richter, and R. Borriss. 2002. Extracellular phytase activity of *Bacillus amyloliquefaciens* FZB45 contributes to its plant-growth-promoting effect. Microbiology **148**:2097–2109.
49) Igual, J.M., A. Valverde, E. Cervantes, and E. Velásquez. 2001. Phosphate-solubilizing bacteria as inoculants for agriculture: use of updated molecular techniques in their study. Agronomie **21**:561–568.
50) In, M.J., E.S. Jang, Y.J. Kim, and N.S. Oh. 2004. Purification and properties of an extracellular acid phytase from *Pseudomonas fragi* Y9451. J. Microbiol. Biotechnol. **14**:1004–1008.
51) Irving, G.C.J., and D.J. Cosgrove. 1971. Inositol phosphate phosphatases of microbiological origin: some properties of a partially purified bacterial (*Pseudomonas* sp.) phytase. Austr. J. Biol. Sci. **24**:547–557.
52) Irving, G.C.J. 1980. Phytase, p. 85–117. *In* D.J. Cosgrove (ed.), Inositol Phosphates. Elsevier Ltd., Amsterdam.
53) Jakobsen, I., M.E. Leggett, and A.E. Richardson. 2005. Rhizosphere microorganisms and plant phosphorus uptake. Agronomy **46**:437–494.
54) Jareonkitmongkol, S., M. Ohya, R. Watanabe, H. Takagi, and S. Nakamori. 1997. Partial purification of phytase from a soil isolate bacterium, *Klebsiella oxytoca* MO-3. J. Ferment. Bioeng. **83**:393–394.
55) Jia, Z.C., S. Golovan, Q.L. Ye, and C.W. Forsberg. 1998. Purification, crystallization and preliminary X-ray analysis of the *Escherichia coli* phytase. Acta Crystallogr., Sect. D:Biol. Crystallogr. **54**:647–649.
56) Jorquera, M., M. Hernández, Z. Rengel, P. Marschner, and M.L. Mora. 2008. Isolation of culturable phosphobacteria with both phytate-mineralization and phosphate-solubilization activity from the rhizosphere of plants grown in a volcanic soil. Biol. Fertil. Soils. In press.
57) Kerovuo, J., M. Lauraeus, P. Nurminen, N. Kalkkinen, and J. Apajalahti. 1998. Isolation, characterization, molecular gene cloning, and sequencing of a novel phytase from *Bacillus subtilis*. Appl. Environ. Microbiol. **64**:2079–2085.
58) Kerovuo, J., I. Lappalainen, and T. Reinikainen. 2000. The metal dependence of Bacillus subtilis phytase. Biochem. Biophys. Res. Comm. **268**:365–369.
59) Kim, Y.O., J.K. Lee, H.K. Kim, J.H. Yu, and T.K. Oh. 1998. Cloning of the thermostable phytase gene (*phy*) from *Bacillus* sp. DS11 and its over-expression in *Escherichia coli*. FEMS Microbiol. Let. **162**:185–191.
60) Kim, D.H., B.C. Oh, W.C. Choi, J.K. Lee, and T.K. Oh. 1999. Enzymatic evaluation of *Bacillus amyloliquefaciens* phytase as a feed additive. Biotechnol. Let. **21**:925–927.
61) Kim, O.Y., J.K. Lee, B.C. Oh, and T.K. Oh. 1999. High-level expression of a recombinant thermostable phytase in *Bacillus subtilis*. Biosci. Biotechnol. Biochem. **63**:2205–2207.
62) Kim, H.W., Y.O. Kim, J.H. Lee, K.K. Kim, and Y.J. Kim. 2003. Isolation and characterization of a phytase with improved properties from *Citrobacter braakii*. Biotechnol. Let. **25**:1231–1234.
63) Kloepper, J.W., J. Leong, M. Teintze, and M.N. Schroth. 1989. Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. Nature **268**:885–886.
64) Knowlton, K.F., J.S. Radcliffe, C.L. Novak, and D.A. Emmerson. 2004. Animal management to reduce phosphorus losses to the environment. J. Anim. Sci. **82**(E. Suppl.):173–195.
65) Konietzny, U., and R. Greiner. 2002. Molecular and catalytic properties of phytate-degrading enzymes (phytases). Int. J. Food Sci. Technol. **37**:791–812.
66) Konietzny, U., and R. Greiner. 2004. Bacterial phytase: potential application, in vivo function and regulation of its synthesis. Brazilian J. Microbiol. **35**:11–18.
67) Kretz, K. 1999. Phytase from *Escherichia coli* B and its use in animal feed. PCT Int. Appl. WO 99 08, 539, Feb. 25.
68) Lan, G.Q., N. Abdullah, S. Jalaludin, and Y.W. Ho. 2002. Culture conditions influencing phytase production of *Mitsuokella jalaludinii*, a new bacterial species from the rumen of cattle. J. Appl. Microbiol. **93**:668–674.
69) Lei, X.G., and Stahl. 2001. Biotechnological development of effective phytases for mineral nutrition and environmental protection. Appl. Microbiol. Biotechnol. **57**:474–481.
70) Lei, X.G., and J.M. Porres. 2003. Phytase enzymology, applications, and biotechnology. Biotechnol. Lett. **25**:1787–1794.
71) Lerat, E., V. Daubin, and N.A. Moran. 2003. From gene tree to

organismal phylogeny in prokaryotes: The case of the γ-proteobacteria. PLoS Biol. **1**:101–109.
72) Liebert, F., and L. Portz. 2005. Nutrient utilization of Nile tilapia *Oreochromis niloticus* fed plant based low phosphorus diets supplemented with graded levels of different sources of microbial phytase. Aquaculture **248**:111–119.
73) Lim, D., S. Golovan, C.W. Forsberg, and Z.C. Jia. 2000. Crystal structures of *Escherichia coli* phytase and its complex with phytate. Nat. Struct. Biol. **7**:108–113.
74) Lim, B.L., P. Yeung, C. Cheng, and J.E. Hill. 2007. Distribution and diversity of phytate-mineralizing bacteria. ISME J. **1**:321–330.
75) Lott, J.N.A., I. Ockenden, V. Raboy, and G.D. Batten. 2000. Phytic acid and phosphorus in crop seeds and fruits: a global estimated. Seed Sci. Res. **10**:11–33.
76) Mehta, S., and C.S. Nautiyal. 2001. An efficient method for qualitative screening of phosphate-solubilizing bacteria. Curr. Microbiol. **43**:51–56.
77) Mullaney, E.J., and A.H. Ullah. 2003. The term phytase comprises several different classes of enzymes. Biochem. Biophys. Res. Commun. **312**:179–184.
78) Mullen, M.D. 2005. Phosphorus in soils: biological interactions, p. 210–215. *In* D. Hillel (ed.), Encyclopedia of Soils in the Environment. Elsevier Ltd., Oxford.
79) National Research Council (NRC). 1993. Nutrient Requirements of Fish. National Academic, Press, Washington, D.C., USA.
80) Oberleas D. 1983. The role of phytate in zinc bioavailability and homeostasis, p. 145–158. *In* G.E. Inglett (ed.), Nutritional Bioavailability of Zinc. American Chemical Society, Washington, D.C.
81) Okazaku, S., N. Nukui, M. Sugawara, and K. Minamisawa. 2004. Rhizobial strategies to enhance symbiotic interactions: Rizobitoxine and 1-aminocyclopropane-1-carboxylate deaminase. Microb. Environ. **19**:99–111.
82) Pandey, A., G. Szakacs, C.R. Soccol, J.A. Rodriguez-Leon, and V.T. Soccol. 2001. Production, purification and properties of microbial phytases. Bioresource Technol. **77**:203–214.
83) Powar, V.K., and V. Jagannathan. 1982. Purification and properties of phytate-specific phosphatase from *Bacillus subtilis*. J. Bacteriol **151**:1102–1108.
84) Quiquampoix, H., and D. Mousain. 2005. Enzymatic hydrolysis of organic phophorus, p. 89–112. *In* B.L. Turner, E. Frossard, and D. Baldwin. (ed.), Organic Phosphorus in the Environment. CABI Publishing, Wallingford, UK.
85) Reyes, I., A. Valery, and Z. Valduz. 2006. Phosphate-solubilizing microorganisms isolated from rhizospheric and bulk soils of colonizer plants at an abandoned rock phosphate mine. Plant Soil **287**:69–75.
86) Richardson, A.E., and P.A. Hadobas. 1997. Soil isolates of *Pseudomonas* spp. that utilize inositol phosphates. Can. J. Microbiol. **43**:509–516.
87) Richardson, A.E. 2001. Prospect for using microorganisms to improve the acquisition of phosphorus by plants. Aust. J. Plant Physiol. **28**:897–906.
88) Richardson, A.E., T.S. George, M. Hens, and R.J. Simpson. 2005. Utilization of soil organic phosphorus by higher plants, p. 165–184. *In* B.L. Turner, E. Frossard, and D. Baldwin. (ed.), Organic Phosphorus in the Environment. CABI Publishing, Wallingford, UK.
89) Rodriguez, E., J.M. Porres, Y. Han, and X.G. Lei. 1999. Different sensitivity of recombinant *Aspergillus niger* phytase (r-phyA) and *Escherichia coli* pH 2.5 acid phosphatase (r-AppA) to trypsin and pepsin in vitro. Arch. Biochem. Biophys. **365**:262–267.
90) Rodríguez, H., R. Fraga, T. González, and Y. Bashan. 2006. Genetics of phosphate solubilization and its potential applications for improving plant growth-promoting bacteria. Plant Soil **287**:15–21.
91) Rosen, G. 2003. Microbial phytase in broiler nutrition, p. 105–117. *In* P.C. Garnsworthy, and J. Wiseman (ed.), Recent Advances in Animal Nutrition. Nottingham University Press, Nottingham, UK.
92) Sajidan, A., A. Farouk, R. Greiner, P. Jungblut, E.-C. Müller, and R. Borriss. 2004. Molecular and physiological characterization of a 3-phytase from soil bacterium *Klebsiella* sp. ASR1. Appl. Micriobiol. Biotechnol. **65**:110–118.
93) Sajjadi, M., and C.G. Carter. 2004. Effect of phytic acid and phytase on feed intake, growth, digestibility and tripsin activity in Atlantic Salmon (*Salmo salar* L.). Aquacult. Nutr. **10**:135–142.
94) Sandberg, A.-S., and T. Andlid. 2002. Phytogenic and microbial phytases in human nutrition. Int. J. Food Sci. Technol. **37**:823–834.
95) Schaefer, A., and W.M. Koppe. 1995. Effect of a microbial phytase on utilization of native phosphorus by carp in a diet based on soybean meal. Water Sci. Technol. **31**:149–155.
96) Schenk, G., M.L.J. Korsinczky, D.A. Hume, S. Hamilton, and J. DeJersey. 2000. Purple acid phosphatases from bacteria: similarities to mammalian and plant enzymes. Gene **255**:419–424.
97) Schroder, B., G. Breves, and M. Rodehutscord. 1996. Mechanisms of intestinal phosphorus absorption and availability of dietary phosphorus in pigs. Dtsch. Tieraerztl. Wochenschr. **103**:209–214.
98) Seshadri, R., S.A. Kravitz, L. Smarr, P. Gilna, and M. Frazier. 2007. CAMERA: a community resource for metagenomics. PLoS Biol. **5**:394–397.
99) Selle, P.H., and V. Ravindran. 2007. Microbial phytase in poultry nutrition. Animal Feed Sci. Technol. **135**:1–41.
100) Shah, V., and L.J. Parekh. 1990. Phytase from *Klebsiella* sp. No. PG-2: purification and properties. Ind. J. Biochem. Biophys. **27**:98–102.
101) Shang, C., J.W.B. Stewart, and P.M. Huang. 1992. pH effect on kinetics of absorption of organic and inorganic phosphates by short-range ordered aluminium and iron precipitates. Geoderma **53**:1–14.
102) Sharpley, A.E. 2000. Agriculture and Phosphorus Management: The Chesapeake Bay. Boca Raton, FL, USA: Lewis Publishers.
103) Shimizu, M. 1992. Purification and characterization of phytase from *Bacillus subtilis* (Natto) N-77. Biosci. Biotechnol. Biochem. **56**:1266–1269.
104) Sims, J.T., A.C. Edwards, O.F. Schoumans, and R.R. Simard. 2000. Integrating soil phosphorus testing into environmentally based agricultural management practices. J. Environ. Qual. **29**:60–71.
105) Smernik, R.J., and W.J. Dougherty. 2007. Identification of phytate in phosphorus-31 nuclear magnetic resonance spectra: the need for spiking. Soil Sci. Soc. Am. J. **71**:1045–1050.
106) Sreeramulu, G., D.S. Srinavasa, K. Nand, and R. Joseph. 1996. *Lactobacillus amylovorus* as a phytase producer in submerged culture. Let. Appl. Microbiol. **23**:385–388.
107) Sunitha, K., J.K. Lee, and T.K. Oh. 1999. Optimization of medium component for phytase production by *E. coli* using response surface methodology. Bioprocess Eng. **21**:477–481.
108) Tambe, S.M., G.S. Kaklij, S.M. Kelkar, and L.J. Parekh. 1994. 2 distinct molecular forms of phytase from *Klebsiella aerogenes* evidence for unusually small active enzyme peptide. J. Ferment. Bioeng. **77**:23–27.
109) Thompson, J.D., D.G. Higgins, and T.J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matix choice. Nucleic Acids Res. **22**:4673–4680.
110) Timmis, K.N. 2002. *Pseudomonas putida*: a cosmopolitan opportunist *par excellence*. Environ. Microbiol. **4**:779–781.
111) Toor, G., S. Hunger, J.D. Peak, J.T. Sims, and D.L. Sparks. 2006. Advances in the characterization of phosphorus in organic wastes: Environmental and agronomic applications. Adv. Agron. **89**:1–72.
112) Turner, B.L., and P.M. Haygarth. 2000. Phosphorus forms and concentrations in leachate under four grassland soil types. Soil Sci. Soc. Am. J. **64**:1090–1097.
113) Turner, B.L., M.J. Papházy, P.M. Haygarth, and I.D. McKelvie. 2002. Inositol phosphates in the environment. Philos. T. Roy. Soc.B **357**: 449–469.
114) Turner, B.L., N. Mahieu, and L.M. Condron. 2003. Quantification of *myo*-inositol hexakisphosphate in alkaline soil extracts by solution 31P NMR spectroscopy and spectral deconvolution. Soil Sci. **168**:469–478.
115) Tye, A.J., F.K. Siu, T.Y. Leung, and B.L. Lim. 2002. Molecular cloning and the biochemical characterization of two novel phytases from *Bacillus subtilis* 168 and *Bacillus licheniformis*. Appl. Microbiol. Biotechnol. **59**:190–197.
116) Unno, Y., K. Okubo, J. Wasaki, T. Shinano, and M. Osaki. 2005. Plant growth promotion abilities and microscale bacterial dynamics in the rhizosphere of Lupin analysed by phytate utilization ability. Environ. Microbiol. **7**:396–404.
117) Urbano, G., M. Lopez-Jurado, P. Aranda, C. Vidal-Valverde, E. Tenorio, and J. Porres. 2000. The role of phytic acid in legumes: antinutrient or beneficial function? J. Physiol. Biochem. **56**:283–294.
118) Vats, P., and U.C. Banerjee. 2004. Production studies and catalytic

properties of phytases (*myo*-inositol-hexakis-phosphate phosphohydrolases): an overview. Enzyme Microb. Technol. **35**:3–14.

119) Vielma, J., K. Ruohonen, and M. Peisker. 2002. Dephytinization of two soy proteins increases phosphorus and protein utilization by rainbow trout, *Oncorhynchus mykiss*. Aquaculture **204**:145–156.

120) Vohra, A., and T. Satyanarayana. 2003. Phytases: microbial sources, production, purification, and potential biotechnological applications. Crit. Rev. Biotechnol. **23**:29–60.

121) Wang, X., S. Upatham, W. Panbangred, D. Isarangkul, P. Summpunn, S. Wiyakrutta, and V. Meevootisomd. 2004. Purification, characterization, gene cloning and sequence analysis of a phytase from *Klebsiella pneumoniae* subsp. *pneumoniae* XY-5. ScienceAsia **30**:383–390.

122) Wyss, M., R. Brugger, A. Kronenberger, Roland Rémy, R. Fimbel, G. Oesterhelt, M. Lehmann, and A.P.G.M. Van Loon. 1999. Biochemical characterization of fungal phytases (*myo*-Inositol hexakisphosphate phosphohydrolases): catalytic properties. Appl. Environ. Microbiol. **65**:367–373.

123) Yan, W., and R.C. Reigh. 2002. Effect of fungal phytase on utilization of dietary protein and minerals, and dephosphorylation of phytic acid in the alimentary tract of channel catfish *Ictalurus punctatus* fed and all-plant protein diet. J. World Aquacult. Soc. **33**:10–22.

124) Yanke, L.J., H.D. Bae, L.B. Selinger, and K.J. Cheng. 1998. Phytase activity of anaerobic ruminal bacteria. Microbiology **144**:1565–1573.

125) Yanke, L.J., L.B. Selinger, and K.J. Cheng. 1999. Phytase activity of *Selenomonas ruminantium*: a preliminary characterization. Let. Appl. Microbiol. **29**:20–25.

126) Yoon, S.J., Y.J. Choi, H.K. Min, K.K. Cho, J.W. Kim, S.C. Lee, and Y.H. Jung. 1996. Isolation and identification of phytase-producing bacterium, *Enterobacter* sp. 4, and enzymatic properties of phytase enzyme. Enzyme Microb. Technol. **18**:449–454.

127) Yu, F.N., and D.Z. Wang. 2000. Effects of supplemental phytase on growth and the utilization of phosphorus by crucian carp *Carassius carassius*. J. Fish Sci. Chin. **7**:106–109.

128) Zakria, M., J. Njoloma, Y. Saeki, and S. Asao. 2007. Colonization and nitrogen-fixing ability of *Herbaspirillum* sp. Strain B501 *gfp*1 and assessment of its growth-promoting ability in cultivated rice. Microb. Environ. **22**:197–206.

129) Zinin, N.V., A.V. Serkina, M.S. Gelfand, A.B. Shevelev, and S.P. Sineoky. 2004. Gene cloning, expression and characterization of novel phytase from *Obesumbacterium proteus*. FEMS Microbiol. Let. **236**:283–290.

Olempska – Beer,
Z.S

1947

Available online at www.sciencedirect.com
SCIENCE DIRECT
Regulatory Toxicology and Pharmacology 45 (2006) 144–158
Regulatory Toxicology and Pharmacology
www.elsevier.com/locate/yrtph

Food-processing enzymes from recombinant microorganisms—a review ☆

Zofia S. Olempska-Beer *, Robert I. Merker, Mary D. Ditto, Michael J. DiNovi

U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, Office of Food Additive Safety, HFS-255, 5100 Paint Branch Parkway, College Park, MD 20740, USA

Received 6 March 2006

Abstract

Enzymes are commonly used in food processing and in the production of food ingredients. Enzymes traditionally isolated from culturable microorganisms, plants, and mammalian tissues are often not well-adapted to the conditions used in modern food production methods. The use of recombinant DNA technology has made it possible to manufacture novel enzymes suitable for specific food-processing conditions. Such enzymes may be discovered by screening microorganisms sampled from diverse environments or developed by modification of known enzymes using modern methods of protein engineering or molecular evolution. As a result, several important food-processing enzymes such as amylases and lipases with properties tailored to particular food applications have become available. Another important achievement is improvement of microbial production strains. For example, several microbial strains recently developed for enzyme production have been engineered to increase enzyme yield by deleting native genes encoding extracellular proteases. Moreover, certain fungal production strains have been modified to reduce or eliminate their potential for production of toxic secondary metabolites. In this article, we discuss the safety of microorganisms used as hosts for enzyme-encoding genes, the construction of recombinant production strains, and methods of improving enzyme properties. We also briefly describe the manufacture and safety assessment of enzyme preparations and summarize options for submitting information on enzyme preparations to the US Food and Drug Administration.

Keywords: Review; Food-processing enzymes; Recombinant microorganisms; FDA

1. Introduction

Enzymes occur in all living organisms and catalyze biochemical reactions necessary to support life. Enzymes are ubiquitous in fresh and processed food and are consumed every day. Like other dietary proteins, enzymes are degraded and metabolized after ingestion. Enzymes naturally present in the human diet have not been associated with toxicity and are considered intrinsically safe.

The industrial production of enzymes for use in food processing dates back to 1874, when Danish scientist Christian Hansen extracted rennin (chymosin) from calves' stomachs for use in cheese manufacturing (Nielsen et al., 1994). Chymosin is now produced from microorganisms that contain the bovine prochymosin gene introduced through recombinant deoxyribonucleic acid (rDNA) techniques. Bovine chymosin expressed in *Escherichia coli* K-12 became the first recombinant enzyme approved for use in food by the US Food and Drug Administration (FDA) (Flamm, 1991).

Many enzymes currently used in food processing are derived from recombinant microorganisms. Enzyme manufacturers take advantage of new genetic techniques to develop and manufacture enzymes with improved properties. Such enzymes often originate from microorganisms that cannot be easily cultured under laboratory or

☆ The opinions expressed in this article are the authors' personal opinions and they do not necessarily reflect those of FDA, DHHS, or the Federal Government.

* Corresponding author. Fax: +1 301 436 2964.
E-mail address: zofia.olempskabeer@fda.hhs.gov (Z.S. Olempska-Beer).

0273-2300/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.yrtph.2006.05.001

industrial conditions. By judicious selection of host microorganisms, recombinant production strains can be constructed to allow efficient production of enzymes that are substantially free of undesirable enzymes or other microbial metabolites.

The increasing sophistication of food processing created a demand for a broad variety of food processing enzymes with characteristics compatible with food processing conditions. For example, commonly used sweeteners such as glucose or fructose syrups are typically produced from corn starch using hydrolytic enzymes. In the first step of starch hydrolysis, starch is liquefied with α-amylase by heating at 105 °C for 2–5 min followed by 1–2 h at 90–100 °C. With the advent of rDNA technology, it became possible to engineer α-amylases with increased heat stability and improved compatibility with other parameters of the liquefaction process. These improvements were accomplished by introducing changes in the α-amylase amino acid sequences through DNA sequence modifications of the α-amylase genes. Other enzymes currently used in food processing have also been improved using rDNA techniques.

Enzymes used in food processing are sold as enzyme preparations. An enzyme preparation typically contains the enzyme of interest and several added substances such as diluents, preservatives, and stabilizers. The added materials are usually well-known substances suitable for use in food. Enzyme preparations may also contain other enzymes and metabolites from the production organism and the residues of raw materials used in fermentation media and during isolation and purification of the enzyme. All these materials are expected to be of appropriate purity consistent with current good manufacturing practice (cGMP).

The safety evaluation of food processing enzymes from recombinant microorganisms has been extensively discussed in the literature (IFBC, 1990; Pariza and Johnson, 2001; Jonas et al., 1996) and in guidance documents issued by regulatory authorities and international organizations, for example, by the Scientific Committee for Food (SCF, 1992). In principle, the same safety considerations apply to enzymes derived from native and recombinant microorganisms. The key component in evaluating enzyme safety is the safety assessment of the production strain, in particular, its pathogenic and toxigenic potential (Pariza and Johnson, 2001). Although neither pathogenic nor toxigenic microorganisms are intentionally used in the production of food-processing enzymes, certain fungi traditionally used as sources of enzymes have been found to produce low levels of toxic secondary metabolites under fermentation conditions conducive to the synthesis of these compounds. Some of these microorganisms are now used as sources of recombinant enzymes.

In this article, we will review information on food-processing enzymes from recombinant microorganisms. We will discuss safety-related characteristics of the host microorganisms, construction of recombinant production strains, and methods of improving enzyme properties. We will also briefly describe the manufacture and safety assessment of enzyme preparations and summarize options for submitting information on enzyme preparations to FDA. We will rely on published sources, nonconfidential documents submitted to the agency, which may be obtained by the public through the Freedom of Information Act, and FDA documents published in the Federal Register or the agency's web site.

2. FDA review of submissions on enzyme preparations

Enzyme preparations can be regulated as secondary direct food additives in Title 21 of the Code of Federal Regulations (21 CFR), section 173. To establish a regulation for an enzyme preparation, a food additive petition must be submitted. In the past, FDA also reviewed generally recognized as safe (GRAS) affirmation petitions for enzyme preparations. A successful review of a GRAS affirmation petition resulted in a regulation in 21 CFR, section 184. The GRAS affirmation process is being replaced with a voluntary notification program under the agency's proposed regulation (Proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; substances generally recognized as safe (GRAS))). The successful notification process results in an FDA letter to the notifier stating that FDA has no questions regarding the notifier's conclusion that the use of the enzyme is GRAS. The GRAS notification program is described in a recent regulatory review (Gaynor, 2006).

Since 1997, FDA has reviewed more than 35 GRAS notices for enzyme preparations derived from native and recombinant microorganisms. The list of all GRAS notices submitted to FDA, including those for enzyme preparations, can be viewed at the agency's web site (http://www.cfsan.fda.gov/~rdb/opa-gras.html). The site provides links to FDA letters issued at the completion of the notification process.

3. Submissions on enzymes from recombinant microorganisms

In the last two decades, FDA has received a number of petitions and GRAS notices on food-processing enzymes derived from recombinant microorganisms. These enzymes are listed in Table 1 with their source microorganisms and references to FDA regulations, GRAS affirmation petitions, and GRAS notices. Lists of commercial enzymes used in food processing can be found at the web sites of the Enzyme Technical Association (http://www.enzymetechnicalassoc.org) and the Association of Manufacturers and Formulators of Enzyme Products (http://www.amfep.org). The scope of this review is limited to enzymes and source microorganisms listed in Table 1.

4. Steps in the development of recombinant production strains

Industrial production of recombinant enzymes is preceded by an extensive research and development phase that

Table 1
Enzymes from recombinant microorganisms (based on FDA regulations, GRAS affirmation petitions, and GRAS notices)

Source microorganism	Enzyme	Reference[a]
Aspergillus niger	Phytase	GRASP 2G0381
	Chymosin	21 CFR 184.1685
	Lipase	GRN 158
Aspergillus oryzae	Esterase–lipase	GRASP 7G0323
	Aspartic proteinase	GRN 34
	Glucose oxidase	GRN 106
	Laccase	GRN 122
	Lipase	GRN 43; GRN 75; GRN 103
	Pectin esterase	GRN 8
	Phospholipase A1	GRN 142
Bacillus licheniformis	α-amylase	GRASP 0G0363; GRN 22; GRN 24; GRN 79
	Pullulanase	GRN 72
Bacillus subtilis	α-acetolactate decarboxylase	21 CFR 173.115
	α-amylase	GRASP 4G0293; GRASP 7G0328
	Maltogenic amylase	GRASP 7G0326
	Pullulanase	GRN 20
Escherichia coli K-12	Chymosin	21 CFR 184.1685
Fusarium venenatum	Xylanase	GRN 54
Kluyveromyces marxianus var. *lactis*	Chymosin	21 CFR 184.1685
Pseudomonas fluorescens Biovar I	α-amylase	GRN 126
Trichoderma reesei	Pectin lyase	GRN 32

[a] GRASP is an acronym for a GRAS affirmation petition. The GRAS affirmation petitions listed in this Table have not resulted in regulations because the agency initiated the GRAS notification program. Some GRAS affirmation petitions were converted to GRAS notices (GRNs). A list of GRAS notices can be viewed at http://www.cfsan.fda.gov/~rdb/opa-gras.html. 21 CFR means Title 21 of the Code of Federal Regulations. Each reference to 21 CFR includes the regulation number.

culminates in the construction of a successful production strain. This process typically involves the following stages: (1) development of the host (recipient) strain; (2) construction of the expression vector; (3) transformation of the host strain; (4) identification of the best recombinant strain; (5) additional improvements; and (6) characterization of the production strain. Each of these steps is dictated by specific circumstances related to the identity and properties of the host organism and availability of genetic methods suitable for its modification and transformation. These steps will be described below and illustrated with examples.

5. Characteristics of host strains

As shown in Table 1, most host strains used to develop production strains for food-processing enzymes have been derived from a relatively small number of bacterial and fungal species primarily *B. subtilis*, *B. licheniformis*, *A. niger*, or *A. oryzae*. These microorganisms have a long history of use as safe sources of native enzymes and a proven record of efficient growth under industrial production conditions. They are also amenable to genetic manipulations and known for their ability to secrete ample quantities of enzymes into fermentation media. These characteristics make these microorganisms particularly desirable for use as hosts for a variety of heterologous enzymes. Several microorganisms with no history of use in the industrial production of native enzymes, such as *E. coli* K-12, *F. venenatum*, and *P. fluorescens*, have also been successfully utilized as hosts for expression of food-processing enzymes.

Although the majority of host microorganisms used to-date secrete enzymes to the fermentation media, the Gram-negative bacteria *E. coli* K-12 (source of chymosin) and *P. fluorescens* Biovar I (source of α-amylase) accumulate rather than secrete heterologous enzymes. Isolation and purification of the accumulated enzymes generally involve more steps during the production process than purification of the secreted enzymes.

The wild-type strains of several host microorganisms produce a variety of extracellular enzymes. Such enzymes may be carried over to the final enzyme preparation and catalyze undesirable reactions in food. Extracellular proteases are particularly troublesome because they tend to degrade heterologous enzymes. To increase enzyme yield, protease-deficient host strains have been constructed. Other modifications include developing sporulation-deficient mutant strains.

Microorganisms used as hosts for recombinant enzymes listed in Table 1 are recognized as nonpathogenic (i.e., they do not cause disease in healthy humans and animals). It is less clear whether all of these microorganisms should also be described as nontoxigenic. Some of these microorganisms, most notably *A. niger*, *A. oryzae*, and *F. venenatum*, may produce low levels of toxic secondary metabolites under certain cultivation conditions. Pariza and Foster (1983) defined the nontoxigenic organism as one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure. Under this definition, the *A. niger*, *A. oryzae*, and *F. venenatum* production strains are sometimes described as nontoxigenic in submissions to FDA. Most literature sources cited in this review do not refer to these microorganisms as nontoxigenic.

6. Bacterial host strains

6.1. Bacillus subtilis and its relatives

As shown in Table 1, several enzymes important for food processing have been recently derived from recombinant strains of the Gram-positive bacteria *B. subtilis* and *B. licheniformis*. *B. subtilis* has been used for several decades as a source of food-processing and industrial enzymes, mainly α-amylases and proteases. Of particular importance is *B. subtilis* strain 168, a well-known wild-type strain from which numerous strains widely used in research

and industrial applications were developed. Its genome has been recently sequenced (Kunst et al., 1997). Strain 168 is the progenitor of many *B. subtilis* strains that have been used as sources of food-processing enzymes. The safety of recombinant enzymes derived from *B. subtilis* is documented in petitions and GRAS notices submitted to FDA (see Table 1) and in relevant publications (for example, see Zeman and McCrea, 1985; Andersen et al., 1987; MacKenzie et al., 1989; de Boer et al., 1993). Other *Bacillus* species, including *B. licheniformis*, *B. amyloliquefaciens*, and *B. stearothermophilus* (recently reclassified as *Geobacillus stearothermophilus*; Nazina et al., 2001), have also emerged as safe sources of food-processing enzymes, primarily α-amylases. In the last decade, *B. licheniformis* and *B. amyloliquefaciens* have been successfully adapted for use as hosts for expression of recombinant enzymes. Complete genome sequences of two industrial *B. licheniformis* strains were determined and found largely homologous to the sequence of *B. subtilis* but distinct from the sequences of *B. cereus* and *B. anthracis*, which are human pathogens (Ray et al., 2004; Veith et al., 2004). *B. cereus* produces several toxins that cause food poisoning and *B. anthracis* causes anthrax.

The safety of *B. amyloliqueafaciens*, *B. licheniformis*, and *B. subtilis* was discussed in several recent reviews (de Boer and Diderichsen, 1991; de Boer et al., 1994; Pedersen et al., 2002). Pedersen et al. (2002) evaluated the cytotoxic potential of several industrial strains of these species used by Novozymes A/S in the production of enzymes. The strains were tested for cytotoxicity against Chinese hamster ovary K1 (CHO-K1) cells and for production of *B. cereus* hemolytic and nonhemolytic enterotoxins using immunological assays. The tested strains were nontoxic to CHO-K1 cells and did not react with antibodies against *B. cereus* enterotoxins. In addition, the database consisting of the DNA sequences from *B. subtilis* (full sequence) and *B. licheniformis* (96% of all genes), was searched for genes homologous to those encoding known *B. cereus* protein toxins. The search revealed no genes with homology to genes encoding the *B. cereus* protein toxins. Based on these studies, Pedersen et al. (2002) concluded that the tested strains do not produce *B. cereus*-like toxins or any other secondary metabolites with cytotoxic potential and are safe to use.

The safety of *B. subtilis* and *B. licheniformis* was evaluated by the U.S. Environmental Protection Agency. Both microorganisms were exempted from EPA review under the Toxic Substances Control Act (TSCA) (EPA, 1997). *B. subtilis* and related species are also used in the production of industrial enzymes, insecticidal and pharmaceutical proteins, antibiotics, purine nucleotides used as flavor enhancers, and other compounds with food, medical, and industrial applications (Harwood, 1992; Schallmey et al., 2004; Westers et al., 2004).

The wild-type strains of *Bacillus* species sporulate in response to nutrient limitations. While some commercially important compounds, such as *B. thuringiensis* pesticidal proteins, are produced concomitantly with sporulation (Harwood, 1992), the production of food-processing enzymes is hampered by sporulation. Consequently, nonsporulating mutants have been developed for use as host strains for recombinant enzymes.

One advantage of using *Bacillus* species as hosts for large scale production of heterologous enzymes and other proteins is their ability to secrete proteins directly into the fermentation medium (Simonen and Palva, 1993). However, the production of several native extracellular proteases, which tend to degrade secreted heterologous proteins, has been a major problem in using *Bacillus* species in the production of recombinant enzymes. To avoid enzyme degradation by extracellular proteases and to increase enzyme yield, enzyme manufacturers developed protease-deficient mutant strains.

Bacillus strains that are nonsporulating and extracellular protease-deficient are now routinely used as hosts for recombinant enzymes. In some instances, these strains are modified by introducing additional mutations. For example, to avoid the use of an antibiotic resistance marker for selection of cells transformed with rDNA, an auxotrophic mutant can be isolated that requires a specific amino acid (or other nutrient) for growth. The auxotrophic mutation is then complemented by providing an active copy of the mutated chromosomal gene on the transformation vector. The cells that acquire the vector attain prototrophy and regain the ability to grow in a medium without the required nutrient.

6.2. *Escherichia coli K-12*

In 1990, FDA affirmed as GRAS the chymosin enzyme preparation derived from *E. coli* K-12 (21 CFR 184.1685; Flamm, 1991). Chymosin is a milk-clotting enzyme also known as rennin. The FDA action was a milestone in the history of food-processing enzymes, because chymosin was the first recombinant enzyme regulated by the U.S. government for use in cheese and other dairy products. The chymosin production strain contains the bovine prochymosin gene. Prochymosin accumulates within the *E. coli* K-12 cells in the form of inclusion bodies. The cells are subsequently lysed and the inclusion bodies are isolated and solubilized. The prochymosin present in the solution is purified and converted to chymosin by acid treatment.

The safety of chymosin preparation was primarily based on published evidence that *E. coli* K-12 has been used as a laboratory organism for over 30 years without reported incidents of infection and that it does not produce toxins that cause illness by ingestion, such as Shiga-like toxin produced by certain toxigenic strains of *E. coli*. *E. coli* K-12 is one of the most extensively studied bacteria. Its genome was sequenced in 1997 (Blattner et al., 1997). *E. coli* K-12 has a history of safe use in the production of specialty chemicals and human drugs and was exempted from EPA review under TSCA (EPA, 1997).

6.3. *Pseudomonas fluorescens*

FDA recently reviewed a GRAS notice on the thermostable α-amylase from the recombinant *P. fluorescens*

Biovar I strain (GRN 126). Since *P. fluorescens* had no previous history of use as a source of enzymes or other compounds intended for use in food, the sponsor of the GRAS notice, Innovase, provided extensive information on the safety of *P. fluorescens*. The organism is a Gram-negative soil bacterium not known to cause disease in healthy humans. It is ubiquitous in the environment and is likely to have been consumed along with raw fruits and vegetables. The genome of *P. fluorescens* (strain Pf-5) was sequenced (Paulsen et al., 2005).

The wild-type strain MB101 that was used as a host for the recombinant α-amylase, was originally isolated from lettuce grown on a California farm. The strain has been utilized since 1989 for the large scale production of *Bacillus thuringiensis* insecticidal protein (B.t. toxin) for agricultural applications (Landry et al., 2003). Innovase characterized the MB101 strain at the genetic and phenotypic levels, and conducted studies in which mice were exposed to high oral doses of live bacteria. Innovase concluded that strain MB101 is nonpathogenic and is considered safe (Landry et al., 2003). Like *E. coli* K-12, *P. fluorescens* accumulates heterologous proteins intracellularly as inclusion bodies.

7. Fungal host strains

7.1. Aspergillus oryzae and Aspergillus niger

A. oryzae and *A. niger* are filamentous fungi well-known for their use in food production. *A. oryzae* has been used for millennia as a source of *koji* mold used in the production of fermented foods including soy sauce, soybean paste miso, and rice wine sake. *A. niger* is widely used for production of citric acid, which was affirmed by FDA as GRAS (21 CFR 184.1033). Both *A. oryzae* and *A. niger* have a long history of use as sources of enzymes used in baking, brewing, and other food applications. Several enzyme preparations derived from these microorganisms were recognized by FDA as GRAS in opinion letters issued in the early 1960s (http://www.cfsan.fda.gov/~dms/opa-enzy.html). Subsequently, a number of enzyme preparations from both natural and rDNA strains of *A. oryzae* and *A. niger* were regulated in 21 CFR either as secondary direct food additives or GRAS food ingredients, or evaluated under the proposed GRAS notification program. The *A. oryzae* genome sequence was recently determined by the Japanese *A. oryzae* genome consortium (Machida et al., 2005).

Based on the historical uses of *A. oryzae* and *A. niger* in the production of fermented foods, both organisms have traditionally been regarded as nonpathogenic and nontoxigenic, while recognizing that certain strains of both species may produce low levels of toxic secondary metabolites such as mycotoxins. In recent literature, these microorganisms continue to be described as nonpathogenic, but are rarely referred to as nontoxigenic.

The techniques for transformation of *A. niger* and *A. oryzae* with rDNA were pioneered in the late 1980s and early 1990s. Since then, both microorganisms have been successfully used as hosts for expression of heterologous enzymes. The industrial success of *A. oryzae* and *A. niger* prompted new studies and discussions on their capability to produce mycotoxins. We will summarize below recent literature on the toxigenic potential of *A. oryzae* and *A. niger* including methods of avoiding mycotoxin synthesis during industrial fermentations, and genetic modifications aimed at creating mutant strains that can no longer produce mycotoxins.

7.1.1. Safety of A. oryzae

Barbesgaard et al. (1992) reviewed information on the taxonomy, ecology, industrial use, and safety of *A. oryzae*. The authors concluded that *A. oryzae* is not pathogenic in healthy humans and described the microorganism as being a member of the *A. flavus* group that lost the ability to produce certain metabolites, e.g., aflatoxins, through thousands of years of cultivation. Several recent studies showed that certain strains of *A. oryzae*, including some strains used in the production of *koji* molds, contain structural and regulatory genes necessary for aflatoxin biosynthesis. Nevertheless, these strains appeared to be incapable of producing aflatoxins even in aflatoxin-inducing media (Kusumoto et al., 1998a; Kusumoto et al., 1998b; Watson et al., 1999; van den Broek et al., 2001). The molecular analysis of the inactive aflatoxin biosynthesis gene cluster in *A. oryzae* industrial strains revealed the presence of mutations that prevent the expression of certain genes necessary for aflatoxin production (Tominaga et al., 2006).

Certain strains of *A. oryzae* can produce low levels of mycotoxins with low-to-moderate toxicity: 3-β-nitropropionic acid, kojic acid, and cyclopiazonic acid (Barbesgaard et al., 1992; Blumenthal, 2004). Information on the toxicology and occurrence in food of these mycotoxins can be found in review articles by Burdock and Flamm (2000) and Burdock et al. (2001a,b). According to publications dating back to the 1950s and 1960s (references in Blumenthal, 2004), *A. oryzae* may also produce several other secondary metabolites including maltoryzine and violacetin. These metabolites have not been reported to be produced by the *A. oryzae* strains used as sources of enzymes.

EPA evaluated the safety of *A. oryzae* under TSCA for use as a recipient microorganism in the production of industrial compounds such as enzymes used in detergent formulations (EPA, 1997). EPA concluded that "Risks from use of the recipient microorganism *A. oryzae* are low. *A. oryzae* has a long history of commercial use. While some strains of *A. oryzae* are known to produce mycotoxins, these mycotoxins are not highly toxic to humans and their production under usual commercial conditions does not appear to pose a significant risk to human health." Based on this assessment, EPA exempted *A. oryzae* from its review.

7.1.2. A. oryzae host strains with reduced toxigenic potential

Table 1 includes several recombinant enzymes derived from *A. oryzae*. Most of these enzymes are derived from the

A. oryzae production strains that are descendants of the wild type strain IFO 4177, also known as strain A1560. Enzyme preparations derived from strain A1560 or its rDNA derivatives were tested and shown to be safe for their intended uses in food. Strain A1560 is capable of producing low levels of 3-β-nitropropionic acid, kojic acid, and cyclopiazonic acid, especially when cultivated in media conducive to the synthesis of these compounds. Strain A1560 is also known to contain nonfunctional genes related to aflatoxin synthesis (GRN 142). Strain A1560 has been recently modified by site-directed disruption of three endogenous TAKA-amylase genes, one alkaline protease gene, and one neutral metalloprotease gene. The modified strain was subsequently subjected to classical mutagenesis and selection to reduce its toxigenic potential. As a result, a mutant strain was isolated (designated BECh2) from which the aflatoxin gene cluster and the genes involved in the synthesis of cyclopiazonic acid were deleted. The strain also contains a mutation that results in reduced kojic acid production under inducing conditions. The BECh2 strain has been used as a host for expression of triacylglycerol lipase (GRN 103), glucose oxidase (GRN 106), and phospholipase A1 (GRN 142).

7.1.3. Safety of A. niger

A. niger has been used for several decades in the production of citric acid and food-processing enzymes. Citric acid derived from several sources including *A. niger* is regulated as a GRAS food ingredient (21 CFR 184.1033). FDA recognized as GRAS several enzymes from *A. niger* in opinion letters issued in the early 1960s (http://www.cfsan.fda.gov/~dms/opa-enzy.html). Carbohydrase and cellulase from *A. niger* are regulated as secondary direct food additives (21 CFR 173.120) and recombinant chymosin derived from *A. niger* var. *awamori* is regulated as a GRAS food ingredient (21 CFR 184.1685).

The safety of *A. niger* has been re-visited in several recent reviews (Schuster et al., 2002; van Dijck et al., 2003; Blumenthal, 2004). According to these reviews, a relatively small number (3%–10%) of known *A. niger* strains produce the nephrotoxic and carcinogenic mycotoxin ochratoxin A under certain fermentation conditions. Schuster et al. (2002) recommend testing *A. niger* isolates for their potential to produce ochratoxin A at the start of the development of the production process for enzymes used in food processing. Some strains of *A. niger* can also produce other secondary metabolites that are not considered to be mycotoxins, including nigragillin, nigerazine B, malformins (cyclic peptides), naphtho-γ-pyrones, and oxalic acid. The chemical nature and toxicity of these metabolites are discussed by Schuster et al. (2002) and Blumenthal (2004). Certain strains of *A. niger* produce a proteinaceous hemolysin, nigerlysin, when incubated on sheep's blood agar. Purified nigerlysin was toxic to mouse neuronal cells in culture (Donohue et al., 2006). Early reports of aflatoxin production by *A. niger* have not been confirmed by later studies (Schuster et al., 2002).

EPA exempted *A. niger* from its review under TSCA (EPA, 1997). EPA concluded that despite the widespread human exposure to *A. niger*, there are only several reports of aspergillosis with *A. niger* and that *A. niger* is not a significant human pathogen. EPA also concluded that, although *A. niger* is capable of producing several mycotoxins, mycotoxin production appears to be controlled by the conditions of fermentation.

7.1.4. A. niger host strains developed for targeted DNA integration

van Dijck et al. (2003) describe the development of *A. niger* host strains that allow targeted introduction of heterologous genes into the host genome. The intention of these modifications was to address concerns that random DNA integration may perturb metabolic pathways and increase the production of toxic metabolites. The novel host strains were developed from strain DS03043, which was derived from the ancestral *A. niger* strain NRRL3122 using classical genetic methods of mutagenesis and selection. The DS03043 strain was used as a host for expression of heterologous enzymes such as phytase and xylanase derived from other *A. niger* strains. The strain contains seven copies of the glucoamylase-encoding gene *gla*A. All seven copies of the *gla*A gene were deleted from the DS03043 strain and the resulting strain was used to construct several production strains that contain genes encoding desired enzymes integrated at the deleted loci. At each locus, several copies of the intended gene were integrated to achieve high expression of the target enzyme. All these strains were tested under conditions optimal for mycotoxin production and showed a pattern of secondary metabolites similar to that of strain DS03043 and the ancestral strain NRRL3122, which can produce secondary metabolites, such as nigragillin and naphtho-γ-pyrones, under stress conditions. The strains were also tested under conditions representative of large-scale production conditions. None of the secondary metabolites normally produced by the strains under stress conditions were detected either in broth samples or the final enzyme products.

7.1.5. General recommendations for safe use of A. oryzae and A. niger

As discussed above, certain strains of *A. oryzae* and *A. niger* used for enzyme production have the potential for producing mycotoxins or other secondary metabolites with varying degrees of toxicity. The formation of these substances usually occurs under stress conditions and can be avoided by controlling the fermentation process. Several precautionary measures have been discussed in the literature (Schuster et al., 2002; Blumenthal, 2004), which may be summarized as follows: (1) whenever possible, the production strains or host strains should be chosen or developed from strains that have a history of safe use and were examined for their ability to produce mycotoxins under industrial fermentation conditions; (2) new isolates or uncharacterized strains should be thoroughly examined

and tested for mycotoxin production capability before they are developed as production or host organisms; and (3) if a strain has the potential to produce mycotoxins, a control system should be implemented to assure that the mycotoxins do not end up in the enzyme preparation at toxicologically-significant levels; the manufacturing process should be carefully designed, operated, and monitored, and the enzyme preparation should be routinely tested for relevant mycotoxins.

7.2. *Fusarium venenatum*

In 2001, FDA reviewed a GRAS notice for the xylanase enzyme preparation derived from a recombinant strain of the filamentous fungus *F. venenatum* containing the xylanase gene from *Thermomyces lanuginosus* (GRN 54). The *F. venenatum* strain that was used as a host for expression of xylanase is a descendant of the wild-type strain A3/5. The A3/5 strain was isolated in 1968 from a soil sample in the United Kingdom. It was initially identified as *Fusarium graminearum* Schwabe and deposited in several international culture depositories including the American Type Culture Collection (Manassas, VA, USA) where the strain was designated as ATCC 20334. The A3/5 strain was subsequently reclassified as *F. venenatum* based on morphological, molecular, and mycotoxin data (O'Donnell et al., 1998; Yoder and Christianson, 1998).

F. venenatum has been used as a source of mycoprotein, a protein-rich product sold for use in food in the U.K. since 1985 under the trade name "Quorn." In recent years, mycoprotein has also been commercialized in several European countries (Wiebe, 2002) and the US. In 2001, FDA reviewed a GRAS notice submitted by the manufacturer of mycoprotein, Marlow Foods, Inc. (UK) notifying the agency that, based on the opinion of qualified experts, mycoprotein is generally recognized as safe for use in food as a meat replacer (GRN 91). Mycoprotein is currently manufactured from the *F. venenatum* strain deposited in ATCC under the Accession No. PTA-2684. The mycoprotein production strain PTA-2684 and the previously deposited strain ATCC 20334 are both derived from the original isolate A3/5.

F. venenatum is not known to be pathogenic. However, studies on secondary metabolite profiles conducted with strain A3/5 and its derivatives revealed that *F. venenatum* is capable of producing the mycotoxins, trichothecenes, culmorins, and fusarins, and a cyclic peptide enniatin B under conditions optimized for the production of these compounds (O'Donnell et al., 1998; Miller and MacKenzie, 2000; Song et al., 2004; GRN 54). Mycotoxin production by *F. venenatum* can be avoided by controlling the fermentation conditions. For example, mycoprotein is manufactured under conditions that are not conducive to mycotoxin synthesis (Johnstone, 1998). To confirm the absence of mycotoxin production, mycoprotein is regularly tested for representative trichothecenes and fusarins (GRN 91).

The *F. venenatum* strain used for production of *T. lanuginosus* xylanase was derived from strain MLY3 (GRN 54). The MLY3 strain is a spontaneous mutant of *F. venenatum* strain CC1-3 which, in turn, is a morphological mutant that arose spontaneously during mycoprotein production. Thus, the MLY3 strain is a descendant of the original strain A3/5. Although the MLY3 strain has been shown to produce a trichothecene diacetoxyscirpenol, it was modified during the construction of the xylanase production strain to delete the *tri5* gene encoding trichodiene synthase, the enzyme that catalyzes the first step in the trichothecene biosynthetic pathway (GRN 54; Royer et al., 1999). The resulting xylanase production strain LyMC4.B was tested for mycotoxin production and confirmed to have lost the capacity of producing trichothecenes. However, the strain produced low levels (μg/l) of culmorins and trace levels of enniatin B under conditions inducing the synthesis of these compounds (Miller and MacKenzie, 2000; GRN 54). Although fusarin C may also be produced by strain LyMC4.B under inducing conditions, there was no indication of its synthesis under industrial fermentation conditions used in xylanase production (GRN 54).

Culmorins are structurally related sesquiterpenes found in grains contaminated by *Fusarium graminearum* and related fungi. They are not mutagenic in the Ames test and show either no or low toxicity in other studies (Pedersen and Miller, 1999). Fusarin C has not been thoroughly studied, because it is labile upon exposure to light and heat. Published toxicological studies show that fusarin C is a mutagen but its carcinogenic potential is unclear (Gelderblom et al., 1984, 1986; Lu and Jeffrey, 1993; IARC, 1993).

Enniatin B is one of several enniatins produced by certain species of the genus *Fusarium*. Enniatins are cyclic peptides that exhibit antibacterial and insecticidal activities. Very few studies on mammalian toxicity of enniatins have been performed. The existing data indicate that the toxicity of enniatins to higher animals is low (GRN 54 and references therein).

Based on its low toxigenic potential, lack of toxin production under industrial fermentation conditions, and well-characterized inserted DNA, strain LyMC4.B was considered to be a safe source of xylanase (GRN 54). The safety of the xylanase preparation was evaluated and confirmed (Pedersen and Broadmeadow, 2000). *F. venenatum* has also been used for expression of other enzymes including serine carboxypeptidase from *A. oryzae* (Blinkovsky et al., 1999), aminopeptidase from *A. oryzae* (Blinkovsky et al., 2000), glucoamylase from *A. niger* (Gordon et al., 2001), and lactose oxidase from *Microdochium nivale* (Ahmad et al., 2004). Of all these enzymes, only xylanase and lactose oxidase were derived from the production strains that do not contain the trichodiene synthase gene.

7.3. *Kluyveromyces marxianus var.lactis*

In 1992, FDA affirmed as GRAS the chymosin enzyme preparation derived from a genetically modified strain of

yeast *K. marxianus* var. *lactis* (21 CFR 184.1685). *K. marxianus* var. *lactis* strain SL56 was used as a host for the bovine prochymosin gene because of its well-characterized fermentation properties and its ability to secrete high levels of prochymosin to the fermentation medium (van den Berg et al., 1990). Prochymosin is converted to active chymosin by pH adjustment.

K. marxianus var. *lactis* was once known as *Saccharomyces lactis* and subsequently as *Kluyveromyces lactis*. The current classification, *K. marxianus* var. *lactis*, was established in 1984 (van der Walt and Johannsen, 1984). The safety of *K. marxianus* var. *lactis* is well-documented. The organism has been used for many years as a source of lactase used for conversion of lactose to galactose and glucose in milk and milk products. The lactase enzyme preparation from *K. lactis* was affirmed by FDA as GRAS (21 CFR 184.1388) in 1984. The agency reviewed the safety of *K. marxianus* var. *lactis* in relation to the regulations for lactase and chymosin enzyme preparations and concluded that the organism is nonpathogenic and nontoxigenic and is a safe source of both enzyme preparations.

7.4. Trichoderma reesei

T. reesei is a filamentous fungus well-known as a source of enzymes that hydrolyze cellulose and hemicellulose. *T. reesei* has also emerged as a host organism for expression of heterologous enzymes. FDA has reviewed a GRAS notice on the pectin lyase preparation from a *T. reesei* strain containing the pectin lyase gene from *A. niger* var. *awamori* (GRN 34).

T. reesei was first isolated from cotton canvas in the Solomon Islands in 1944 (Kuhls et al., 1996). The original isolate, QM6a, is the parent of practically all *T. reesei* industrial production strains (Nevalainen et al., 1994). During the 1980s, *T. reesei* was considered to be identical to *Trichoderma longibrachiatum*. More recent studies suggest that *T. reesei* is the asexual form of a tropical fungus, *Hypocrea jecorina* (Kuhls et al., 1996).

In 1999, FDA affirmed as GRAS the cellulase enzyme preparation derived from a nonpathogenic and nontoxicogenic strain of *T. longibrachiatum* (now known as *T. reesei*) (21 CFR 184.1250). Cellulases from *T. reesei* have been used safely in food, animal feed, and pharmaceuticals since the 1960s (reviewed in Nevalainen et al., 1994). Major food applications of *Trichoderma* cellulases include baking, malting, and grain alcohol production. A comprehensive review of *Trichoderma* enzymes used in food and feed can be found in a review by Gallante et al. (1998). Penttilä et al. (2004) reviewed the molecular biology of *Trichoderma* and various aspects of its use as a host for production of heterologous enzymes. Watts et al. (1988) reported that a strain of *T. reesei* produced two antifungal metabolites, one of which was identified as trichodermin, a trichothecene mycotoxin. However, Blumenthal (2004) cites another opinion (van Dijck, personal communication) that among *Trichoderma* species, the only producer of trichodermin is a strain from *T. harzianum*.

8. Construction of recombinant production strains

8.1. Expression vectors

Genes encoding recombinant enzymes are typically introduced into host strains using expression vectors. An expression vector is a DNA plasmid that carries the expression cassette. Essential components of the expression cassette include a promoter, the gene encoding the desired enzyme, and a terminator. The promoter and terminator are regulatory sequences that control the transcription of the enzyme-encoding gene. Expression vectors also contain DNA derived from bacterial plasmids. Generally, well characterized, commercially-available plasmids are used to construct specific expression vectors. Most commonly used plasmids are pUB110, pUC18, and pUC19.

Plasmid pUB110 was originally isolated from *Staphylococcus aureus* (Keggins et al., 1978) and was subsequently sequenced by McKenzie et al. (1986, 1987). The plasmid replicates in *B. subtilis* and is used in the construction of expression vectors for production of enzymes in *B. subtilis* and other *Bacillus* species. The plasmid carries the *kan*^{r} (kanamycin or neomycin resistance) gene, also known as the *neo* or *nptII* gene, and the *phl* (phleomycin resistance) gene. The plasmid also carries two other genes, one encoding the primary replication initiation protein (ORF alpha) which initiates the copying of the plasmid, and the other encoding the mobilization protein (ORF beta), which enables the mobilization of the plasmid for transfer from one *Bacillus* strain to another (Selinger et al., 1990). The mobilization gene is routinely deleted during construction of the expression vectors to avoid vector instability. The *kan*^{r} and *phl* genes are useful as selectable markers during construction of the transformation vector. However, they are not always carried over to the final expression vector. Most expression vectors contain either the *kan*^{r} or the *phl* gene and some expression vectors contain neither gene. In the latter case, bacterial transformants are selected either on the basis of enzyme activity or the complementation of an auxotrophic mutation in the host strain.

The pUC18 and pUC19 plasmids were developed for cloning in *E. coli* (Yanish-Perron et al., 1985). They contain an origin of replication active in *E. coli* and the *amp*^{r} gene (also known as the *bla* gene) that confers ampicillin resistance. The pUC18 and pUC19 plasmids are used in the construction of certain bacterial and fungal expression vectors in *E. coli* using the *amp*^{r} gene as a selectable marker. Because the *amp*^{r} gene is under the control of a bacterial promoter and is not expressed in fungal hosts, fungal expression vectors must also contain a selectable marker gene suitable for selection in fungi. A commonly used selectable marker is the *amdS* gene (see Section 8.3 for more information). In recently developed fungal expression vectors the *amp*^{r} gene has been replaced with the *URA3*

gene from *Saccharomyces cerevisiae* (baker's yeast), which complements the *pyrF* mutation in the *E. coli* strain used as an intermediate host for vector construction (see Section 8.3 for more information).

There are other bacterial plasmids (described in the literature and petitions or GRAS notices submitted to FDA) that have been utilized in the construction of expression vectors for production of recombinant enzymes. In some instances, fragments derived from two or more plasmids are combined. Most expression vectors carry their own selectable marker gene. However, in some instances, two separate vectors are constructed; one carries the expression cassette, while the other carries the selectable marker gene. In such a case, the host strain is co-transformed with both vectors.

An interesting plasmid system was developed for inducible production of a thermostable α-amylase in a Gram-negative bacterium, *Pseudomonas fluorescens* (Richardson et al., 2002; GRN 126). The expression vector carries the α-amylase gene under the control of an inducible *tac* promoter. The auxiliary plasmid carries the *lacI* gene from *E. coli*. The *lacI* gene encodes the LacI repressor protein, which binds to the *tac* promoter and inhibits α-amylase expression. Only after the desired cell growth has been achieved is production of α-amylase induced by addition to the fermentation medium of the lactose analog isopropyl-thio-β-D-galactopyranoside (IPTG), which prevents binding of the LacI repressor to the *tac* promoter.

Bacterial expression vectors may be designed either for integration into the host chromosome or for extrachromosomal (autonomous) replication. Autonomously replicating bacterial expression vectors contain origins of replication compatible with the host bacterium and replicate at multiple copies per cell to assure high production of the target enzyme. Expression vectors used for enzyme production in yeasts and filamentous fungi are usually designed for integration into the host genome. Most frequently, the complete expression vector is integrated into the fungal host genome. Alternatively, the expression vector is cut with a restriction enzyme and a vector fragment containing the expression cassette and a selectable marker gene is transformed into the host.

In recent years, techniques for integration of vector DNA at multiple loci within the host genome have been developed. In such a case, the DNA intended for integration contains DNA sequences homologous to the DNA sequences of a host gene. Depending on the specific host/vector combination, vector DNA that carries either both the expression cassette and a selectable marker gene or only the expression cassette is used for transformation. The expression cassette may be directed into predetermined loci in the host genome and may either replace a dispensable host gene or integrate in the vicinity of the host gene without affecting its function. An example of targeted integration at several loci is the construction of a *B. licheniformis* strain for production of the thermostable α-amylase. Three copies of the gene encoding thermostable α-amylase flanked with appropriate DNA sequences homologous to the host DNA were introduced into the host strain by homologous recombination at the *amyL* (α-amylase), *xyl* (xylose isomerase), and *gnt* (gluconate permease) loci. The introduced DNA sequences contained only the α-amylase gene and *B. licheniformis* chromosomal DNA sequences (GRN 79).

8.2. Expression cassettes

The expression cassette is the essential genetic element that must be present in the production microorganism. The simplest expression cassette carries the gene of interest placed under the control of regulatory sequences, the promoter and terminator. Heterologous genes are usually placed under the control of promoters and terminators derived from genes native to the host microorganism or related species. For example, bacterial genes encoding enzymes expressed in Bacilli are usually placed under the control of regulatory sequences derived from *Bacillus* species. Likewise, fungal genes are placed under the control of fungal promoters and terminators derived either from the host species or closely related species.

Promoter strength is essential for achieving efficient expression of the target enzyme. Examples of strong promoters used in *Bacillus* species include promoters of the *amyL* (*B. licheniformis* α-amylase) gene and *amyM* (*B. stearothermophilus* maltogenic amylase) gene (GRN 79; GRASP 7G0326). Heterologous genes expressed in *A. oryzae* are frequently placed under the control of the TAKA amylase promoter from *A. oryzae* (Christensen et al., 1988; GRN 34; GRN 43; GRN 122). Increasing knowledge about promoter function has recently led to the development of improved promoters by introducing mutations into promoter sequences, or by fusing sequences derived from two or more promoters and creating tandem promoters or hybrid promoters. An example of a modified promoter is the Pna2/TPA promoter used for expression of several enzymes in *A. oryzae* and *A. niger* (GRNs 75, 103, 106, and 158). The Pna2/TPI promoter is the neutral amylase II promoter from *A. niger*, in which the 5′ nontranslated part has been replaced with the corresponding part of the *A. nidulans* triose phosphate isomerase (TPI) promoter. An even more complex promoter was developed for expression of phospholipase A1 in *A. oryzae* (GRN 142). It consists of a modified Pna2 promoter from which the TATA box has been removed, and the Pna2/TPI promoter described above. In some instances, it is advantageous to use inducible promoters that are activated by the addition of an inducer to the fermentation medium. An example of an inducible promoter that controls the expression of the thermostable α-amylase gene in *P. fluorescens* is described in Section 8.1.

8.3. Transformation and identification of transformed cells

The technique used to transfer vector DNA into host cells depends on the properties of the host and the nature of

the expression vector. For enzyme production, bacterial hosts have been transformed with vector DNA using conjugation (cell-to-cell contact, in which protein pili on cell surfaces mediate direct transfer of DNA), electroporation, DNA uptake by competent cells (known as bacterial transformation), or vector incubation with protoplasts, i.e., cells from which cell walls were chemically removed. Yeasts and filamentous fungi are usually transformed by incubating DNA with protoplasts.

As discussed in Section 8.1, most expression vectors used in enzyme production contain one or more genes that enable selection of transformed cells. Selection of bacterial transformants is usually conducted using either antibiotic resistance selectable marker genes or by complementing a chromosomal auxotrophic mutation with the functional gene provided on the expression vector. In some instances, antibiotic resistance markers are deleted in the final construction steps of bacterial expression vectors. In such cases, selection is conducted by screening microbial colonies for enzyme activity (GRASP 4G0293). Positive selection methods are used when a gene function is restored as a result of DNA integration. For example, the α-amylase activity is restored in the α-amylase-deficient host strain transformed with a recombinant α-amylase gene (see, for example, GRN 79).

Most expression vectors used to-date for production of enzymes in Bacilli contain the *kan*^{r} gene which encodes an enzyme, aminoglycoside 3′-phosphotransferase II, known either as APH(3′)II or NPTII. APH(3′)II catalyzes the phosphorylation of kanamycin or neomycin thereby inactivating these antibiotics. APH(3′)II has often been used as a selectable marker in the development of bioengineered plants. The safety of the APH(3′)II protein has been discussed in numerous publications including a recent review by Goldstein et al. (2005). FDA issued a regulation for use of the APH(3′)II protein in the development of genetically modified cotton, oilseed rape, and tomatoes (21 CFR 173. 170).

Other antibiotic resistance markers used in enzyme production include the *amp*^{r} gene and the *tet* gene. The *amp*^{r} gene was used for selection of transformants during construction of the *E. coli* K-12 strain for production of bovine chymosin affirmed by FDA as GRAS (21 CFR 184.1685). The *amp*^{r} gene encodes β-lactamase, which catalyzes the hydrolysis of penicillin antibiotics including ampicillin. The *tet* (tetracycline resistance) and *kan*^{r} genes were used as selectable markers in the construction of the *P. fluorescens* strain for production of the thermostable α-amylase (GRN 126). The *tet* gene confers resistance to tetracycline and encodes a membrane-bound protein that pumps tetracycline out of the microbial cell. Antibiotic resistance genes, including those used as selectable markers in the production of enzymes, are widely spread among bacteria.

Methods of manufacturing enzymes include steps to prevent carryover of intact copies of antibiotic resistance genes to the final enzyme products. For example, enzymes secreted to the fermentation broth are separated from microbial cells. Enzymes that accumulate within bacterial cells as inclusion bodies (e.g., chymosin expressed in *E. coli* or α-amylase expressed in *P. fluorescens*) are isolated from the microbial mass and processed to hydrolyze any DNA that may have been released from the production strain (GRASP 8G0337; GRN 126). Enzyme manufacturers often test enzyme preparations for the presence of antibiotic resistance genes using either a DNA transformation assay or assessing the size of the DNA fragments. The results of these tests were provided in submissions to FDA and showed that all tested enzyme preparations contained neither transformable DNA nor full-size copies of the antibiotic resistance genes.

Several selectable markers suitable for use in filamentous fungi are described in the literature (see, for example, Howe, 1995). The selection system based on the *amdS* gene isolated from *Aspergillus nidulans* is favored by enzyme manufacturers because fungi commonly used as hosts, such as *A. niger* and *A. oryzae*, lack an endogenous *amdS* gene. The *amdS* gene encodes acetamidase, an enzyme that catalyzes the hydrolysis of acetamide to acetic acid and ammonia, which the fungus uses as carbon and nitrogen sources, respectively. Consequently, the fungal cells transformed with an expression vector containing the *amdS* gene can grow on acetamide as a sole nitrogen source. The *amdS* gene was initially shown to function as a selectable marker in *A. niger* (Kelly and Hynes, 1985) and subsequently in *A. oryzae* (Christensen et al., 1988). More recently, the *amdS*-based selection system has also been developed for *F. venenatum* (Royer et al., 1995).

Fungal expression vectors often contain the *amp*^{r} gene used for selection in *E. coli*. This marker gene is under the control of a bacterial promoter and is, therefore, not expressed in fungal production strains. As noted in Section 8.1, new fungal expression vectors have been developed without the *amp*^{r} gene (GRN 103; GRN 142; GRN 158). These vectors contain the *URA3* gene from *S. cerevisiae* for selection in *E. coli* and the *amdS* gene for selection in fungi. The *URA3* gene encodes orotidine 5′-monophosphate decarboxylase, the enzyme that catalyzes the last step in the pyrimidine biosynthetic pathway. The *URA3* gene complements the *pyrF* mutation in the *E. coli* strain used as an intermediate host for the construction of the expression vector. The *pyrF* mutant requires uridine for growth. This requirement is alleviated in the *E. coli* transformants carrying the *URA3* gene provided on the expression vector. The *URA3*-based selection system allows the construction of fungal production strains without the use of antibiotic resistance genes.

9. Sources of recombinant enzymes

Recombinant enzymes can be derived from a variety of sources including microorganisms, plants or animal tissue. They are often identical to well-known enzymes with a long history of use in food. For example, chymosin derived from recombinant strains of *E. coli* K-12, *K. marxianus* var.

lactis, and *A. niger* var. *awamori* is identical to that present in animal-derived rennet (21 CFR 184.1685).

Most recombinant enzymes currently used in food are derived from well-characterized culturable microorganisms. However, the development of modern highly-efficient screening techniques facilitated discovery of new enzymes from microorganisms sampled from the environment. In this approach, DNA is isolated directly from the environmental samples and used to create expression libraries in *E. coli* or other suitable expression hosts. The expression libraries are then screened to identify enzymes with desired characteristics (Short, 1997). Modern PCR techniques are used to limit the DNA intended for introduction into the host organism to the sequence encoding the desired enzyme. This approach precludes the transfer of any extraneous or unidentified DNA from the donor organism(s) to the production strain.

Thermophilic enzymes with optimized properties are important in baking and starch processing. Several genes encoding such enzymes, including thermostable α-amylases and xylanases, have been isolated from thermophillic microorganisms and expressed in heterologous production strains. Enzyme properties may also be adapted to specific use conditions by using modern genetic techniques. Site-specific mutagenesis can be used to introduce specific changes in the amino acid sequence of the enzyme. Site-specific mutagenesis is most effective when the three-dimensional structure of the enzyme is known and the relationships between structure and enzyme properties have been elucidated.

In recent years, a powerful approach for improving enzyme properties known as molecular or directed evolution has been developed. The process of molecular evolution consists of several steps, often performed in an iterative manner. In the first step, one or several "parent" genes are chosen. If several genes are used, they are often derived from diversified sources to provide sequence diversity. These genes are subsequently mutagenized in a random manner using techniques such as an error-prone PCR mutagenesis, sequential random mutagenesis, or gene shuffling to create a large number of gene variants. A library of altered genes is then constructed in a suitable host microorganism. The clones are screened using high-throughput methods to identify those expressing improved enzymes. Genes encoding these enzymes are isolated, sequenced and usually recycled through the process until an enzyme with the desired characteristics is identified. Extensive information on directed enzyme evolution is available in published sources such as at F. Arnold's web page (http://www.cheme.caltech.edu/groups/fha/) or in recent reviews, for example, Kirk et al. (2002), van Beilen and Li (2002), Roodveldt et al. (2005), and Yuan et al. (2005).

FDA reviewed several GRAS notices on enzymes improved by using either one or a combination of modern genetic techniques. For example, the α-amylase from *B. licheniformis* present in the mixed carbohydrase and protease enzyme preparation affirmed as GRAS in 1983 (21 CFR 184.1027), was modified by replacing its amino terminus with the corresponding sequence from a *B. amyloliquefaciens* α-amylase and by introducing five additional amino acid substitutions. The modified enzyme is thermostable, active at low pH, and does not require added calcium when used for starch hydrolysis in the production of high fructose corn syrup and other similar sweeteners. The enzyme is produced from a recombinant strain of *B. licheniformis* (GRN 22). Another α-amylase with similar characteristics was developed using molecular evolution from three α-amylases discovered in nature. The microorganisms that produce these α-amylases were identified as belonging to the order Thermococcales within Archaea (previously known as Archaebacteria). The hybrid α-amylase is produced from *P. fluorescens* Biovar I and is intended for use in starch processing and fermentation of ethanol for alcoholic beverages (GRN 126; Richardson et al., 2002; Landry et al., 2003) (see Section 6.3).

10. Fine tuning of the production strain

Recombinant production strains can be further improved using classical mutagenesis. Fungal expression vectors may integrate into the host genome at different loci and various copy numbers. Consequently, the transformation procedure yields a population of transformants that produce different levels of the intended enzyme. These transformants are subsequently grown under different conditions and assessed for enzyme expression and other characteristics. Once a satisfactory transformant is identified, it can be subjected to mutagenesis using either a chemical mutagen or UV or ionizing radiation. Subsequently, the population of mutants is screened for enzyme yield to identify the best performer (Novozymes, 2005). For example, the production of laccase from *Myceliophthora thermophila* expressed in *A. oryzae* was increased by chemical mutagenesis and selection (GRN 122; WHO, 2004).

11. Fermentation and processing

Microbial enzymes, whether native or recombinant, are manufactured by controlled fermentation of the production strains. In most instances, the fermentation is conducted as a batch process in large-scale aerated fermenters under strictly controlled fermentation parameters such as temperature, pH, and aeration. The culture is periodically tested to assure the absence of microbial contaminants. Fermentation media contain nutrients and compounds that facilitate the fermentation process. Commonly used media components include dextrose, corn steep liquor, starch, soybean meal, yeast extract, ammonia, urea, and minerals such as phosphates, chlorides or carbonates. Other components may include antifoaming agents and acid or alkali for pH adjustment. For optimal production, the composition of the fermentation medium must satisfy the nutritional requirements of the production strain.

Most recombinant enzymes manufactured today are secreted to the fermentation medium. After the fermentation has been completed, the fermentation broth is separated from the cellular debris using flocculation and filtration. The enzyme is subsequently concentrated by ultrafiltration or a combination of ultrafiltration and evaporation. Enzymes that accumulate within cells are isolated from the cellular mass, solubilized, and concentrated. The enzyme concentrate is then sterilized by germ filtration and formulated with compounds such as sucrose, maltose, maltodextrin, potassium sorbate, or sodium benzoate. For certain food applications, enzymes may also be formulated as granulates or tablets or immobilized using solid support materials. The final product is commonly referred to as an enzyme preparation. Enzyme preparations may contain, in addition to the formulating aids and the enzyme of interest, metabolites derived from the production microorganism or compounds used in fermentation and processing.

12. Assessment of the enzyme preparation

Enzymes are used in food processing at very low levels. Often, they are either not carried over to food as consumed or are inactivated during cooking or baking. Exposure to enzyme preparations used in food processing is typically calculated on the basis of total organic solids (TOS). TOS includes the enzyme itself as well as other organic material that originated from the production organism and enzyme processing. Enzyme preparations are tested according to generally accepted procedures discussed in several publications and guidance documents (IFBC, 1990; SCF, 1992; Pariza and Johnson, 2001). The test material is usually the concentrated enzyme before formulation. The final formulated enzyme product is assessed for compliance with specifications established for enzyme preparations by the Food Chemicals Codex (FCC, 2004) and JECFA (2001).

Some enzyme preparations derived from recombinant microorganisms were tested for the presence of transformable DNA (i.e., DNA that can be taken up by competent bacteria) encoding antibiotic resistance markers. So far, no such DNA was detected in enzyme preparations described in dossiers submitted to FDA. The number of enzyme preparations derived from recombinant strains that do not contain antibiotic resistance markers is increasing. Such enzyme preparations are not expected to be tested for transformable DNA encoding antibiotic resistance genes.

13. Conclusions

Enzymes found in nature have been used in the production of fermented foods for millennia. The production of enzyme preparations isolated from natural sources dates back to the late 19th century. The developments in molecular genetics and cell biology in the last four decades have reshaped enzyme production. It became possible to clone genes encoding enzymes and express them in host microorganisms that are well-adapted to large-scale industrial fermentation. Enzyme yield could be substantially increased by using efficient promoters and introducing multiple copies of the enzyme-encoding gene. It also became possible to tailor enzyme properties to food-processing conditions such as temperature or pH. This has been accomplished by modifying the amino acid sequence of the enzyme using either rational design or molecular evolution. As examples, we described α-amylases that were modified for increased thermostability to match the conditions of starch hydrolysis during production of sweeteners from corn.

The safety of enzyme production strains continues to be a focus of attention. The concept of developing safe strain lineages using well-characterized nonpathogenic, nontoxigenic microbial strains, particularly those with a history of safe use in food enzyme manufacture (Pariza and Johnson, 2001) has been embraced by the enzyme industry. Industrial strains of microorganisms used as sources of native enzymes are now often used as hosts for heterologous enzymes.

It has been recognized that certain fungi traditionally used as sources of food-processing enzymes and considered to be nonpathogenic and nontoxigenic, for example, *A. oryzae* or *A. niger*, are capable of producing low levels of certain mycotoxins under fermentation conditions conducive to the synthesis of these compounds. Mycotoxin production by these fungi can be minimized or avoided by controlling the fermentation conditions used in the production of enzymes. Advances in genetics and molecular biology enriched the knowledge about these microorganisms and provided new tools for reducing their toxigenic potential. Some fungal host strains described above have been altered using classical mutagenesis and/or rDNA methods to inactivate or impair their mycotoxin synthetic pathways. Most effective is the inactivation of the entire pathway by deletion or disruption of genes encoding key enzymes involved in mycotoxin synthesis. For example, the *F. venenatum* strain, from which the trichodiene synthase gene was deleted, lost the capacity to produce trichothecene mycotoxins.

Another interesting approach relies on avoiding potential unintended effects of DNA insertion, such as an increase in the levels of secondary metabolites, by targeting the cloned genes into designated chromosomal loci in the host genome. Once it has been shown that the DNA insertion does not affect secondary metabolite pathways, it is assumed that other cloned genes can be safely inserted into the same locus without triggering unintended effects. This strategy is useful for host strains that naturally produce secondary metabolites at toxicologically insignificant levels.

Microbial host strains have also been genetically modified to improve enzyme production. For example, several bacterial and fungal species used as sources of food-processing enzymes naturally produce extracellular proteases that may degrade the target enzyme. Over the years, classical mutagenesis has been effectively employed to generate protease-deficient mutants of these microorganisms. However, upon the identification of genes encoding proteases, it

became possible to generate strains deficient in specific proteases.

Current strain improvement strategies have already contributed to creating more efficient and safer enzyme production strains. This trend will undoubtedly continue as the knowledge about the genetic make-up of microorganisms used for enzyme production expands and new genetic techniques emerge.

Acknowledgment

The authors thank Drs. A. Mattia and R. Martin for their support and helpful comments.

References

Ahmad, S.K., Brinch, D.S., Friis, E.P., Pedersen, P.B., 2004. Toxicological studies on lactose oxidase from *Microdochium nivale* expressed in *Fusarium venenatum*. Regul. Toxicol. Pharmacol. 39, 256–270.

Andersen, J.R., Diderichsen, B.K., Hjortkjaer, R.K., de Boer, A.S., Bootman, J., West, H., Ashby, R., 1987. Determining the safety of maltogenic amylase produced by rDNA technology. J. Food Prot. 50, 521–526.

Barbesgaard, P., Heldt-Hansen, H.P., Diderichsen, B., 1992. On the safety of *Aspergillus oryzae*: a review. Appl. Microbiol. Biotechnol. 36, 569–572.

van Beilen, J.B., Li, Z., 2002. Enzyme technology: an overview. Curr. Opin. Biotechnol. 13, 338–344.

van den Berg, J.A., van der Laken, K.J., van Ooyen, A.J.J., Renniers, T.C.H.M., Rietveld, K., Schaap, A., Brake, A.J., Bishop, R.J., Schultz, K., Moyer, D., Richman, M., Shuster, J.R., 1990. *Kluyveromyces* as a host for heterologous gene expression: expression and secretion of prochymosin. Bio/Technology 8, 135–139.

Blattner, F.R. et al., 1997. The complete genome sequence of *Escherichia coli* K-12. Science 277, 1453–1462.

Blinkovsky, A.M., Byun, T., Brown, K.M., Golightly, E.J., 1999. Purification, characterization, and heterologous expression in *Fusarium venenatum* of a novel serine carboxypeptidase from *Aspergillus oryzae*. Appl. Environ. Microbiol. 65, 3298–3303.

Blinkovsky, A.M., Byun, T., Brown, K.M., Golightly, E.J., Klotz, A.V., 2000. A non-specific aminopeptidase from *Aspergillus*. Biochim. Biophys. Acta 1480, 171–181.

Blumenthal, C.Z., 2004. Production of toxic metabolites in *Aspergillus niger*, *Aspergillus oryzae*, and *Trichoderma reesei*: justification of mycotoxin testing in food grade enzyme preparations derived from the three fungi. Regul. Toxicol. Pharmacol. 39, 214–228.

de Boer, A.S., Diderichsen, B., 1991. On the safety of *Bacillus subtilis* and *B. amyloliquefaciens*: a review. Appl. Microbiol. Biotechnol. 36, 1–4.

de Boer, A.S., Marshall, R., Broadmeadow, A., Hazelden, K., 1993. Toxicological evaluation of acetolactate decarboxylase. J. Food Prot. 56, 510–517.

de Boer, A.S., Priest, F., Diderichsen, B., 1994. On the industrial use of *Bacillus licheniformis*: a review. Appl. Microbiol. Biotechnol. 40, 595–598.

van den Broek, P., Pittet, A., Hajjaj, H., 2001. Aflatoxin genes and the aflatoxigenic potential of Koji moulds. Appl. Microbiol. Biotechnol. 57, 192–199.

Burdock, G.A., Flamm, W.G., 2000. Review article: safety assessment of the mycotoxin cyclopiazonic acid. Int. J. Toxicol. 19, 195–218.

Burdock, G.A., Soni, M.G., Carabin, I.G., 2001a. Evaluation of health aspects of kojic acid in food. Regul. Toxicol. Pharmacol. 33, 80–101.

Burdock, G.A., Carabin, I.G., Soni, M.G., 2001b. Safety assessment of β-nitropropionic acid: a monograph in support of an acceptable daily intake in humans. Food Chem. 75, 1–27.

Christensen, T., Woeldike, H., Boel, E., Mortensen, S.B., Hjortshoej, K., Thim, L., Hansen, M.T., 1988. High level expression of recombinant genes in *Aspergillus oryzae*. Bio/Technology 6, 1419–1422.

van Dijck, P.W.M., Selten, G.C.M., Hempenius, R.A., 2003. On the safety of a new generation of DSM *Aspergillus niger* enzyme production strains. Regul. Toxicol. Pharmacol. 38, 27–35.

Donohue, M., Wei, W., Wu, J., Zawia, N.H., Hud, N., De Jesus, V., Schmechel, D., Hettick, J.M., Beezhold, D.H., Vesper, S., 2006. Characterization of nigerlysin©, hemolysin produced by *Aspergillus niger*, and effect on mouse neuronal cells in vitro. Toxicology 219, 150–155.

Environmental Protection Agency (EPA), 1997. Biotechnology Program Under Toxic Substances Control Act (TSCA). 1996 Final Microbial Assessments (<http://www.epa.gov/biotech_rule/rulesupc.htm>).

Food Chemicals Codex (FCC), 2004. Institute of Medicine of the National Academies. fifth edition. The National Academies Press, Washington, D.C.

Flamm, E.L., 1991. How FDA approved chymosin: a case history. Bio/Technology 9, 349–351.

Gallante, Y.M., De Conti, A., Monteverdi, R., 1998. Application of *Trichoderma* enzymes in the food and feed industries. In: Harman, G., Kubicek, C.P. (Eds.), *Trichoderma & Gliocladium*, Enzymes, Biological Control and Commercial Applications, Vol. 2. Taylor and Francis, London, pp. 327–342.

Gaynor, P., 2006. How U.S. FDA's GRAS notification program works. Food Safety Magazine 11, 16–19.

Gelderblom, W.C.A., Thiel, P.G., Marasas, W.F.O., van der Merwe, K.J., 1984. Natural occurrence of fusarin C, a mutagen produced by *Fusarium moniliforme*, in corn. J. Agric. Food Chem. 32, 1064–1067.

Gelderblom, W.C.A., Thiel, P.G., Jaskiewicz, K., Marasas, W.F., 1986. Investigations on the carcinogenicity of fusarin C—a mutagenic metabolite of *Fusarium moniliforme*. Carcinogenesis 7, 1899–1901.

Goldstein, D.A., Tinland, B., Gilbertson, L.A., Staub, J.M., Bannon, G.A., Goodman, R.E., McCoy, R.L., Silvanovich, A., 2005. Human safety and genetically modified plants: a review of antibiotic resistance markers and future transformation selection technologies. J. Appl. Microbiol. 99, 7–23.

Gordon, C., Thomas, S., Griffen, A., Robson, G.D., Trinci, A.P.J., Wiebe, M.G., 2001. Combined use of growth rate correlated and growth rate independent promoters for recombinant glucoamylase production in *Fusarium venenatum*. FEMS Microbiol. Lett. 194, 229–234.

Harwood, C.R., 1992. *Bacillus subtilis* and its relatives: molecular biological and industrial workhorses. Trends Biotechnol. 10, 247–256.

Howe, C., 1995. Gene Cloning and Manipulation. Cambridge University Press, Cambridge, UK.

International Agency of Research on Cancer (IARC), 1993. Toxins derived from *Fusarium moniliforme*: fumonisins B1 and B2 and fusarin C. IARC Monogr. Eval. Carcinog. Risks Hum. 56, 445 (<http://www-cie.iarc.fr/htdocs/monographs/vol56/11-monil.htm>).

International Food Biotechnology Council (IFBC), 1990. Safety evaluation of foods and food ingredients derived from microorganisms. In: Biotechnologies and Food: Assuring the Safety of Foods Produced by Genetic Modification. Regul. Toxicol. Pharmacol. 12, S114–S128.

Johnstone, L., 1998. Letter to the editor. Fungal Genet. Biol. 25, 75.

Joint FAO/WHO Expert Committee on Food Additives (JECFA), 2001. General Specifications and Considerations for Enzyme Preparations Used in Food Processing. 57th session. Compendium of Food Additive Specifications. FAO Food and Nutrition Paper 52 (Addendum 9).

Jonas, D.A., Antignac, E., Antoine, J.-M., Classen, H.-G., Huggett, A., Knudsen, I., Mahler, J., Ockhuizen, T., Smith, M., Teuber, M., Walker, R., de Vogel, P., 1996. The safety assessment of novel foods. Guidelines prepared by ILSI Europe Novel Food Task Force. Food Chem. Toxicol. 34, 931–940.

Keggins, K.M., Lovett, P.S., Duvall, E.J., 1978. Molegular cloning of genetically active fragments of *Bacillus* DNA in *Bacillus subtilis* and properties of the vector plasmid pUB110. Proc. Natl. Acad. Sci. USA 75, 1423–1427.

Kelly, J.M., Hynes, M.J., 1985. Transformation of *Aspergillus niger* by the *amdS* gene of *Aspergillus nidulans*. EMBO J. 4, 475–479.

Kirk, O., Borchert, T.V., Fuglsang, C.C., 2002. Industrial enzyme applications. Curr. Opin. Biotechnol. 13, 345–351.

Kuhls, K., Lieckfeldt, E., Samuels, G.J., Kovacs, W., Meyer, W., Petrini, O., Gams, W., Börner, T., Kubicek, C.P., 1996. Molecular evidence that the

asexual industrial fungus *Trichoderma reesei* is a clonal derivative of the ascomycete *Hypocrea jecorina*. Proc. Natl. Acad. Sci. USA 93, 7755–7760.

Kunst, F., Ogasawara, N., Moszer, I., Albertini, A.M., Alloni, G., Azevdo, V., et al., 1997. The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. Nature 390, 249–256.

Kusumoto, K.-I., Yabe, K., Nogata, Y., Ohta, H., 1998a. *Aspergillus oryzae* with and without a homolog of aflatoxin biosynthetic gene *ver-1*. Appl. Microbiol. Biotechnol. 50, 98–104.

Kusumoto, K.-I., Yabe, K., Nogata, Y., Ohta, H., 1998b. Transcript of a homolog of *aflR*, a regulatory gene for aflatoxin synthesis in *Aspergillus parasiticus*, was not detected in *Aspergillus oryzae* strains. FEMS Microbiol. Lett. 169, 303–307.

Landry, T.D., Chew, L., Davis, J.W., Frawley, N., Foley, H.H., Stelman, S.J., Thomas, J., Wolt, J., Hanselman, D.S., 2003. Safety evaluation of an α-amylase enzyme preparation derived from the archaeal order *Thermococcales* as expressed in *Pseudomonas fluorescens* biovar I. Regul. Toxicol. Pharmacol. 37, 149–168.

Lu, F-X., Jeffrey, A.M., 1993. Isolation, structural identification, and characterization of a mutagen from *Fusarium moniliforme*. Chem. Res. Toxicol. 6, 91–96.

Machida, M. et al., 2005. Genome sequencing and analysis of *Aspergillus oryzae*. Nature 438, 1157–1161.

MacKenzie, K.M., Petsel, S.R.W., Weltman, R.H., Zeman, N.W., 1989. Subchronic toxicity studies in dogs and *in utero* rats fed diets containing *Bacillus stearothermophilus* α-amylase from a natural or recombinant DNA host. Food Chem.Toxicol. 27, 599–606.

McKenzie, T., Hoshino, T., Tanaka, T., Sueoka, N., 1986. The nucleotide sequence of pUB110: some salient features in relation to replication and its regulation. Plasmid 15, 93–103.

McKenzie, T., Hoshino, T., Tanaka, T., Sueoka, N., 1987. Correction. A revision of the nucleotide sequence and functional map of pUB110. Plasmid 17, 83–85.

Miller, J.D., MacKenzie, S., 2000. Secondary metabolites of *Fusarium venenatum* strains with deletions in the *Tri5* gene encoding trichodiene synthetase. Mycologia 92, 764–771.

Nazina, T.N., Tourova, T.P., Poltaraus, A.B., Novikova, E.V., Grigoryan, A.A., Ivanova, A.E., Lysenko, A.M., Petrunyaka, V.V., Osipov, G.A., Belyaev, S.S., Ivanov, M.V., 2001. Taxonomic study of aerobic thermophilic bacilli: descriptions of *Geobacillus subterraneus* gen. nov., sp. nov. and *Geobacillus uzenensis* sp. nov. from petroleum reservoirs and transfer of *Bacillus stearothermophilus*, *Bacillus thermocatenulatus*, *Bacillus thermoleovorans*, *Bacillus kaustophilus*, *Bacillus thermoglucosidasius* and *Bacillus thermodenitrificans* to *Geobacillus* as the new combinations *G. stearothermophilus*, *G. thermocatenulatus*, *G. thermoleovorans*, *G. kaustophilus*, *G. thermoglucosidasius* and *G. thermodenitrificans*. Int. J. Syst. Evol. Microbiol. 51, 433–446.

Nevalainen, H., Suominen, P., Taimisto, K., 1994. On the safety of *Trichoderma reesei*. J. Biotechnol. 37, 193–200.

Nielsen, P.H., Malmos, H., Damhus, T., Diderichsen, B., Nielsen, H.K., Simonsen, M., Schiff, H.E., Oestergaard, A., Olsen, H.S., Eigtved, P., Nielsen, T.K., 1994. Enzyme applications (industrial). In: fourth ed.. Kirk-Othmer Encyclopedia of Chemical Technology, Vol. 9. John Wiley & Sons, Inc, New York, pp. 567–620.

Novozymes, 2005. <http://www.novozymes.com/cgi-bin/bvisapi.dll/portal.jsp> Search term: "recombinant expression".

O'Donnell, K., Cigelnik, E., Casper, H.H., 1998. Molecular phylogenetic, morphological, and mycotoxin data support reidentification of the Quorn mycoprotein fungus as *Fusarium venenatum*. Fungal Genet. Biol. 23, 57–67.

Pariza, M.W., Foster, E.M., 1983. Determining the safety of enzymes used in food processing. J. Food Protect. 46, 453–468. see also p. 752.

Pariza, M.W., Johnson, E.A., 2001. Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. Regul.Toxicol. Pharmacol. 33, 173–186.

Paulsen, I.T. et al., 2005. Complete genome sequence of the plant commensal *Pseudomonas fluorescence* Pf-5. Nat. Biotechnol. 23, 873–878.

Pedersen, P.B., Bjørnvad, M.E., Rasmussen, M.D., Petersen, J.N., 2002. Cytotoxic potential of industrial strains of *Bacillus* sp. Regul. Toxicol. Pharmacol. 36, 155–161.

Pedersen, P.B., Broadmeadow, A., 2000. Toxicological studies on *Thermomyces lanuginosus* xylanase expressed by *Fusarium venenatum*, intended for use in food. Food Addit. Contam. 17, 739–747.

Pedersen, P.B., Miller, J.D., 1999. The fungal metabolite culmorin and related compounds. Nat. Toxins 7, 305–309.

Penttilä, M., Limón, C., Nevalainen, H., 2004. Molecular biology of *Trichoderma* and biotechnological applications. In: Arora, D.K. (Ed.), Handbook of Fungal Biotechnology. Second edition, revised and expanded. Marcel Dekker, Basel, New York, pp. 413–427.

Ray, M.W. et al., 2004. Complete genome sequence of the industrial bacterium *Bacillus licheniformis* and comparisons with closely related *Bacillus* species. Genome Biol. 5, R77.1–R77.12.

Richardson, T.H., Tan, X., Frey, G., Callen, W., Cabell, M., Lam, D., Macomber, J., Short, J.M., Robertson, D.E., Miller, C., 2002. A novel, high performance enzyme for starch liquefaction. Discovery and optimization of a low pH, thermostable α-amylase. J. Biol. Chem. 277, 26501–26507.

Roodveldt, C., Aharoni, A., Tawfik, D.S., 2005. Directed evolution of proteins for heterologous expression and stability. Curr. Opin. Struct. Biol. 15, 50–56.

Royer, J.C., Moyer, D.L., Reiwitch, S.G., Madden, M.S., Jensen, E.B., Brown, S.H., Yonker, C.C., Johnstone, J.A., Golightly, E.J., Yoder, W.T., Shuster, J.R., 1995. *Fusarium graminearum* A 3/5 as a novel host for heterologous protein production. Bio/Technology 13, 1479–1483.

Royer, J.C., Christianson, L.M., Yoder, W.T., Gambetta, G.A., Klotz, A.V., Morris, C.L., Brody, H., Otani, S., 1999. Deletion of the trichodiene synthase gene of *Fusarium venenatum*: two systems for repeated gene deletions. Fungal Genet. Biol. 28, 68–78.

Schallmey, M., Singh, A., Ward, O.P., 2004. Developments in the use of *Bacillus* species for industrial production. Can. J. Microbiol. 50, 1–17.

Schuster, E., Dunn-Coleman, N., Frisvad, J.C., van Dijck, P.W.M., 2002. On the safety of *Aspergillus niger*—a review. Appl. Microbiol. Biotechnol. 59, 426–435.

Scientific Committee for Food (SCF), 1992. Guidelines for the Presentation of Data on Food Enzymes, 27th series, Ref. No. EUR 14181 EN, pp. 13–22. (<http:/europa.eu.int/comm/food/fs/sc/scf/reports_en.html>).

Selinger, L.B., McGregor, N.F., Khachatourians, G.G., Hynes, M.F., 1990. Mobilization of closely related plasmids pUB110 and pBC16 by *Bacillus* plasmid pXO503 requires *trans*-acting open reading frame β. J. Bacteriol. 172, 3290–3297.

Short, J.M., 1997. Recombinant approaches for accessing biodiversity. Nat. Biotechnol. 15, 1322–1323.

Simonen, M., Palva, I., 1993. Protein secretion in *Bacillus* species. Microbiol. Rev. 57, 109–137.

Song, Z., Cox, R.J., Lazarus, C.M., Simpson, T.J., 2004. Fusarin C biosynthesis in *Fusarium moniliforme* and *Fusarium venenatum*. ChemBioChem. 5, 1196–1203.

Tominaga, M., Lee, Y.-H., Hayashi, R., Suzuki, Y., Yamada, O., Sakamoto, K., Gotoh, K., Akita, O., 2006. Molecular analysis of an inactive aflatoxin biosynthesis gene cluster in *Aspergillus oryzae* RIB strains. Appl. Environ. Microbiol. 72, 484–490.

Veith, B. et al., 2004. The complete genome sequence of *Bacillus licheniformis* DSM13, an organism with great industrial potential. J. Mol. Microbiol. Biotechnol. 7, 204–211.

van der Walt, J.P., Johannsen, E., 1984. Genus 13. *Kluyveromyces* van der Walt emend. van der Walt. In: Kreger-van Rij, N.J.W. (Ed.), The Yeasts: A Taxonomic Study. Third revised and enlarged edition. Elsevier Science Publishers B.V., Amsterdam, pp. 224–251.

Watson, A.J., Fuller, L.J., Jeenes, D.J., Archer, D.B., 1999. Homologs of aflatoxin biosynthesis genes and sequence of *aflR* in *Aspergillus oryzae* and *Aspergillus sojae*. Appl. Environ. Microbiol. 65, 307–310.

Watts, R., Dahiya, J., Chaudhary, K., Tauro, P., 1988. Isolation and characterization of a new antifungal metabolite of *Trichoderma reesei*. Plant Soil 107, 81–84.

Westers, L., Westers, H., Quax, W.J., 2004. *Bacillus subtilis* as a cell factory for pharmaceutical proteins: a biotechnological approach to optimize the host organism. Biochim. Biophys. Acta 1694, 299–310.

World Health Organization (WHO), 2004. "Laccase from *Myceliophthora thermophila* expressed in *Aspergillus oryzae*." WHO Technical Report Series 922. Evaluation of Certain Food Additives and Contaminants. Sixty-first report of the Joint FAO/WHO Expert Committee on Food Additives, pp. 26–28.

Wiebe, M.G., 2002. Myco-protein from *Fusarium venenatum*: a well-established product for human consumption. Appl. Microbiol. Biotechnol. 58, 421–427.

Yanish-Perron, C., Vieira, J., Messing, J., 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33, 103–119.

Yoder, W.T., Christianson, L.M., 1998. Species-specific primers resolve members of *Fusarium* section *Fusarium*. Taxonomic status of the edible "Quorn" fungus reevaluated. Fungal Genet. Biol. 23, 68–80.

Yuan, L., Kurek, I., English, J., Keenan, I., 2005. Laboratory-directed protein evolution. Microbiol. Molec. Biol. Rev. 69, 373–392.

Zeman, N.W., McCrea, J.M., 1985. Alpha-amylase production using a recombinant DNA organism. Cereal Foods World 30, 777–780.

ELSEVIER

Food Chemistry 50 (1994) 133–136

0308-8146/94/$7.00

Total and phytate phosphorus contents of various foods and feedstuffs of plant origin

V. Ravindran,[a]* G. Ravindran[b] & S. Sivalogan[b]

[a]*Department of Animal Science,* [b]*Department of Food Science and Technology, Faculty of Agriculture, University of Peradeniya, Peradeniya, Sri Lanka*

(Received 14 May 1993; revised version received and accepted 31 August 1993)

Samples of 29 common foods and 10 feedstuffs of tropical origin were analysed for their total and phytate phosphorus (P) contents. In cereal grains, oilseeds and grain legumes, high levels of phytic acid were obtained, and phytate P constituted the major portion (60–82%) of total P. The various roots and tubers contained moderate amounts of phytic acid and phytate P accounted for 21–25% of the total P in this food group. Leafy greens contained negligible amounts of phytate P. In rice bran and the various oilseed meals, phytate P constituted 56–77% of the total P. Phytic acid contents were highest for gingelly (3·87%), gingelly meal (3·76%) and rice bran (3·65%).

INTRODUCTION

In most plant materials, a large portion of phosphorus (P) is present in the form of phytate. Phytate is a complex salt of calcium or magnesium with myoinositol (1, 2, 3, 4, 5, 6-hexakis dihydrogen phosphate) and is regarded as the primary storage form of P and inositol in almost all seeds (Cosgrove, 1980). During germination, the phytate is hydrolysed by the phytase present in seeds and serves as a source of inorganic P and cation for the emerging seedling (Williams, 1970).

P in phytate form is either unavailable to or poorly utilised by monogastric animals and humans (Nelson, 1967; Erdman, 1979; Reddy *et al.*, 1982) because they lack the phytase enzyme required to hydrolyse the phytate and release the P. In addition, the phosphoric acid moiety of the phytate molecule has a strong capacity to form complexes with multivalent cations, including calcium, magnesium, zinc, iron, manganese and copper. These phytate–mineral complexes are generally insoluble at physiological pH and hence render the minerals biologically unavailable to monogastric animals and humans. The adverse effects of phytate on mineral bioavailability have been the subject of several excellent reviews (Erdman, 1979; Maga, 1982; Reddy *et al.*, 1982).

Foods and feedstuffs derived from plants play a significant role in the nutrition of humans and animals in tropical regions. As a result, their contribution of phytate P to diets becomes nutritionally important. Whereas the phytate P contents of foods (Common, 1940; McCance & Widdowson, 1960; Reddy *et al.*, 1982) and feedstuffs (Nelson *et al.*, 1968; Kirby & Nelson, 1988) in the temperate regions are well documented, corresponding information on plant materials of tropical origin is limited. The present study was nitiated to assay a variety of tropical foods and feedstuffs for their total and phytate P contents.

MATERIALS AND METHODS

The study included 29 common foods and 10 feedstuffs. Samples, approximately 1 kg in size, were collected from local farms or retail outlets around Peradeniya. Cassava leaf meal and ipil ipil leaf meal were prepared in the laboratory using methods described previously (Ravindran *et al.*, 1986; Ravindran & Wijesiri, 1988).

Samples were dried at 100°C in an oven to constant weight and ground in a Wiley Laboratory mill to pass through a 60 mesh sieve. The ground samples were stored at room temperature in air-tight containers prior to chemical analyses.

All samples were assayed in triplicate for moisture (AOAC, 1975) and for total P using the ammonium vanadate method (Chapman & Pratt, 1961). Phytate P was determined by the colorimetric method of Wheeler and Ferrel (1971) as modified by Reddy *et al.* (1978). In this method, phytate was extracted using trichloroacetic acid and precipitated as ferric phytate. The ferric phytate was converted to ferric hydroxide (precipitate) by the addition of sodium hydroxide and boiling. The ferric hydroxide precipitate was dissolved in dilute hydrochloric acid and the iron content was measured colorimetrically (AOAC, 1975) using *o*-phenanthroline reagent. The phytate P content was calculated from the

* To whom correspondence should be addressed.

iron concentration by assuming a constant Fe : P molecular ratio of 4 : 6 in the precipitate.

RESULTS AND DISCUSSION

The total and phytate P contents of the cereals, roots, tubers, fruit vegetables and leafy greens are summarised in Table 1. Phytate P constituted the major portion of total P in cereal grains. The proportion of phytate P varies from 64–85% of the total P in most cereals, the exceptions being polished rice and finger millet. In polished rice and finger millet, the phytate P accounted for 55 and 58 % of the total P, respectively. The lower phytic acid content of polished rice compared with unpolished rice is due to the removal of bran layers during the polishing process. It is well documented that over 80% of the phytate in rice grain is present in the outer bran layers and aleurone layers of the kernel (O'Dell *et al.*, 1972; De Boland *et al.*, 1975).

The dark-coloured sorghum grains contained somewhat higher levels of phytic acid than the light-coloured grains. The significance of this observation is unclear. It is perhaps of interest to note that the dark-coloured sorghum grains are considered bird-resistant owing to their high tannin contents (Hulse *et al.*, 1980).

The various roots and tubers contained only moderate amounts of phytic acid. In this type of food, phytate P accounted for 21–25% of the total P. Published information on the phytate contents of roots and tubers is limited. The phytate values obtained for cassava roots compare closely with that reported by Jongbloed and Kemme (1990). However, the values determined for potatoes are lower than those obtained by Samotus and Schwimmer (1962). These workers determined that phytate P accounted for up to 35–40% of the total P in mature potato tubers.

Plantains, breadfruit and jak fruit are popular fruit vegetables; widely used for human consumption in tropical regions. They contain moderate amounts of phytic acid. The high level of phytic acid in jak seeds is as expected, since phytate is considered to be the chief storage form of P and inositol in seeds (Cosgrove, 1980). Phytate P constituted 61% of the total P in the jak seed. The two leafy greens analyzed, spinach and sweet potato, contained low amounts of phytic acid. This is in agreement with the observation of Oberleas (1973) that leafy vegetables appear to be essentially devoid of phytate. According to Bieleski (1968), over 70% of the P in plant leaves is found in the form of inorganic P with the remainder in the form of ribonucleic acids, phospholipids and acid-soluble phosphate esters.

Oilseeds had higher levels of phytic acid (Table 2) than cereals. The phytate P content in groundnut and gingelly constituted over 80% of the total P. The phytic acid concentration in gingelly seeds amounted to 3·87% of the dry weight. This value is, however, lower than the values of 4·7–5·2% reported for North American samples of gingelly by De Boland *et al.* (1975) and Toma *et al.* (1979).

The various grain legumes were found to contain 0·60–1·03% phytic acid (Table 2) and these values are

Table 1. Total and phytate phosphorus contents of various cereals, root and tubers and fruit vegetables of tropical origin (mean ± SE)

	No of samples	Phosphorus (g/100 g DM)		Phytate P (as% of) total	Phytic acid (g/100 g DM)[a]
		Total	Phytate		
Cereals					
Maize (*Zea mays*)	4	0·26 ± 0·01	0·22 ± 0·02	84·6	0·78
Rice *Oryza sativa*), brown unpolished	3	0·38 ± 0·03	0·28 ± 0·02	73·7	0·99
Rice, polished	2	0·31 ± 0·02	0·17 ± 0·01	54·8	0·60
Sorghum (*Sorghum vulgare*), dark-coloured seeds	2	0·41 ± 0·01	0·27 ± 0·01	65·9	0·96
Sorghum, light-coloured seeds	3	0·36 ± 0·02	0·23 ± 0·02	63·9	0·82
Foxtail millet (*Setaria italica*)	4	0·27 ± 0·01	0·19 ± 0·02	70·4	0·67
Finger millet (*Eleusine coracana*)	3	0·24 ± 0·01	0·14 ± 0·01	58·3	0·50
Common millet (*Panicum miliaceum*)	6	0·26 ± 0·01	0·17 ± 0·01	65·4	0·60
Roots and Tubers					
Cassave (*Manihot esculenta*), roots	2	0·16 ± 0·01	0·04 ± 0·003	25·0	0·14
Sweet potato (*Ipomea batatas*), tubers	2	0·21 ± 0·01	0·05 ± 0·004	23·8	0·18
Taro (*Colocasia esculenta*), corms	2	0·38 ± 0·01	0·09 ± 0·004	23·7	0·32
Dioscorea (*Dioscorea esculenta*), yam	3	0·12 ± 0·01	0·03 ± 0·008	25·0	0·11
Dioscorea (*Dioscorea alata*), yam	6	0·17 ± 0·01	0·04 ± 0·01	23·5	0·14
Potato (*Solanum tuberosum*), tubers	2	0·24 ± 0·01	0·05 ± 0·002	20·8	0·18
Miscellaneous					
Plantains (*Musa paradisica*), cooking type, unripe, peeled	2	0·12 ± 0·01	0·04 ± 0·008	33·3	0·14
Bread fruit (*Artocarpus altilis*), unripe, peeled	1	0·22	0·04	18·2	0·14
Jak fruit (*Artocarpus heterophyllus*), unripe, peeled	1	0·20	0·04	20·0	0·14
Jak seeds	3	0·31 ± 0·02	0·19 ± 0·02	61·3	0·67
Dates (*Phoenix dactylifera*), fruit, without seeds	2	0·18 ± 0·01	0·04 ± 0·008	22·2	0·14
Spinach (*Basella rubra*), leaves	2	0·44 ± 0·04	0·02 ± 0·004	4·5	0·07
Sweet potato, leaves	2	0·33 ± 0·02	0·02 ± 0·002	6·1	0·07

[a] Calculated phytic acid content assuming 28·20% phosphorus in the molecule.

Table 2. Total and phytate phosphorus contents of some oilseeds and grain legumes (mean ± SE)

	No of samples	Phosphorus (g/100 g DM)		Phytate P (as % of total)	Phytic acid (g/100 g DM)[a]
		Total	Phytate		
Oilseeds					
Soya bean (*Glycine max*)	3	0·60 ± 0·02	0·37 ± 0·01	61·7	1·31
Groundnut (*Arachis hypogea*)	4	0·49 ± 0·02	0·40 ± 0·02	81·6	1·42
Gingelly (*Sesamum indicum*)	3	1·34 ± 0·04	1·09 ± 0·05	81·3	3·87
Grain legumes					
Chick peas (*Cicer arietinum*)	2	0·41 ± 0·01	0·21 ± 0·01	51·2	0·74
Cowpeas (*Vigna unguiculata*)	4	0·39 ± 0·01	0·28 ± 0·02	71·8	0·99
Green gram (*Vigna radiata*)	2	0·38 ± 0·01	0·24 ± 0·01	63·2	0·85
Black gram (*Vigna mungo*)	3	0·39 ± 0·02	0·29 ± 0·01	74·4	1·03
Pigeon peas (*Cajanus cajan*)	3	0·32 ± 0·02	0·24 ± 0·02	75·0	0·85
Lentils (*Lens culinaris*)	2	0·31 ± 0·01	0·20 ± 0·02	64·5	0·71
Winged bean (*Psophocarpus tetragonolobus*)	2	0·33 ± 0·02	0·19 ± 0·02	57·6	0·67
Velvet bean (*Mucuna deeringiana*)	3	0·29 ± 0·02	0·17 ± 0·02	58·6	0·60

[a] Calculated phytic acid content assuming 28·20% phosphorus in the molecule.

similar to those determined for cereal grains (Table 1). In general, phytate P accounted for 60–75% of the total P in grain legume seeds. These results are in close agreement with those reported by Kumar *et al.* (1978) and Reddy and Salunkhe (1980).

The total and phytate P contents of some common feedstuffs are presented in Table 3. Rice bran and wheat bran contained high amounts of phytic acid and, this finding is consistent with the reports that phytic acid in rice and wheat is concentrated in bran layers of the kernels (De Boland *et al.*, 1975; Erdman, 1979). Phytate P accounted for 77 and 50% of the total P, respectively. The relatively lower levels of phytate P in wheat bran were unexpected, but may be due to the reported presence of phytase enzyme activity in wheat bran (Lim & Tate, 1971). The values obtained for rice bran in the present study are similar, whilst those obtained for wheat bran are lower than those reported by Kirby and Nelson (1988).

The various oilseed meals contained high amounts of phytic acid (Table 3). Gingelly meal contained 3·76% phytic acid based on the dry weight which is in agreement with earlier reports (Lease *et al.*, 1960; Cuca & Sunde, 1967; Nelson *et al.*, 1968). In general, about 60–77% of the total P in oilseed meals was found to be in the form of phytate. The leaf meals analysed had low levels of phytate. Similarly, Nelson *et al.* (1968) determined only traces of phytate in dehydrated alfalfa meal.

The present results indicate that the concentration of phytate is dependent on the portion of the plant that is consumed. The various types of seeds (cereals, oilseeds and grain legumes) contained large amounts of phytate, whereas roots, tubers and fruit vegetables had moderate amounts. Low levels of phytate were determined in the leafy green materials.

In developing country situations where cereals and other plant-based foods provide a large proportion of the food consumption, the dietary P intake as phytate will be greater. Although this might theoretically cause profoundly adverse effects on the bioavailability of phosphorus and cationic minerals, such populations do not suffer from nutrient deficiencies as much as would be anticipated (Hegsted, 1968; Hazell, 1985). Popula-

Table 3. Total and phytate phosphorus contents of some feedingstuffs of tropical origin (mean ± SE)

	No of samples	Phosphorus (g/100 g DM)		Phytate P (as % of total)	Phytic acid (g/100 g DM)[a]
		Total	Phytate		
Cereal by-products					
Rice bran	4	1·34 ± 0·03	1·03 ± 0·05	76·9	3·65
Wheat bran	2	1·15 ± 0·02	0·57 ± 0·03	49·6	2·02
Oilseed meals					
Soya bean meal, solvent extracted, dehulled	2	0·63 ± 0·02	0·38 ± 0·01	60·3	1·35
Soya bean meal, expeller extracted, with hulls	3	0·64 ± 0·02	0·39 ± 0·02	60·9	1·38
Coconut (*Cocos nucifera*) meal, expeller extracted	5	0·59 ± 0·03	0·33 ± 0·02	55·9	1·17
Gingelly meal, expeller extracted, with hulls	3	1·37 ± 0·04	1·06 ± 0·04	77·4	3·76
Rubber (*Hevea brasiliensis*) seed meal, expeller extracted	1	0·58	0·35	60·3	1·24
Kapok (*Ceiba pentandra*) seed meal, expeller extracted	1	0·93	0·64	68·8	2·27
Miscellaneous					
Ipil ipil (*Leucaena leucocephala*) leaf meal	1	0·22	0·02	9·1	0·07
Cassava leaf meal	2	0·42 ± 0·01	0·04 ± 0·007	9·5	0·14

[a] Calculated phytic acid content assuming 28·20% phosphorus in the molecule.

tions in developing countries are apparently able to adapt to high phytate intakes by the secretion of phytase enzyme (Lotz *et al.*, 1968) or phytates are likely to be broken down through indigenous food preparation methods. While evidence to support the earlier suggestion is lacking, it is well known that phytic acid contents of foods can be significantly reduced by milling, soaking, germination, cooking, fermentation and leavening (Reddy *et al.*, 1982).

It is, however, relevant to note that the adverse effect of phytates on mineral availability may have been overemphasised. Most foods that contain phytates are also good sources of dietary fibre which are known to have a high affinity for minerals (Reinhold *et al.*, 1975; Harland & Morris, 1985). Unless the phytates and fibre components can be separated and evaluated separately, it may be difficult to attribute the negative effects on mineral availability to phytates alone (Torre *et al.*, 1991).

ACKNOWLEDGEMENTS

This study was funded by a research grant from the International Foundation of Science, Sweden. The assistance of Messrs H. G. D. Perera and A. R. K. Rajapakse during sample collection and laboratory analysis is acknowledged.

REFERENCES

AOAC (1975). *Official Methods of Analysis* (11th edn). Association of Official Analytical Chemists, Washington, DC, USA.

Bieleski, R. L. (1968). Levels of phosphate esters in spirodella. *Plant Physiol.*, **43**, 1297–308.

Chapman, H. H. & Pratt, P. C. (1961). *Methods of Analysis for Soils, Plants and Water*. Division of Agricultural Science, University of California, Davis, CA, USA.

Common, R. H. (1940). The phytic acid content of some poultry feedingstuffs. *The Analyst*, **65**, 79–83.

Cosgrove, D. J. (1980). *Inositol Phosphates: Their Chemistry, Biochemistry and Physiology*. Elsevier Scientific Publishing Company, New York, USA.

Cuca, M. & Sunde, M. L. (1967). The availability of calcium from Mexican and Californian sesame meals. *Poultry Sci.*, **46**, 994–1002.

De Boland, A. R., Garner, G. B. & O'Dell, B. L. (1975). Identification and properties of phytate in cereal grains and oilseed products. *J. Agric. Food Chem.*, **23**, 1186–9.

Erdman Jr, J. W. (1979). Oilseed phytates: nutritional implications. *Am. Oil Chem. Soc. J.*, **56**, 736–41.

Harland, B. F. & Morris, E. R. (1985). Fibre and mineral absorption. In *Dietary Fibre Perspectives: Reviews and Bibliography*, Ed. A. R. Leeds & A. Avenell. John Libby Co. Ltd, London, UK, pp. 72–82.

Hazell, T. (1985). Minerals in foods: dietary sources, chemical forms, interactions bioavailability. *World Rev. Nutr. Diet.*, **46**, 1–123.

Hegsted, D. M. (1968). Present knowledge of calcium, phosphorus and magnesium. *Nutr. Rev.*, **26**, 65–70.

Hulse, J. H., Laing, E. M. & Pearson, O. E. (1980). *Sorghum and Millets: Their Composition and Nutritive Value.*, Academic Press, New York, USA.

Jongbloed, A. W. & Kemme, P. A. (1990). Apparent digestible phosphorus in the feeding of pigs in relation to availability, requirement and environment. 1. Digestible phosphorus in feedstuffs from plant and animal origin. *Neth. J. Agric. Sci.*, **38**, 567–75.

Kirby, L. K. & Nelson, T. S. (1988). Total and phytate phosphorus content of some feed ingredients derived from grains. *Nutr. Rep. Int.*, **37**, 277–80.

Kumar, K. G., Venkataraman, L. V., Jaya, T. V. & Krishnamurthy, K. S. (1978). Cooking characteristics of some germinated legumes: Changes in phytins, Ca, Mg and pectins. *J. Food Sci.*, **43**, 85–8.

Lease, J. G., Barnett, B. D., Lease, E. J. & Turf, D. E. (1960). The biological unavailability to the chick of zinc in a sesame meal ration. *J. Nutr.*, **72**, 66–70.

Lim, P. E. & Tate, M. E. (1971). The phytases. I. Lysolecithin—activated phytase from wheat bran. *Biochem. Biophys. Acta*, **250**, 155–64.

Lotz, M., Zisman, E. & Barter, F. C. (1968) . Evidence for a phosphorus-depletion syndrome in man. *New England J. Med.*, **278**, 409–15.

Maga, J. A. (1982). Phytate: its chemistry, occurrence, interactions, nutritional significance and methods of analysis. *J. Agric. Food Chem.*, **30**, 1–9.

McCance, R. A. & Widdowson, E. M. (1960). *The Composition of Foods*. HMSO, London, UK.

Nelson, T. S. (1967). The utilization of phytate phosphorus by poultry—a review. *Poultry Sci.*, **46**, 862–71.

Nelson, T. S., Ferrara, L. W. & Storer, N. L. (1968). Phytase phosphorus content of feed ingredients derived from plants. *Poultry Sci.*, **47**, 1372–5.

Oberleas, D. (1973). Phytates. In *Toxicants Occurring Naturally in Foods*. National Research Council, National Academy of Sciences, Washington, DC, USA, pp. 363–71.

O'Dell, B. L., De Boland, A. R. & Koirtyohann, S. R. (1972). Distribution of phytate and nutritionally important elements of cereal grains. *J. Agric. Food Chem.*, **20**, 718–21.

Ravindran, V. & Wijesiri, C. J. (1988). *Leucaena leucocephala* leaf meal as an animal feed. I. Composition and nutritive value for growing chicks. *Sri Lankan J. Agric. Sci.*, **25**, 94–100.

Ravindran, V., Kornegay, E. T., Rajaguru, A. S. B., Potter, L. M. & Cherry, J. A. (1986). Cassava leaf meal as a replacement for coconut oil meal in broiler diets. *Poultry Sci.*, **65**, 1720–7.

Reddy, N. R. & Salunkhe, D. K. (1980). Effects of fermentation on phytate phosphorus and minerals of blackgram, rice and blackgram/rice blends. *J. Food. Sci.*, **45**, 1708–12.

Reddy, N. R., Balakrishnan, C. V. & Salunkhe, D. K. (1978). Phytate phosphorus and mineral changes during germination and cooking of blackgram (*Phaseolus mungo* L.) seeds. *J. Food Sci.*, **43**, 540–2.

Reddy, N. R., Sathe, S. K. & Salunkhe, D. K. (1982). Phytates in legumes and cereals. *Adv. Food Res.*, **28**, 1–92.

Reinhold, J. G., Ismail-Beigi, F. & Faradji, B. (1975). Fibre vs phytate as determinant of the availability of calcium, zinc and iron of breadstuffs. *Nutr. Rep. Int.*, **12**, 75–85.

Samotus, B. & Schwimmer, S. (1962). Phytic acid as a phosphorus reservoir in the developing potato tuber. *Nature* (London), **194**, 578–9.

Toma, R. B., Tabekhia, M. M. & Williams, J. D. (1979). Phytate and oxalate contents in sesame seed. *Nutr. Rep. Int.*, **20**, 25–31.

Torre, M., Rodriquez, A. R. & Saura-Calixto, F. (1991). Effects of dietary fiber and phytic acid on mineral availability. *CRC Crit. Rev. Food Sci. Nutr.*, **30**, 1–22.

Wheeler, E. L. & Ferrel, R. E. (1971). A method for phytic acid determination in wheat and wheat fractions. *Cereal Chem.*, **48**, 312–15.

Williams, S. G. (1970). The role of phytic acid in the wheat grain. *Plant Physiol.*, **45**, 376–9.

Rimbach, G.

A049

CURRENT TOPICS IN NUTRACEUTICAL RESEARCH Vol. 6, No. 3, pp. 131-144 2008
ISSN 1540-7535 print , Copyright © 2008 by New Century Health Publishers, LLC
www.newcenturyhealthpublishers.com

EFFECT OF DIETARY PHYTATE AND MICROBIAL PHYTASE ON MINERAL AND TRACE ELEMENT BIOAVAILABILITY - A LITERATURE REVIEW

Gerald Rimbach[1], Josef Pallauf[2], Jennifer Moehring[1], Klaus Kraemer[3] and Anne Marie Minihane[4]

[1]Institute of Human Nutrition and Food Science, Christian Albrechts University Kiel, Germany;
[2]Institute of Animal Nutrition and Nutrition Physiology, Justus Liebig University Giessen, Germany;
[3]Sight and Life, Basel, Switzerland; and [4]Hugh Sinclair Human Nutrition Group, School of Chemistry, Food Biosciences, and Pharmacy, University of Reading, Reading,UK

[Received August 29, 2008; Accepted September 25, 2008]

ABSTRACT: *Phytic acid (PA) is the main phosphorus storage compound in cereals, legumes and oil seeds. In human populations where phytate-rich cereals such as wheat, maize and rice are a staple food, phytate may lead to mineral and trace element deficiency. Zinc appears to be the trace element whose bioavailability is most influenced by PA. Furthermore, several studies in humans as well as in monogastric animals clearly indicate an inhibition of non-haem iron absorption at marginal iron supply due to phytic acid. In fact PA seems to be, at least partly, responsible for the low absorption efficiency and high incidence of iron deficiency anaemia evident in most developing countries, where largely vegetarian diets are consumed. Microbial phytases have provided a realistic means of improving mineral availability from traditionally high-phytate diets. In fact it has been consistently shown that Aspergillus phytases significantly enhance the absorption of calcium, magnesium and zinc in pigs and rats. Furthermore there are a few studies in humans indicating an improvement of iron bioavailability due to microbial phytase.*

KEY WORDS: Iron, Minerals, Phytase, Phytic Acid, Trace Elements, Zinc

Corresponding Author: Prof. G. Rimbach, Institute of Human Nutrition and Food Science, Christian Albrechts University Kiel, Germany; Tel: +49 431 880-2583; Fax: +49 431 880-2628; Email: rimbach@foodsci.uni-kiel.de

INTRODUCTION

In recent years there has been a considerable increase in the scientific literature concerning the nutritional significance of phytic acid (PA) and phytase in human and animal nutrition. The present review article gives an overview of the effect of dietary PA on the bioavailability of minerals and trace elements. Furthermore, a major focus is a review of experimental results on the physiological relevance of microbial phytase in the nutrition of monogastric animals and humans. Studies over the past 15 to 20 years on the effect of microbial phytase on the bioavailability of calcium, magnesium, iron, zinc, copper and cadmium are reviewed.

Definition and occurrence of phytogenic and microbial phytase

The definitions of phytic acid, phytate and phytase are presented in Figure 1. In the literature synonyms are often used for some of the terms and this easily leads to misunderstandings. Phytic acid is, as a free acid, a hexa phosphorus acid ester of the 6-hydroxyl group cyclic alcohol myo-inositol, which can be synthesized within the animal organism, but has an essential characteristic for certain microorganisms. The correct chemical term for phytic acid is myo-inositol 1,2,3,4,5,6 hexakis dihydrogen phosphate (IUPAC-IUB 1978). The salts of phytic acid are described as phytates. The international abbreviation PA stands not only for phytic acid, but also for phytate. More accurately, phytate is a mixed potassium, magnesium and calcium salt of phytic acid that is present as a chelate and storage form for phosphorus in cereals, legumes and oilseeds.

FIGURE 1. Definitions of phytic acid, phytate and phytase

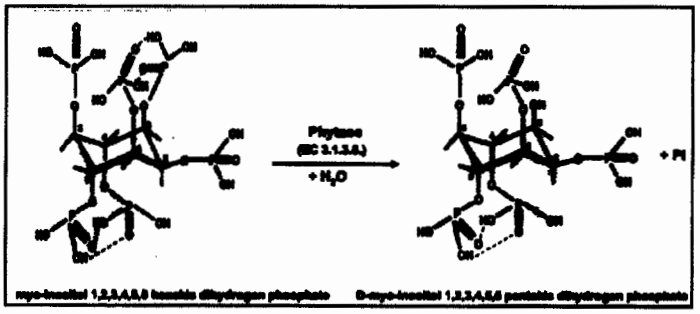
Phytic acid:	Myo-inositol 1,2,3,4,5,6, hexakis dihydrogen phosphate International abbreviation: PA (phytic acid)
Phytate:	Salt of phytic acid
Phytase:	Enzyme that catalyses the hydrolytic phosphate splitting of phytic (IP6) to lower inositol phosphate esters (IP5-IP1) and inorganic phosphate (PI) a) 3-phytase (E.C. 3.1.3.8) microbial origin b) 6-phytase (E.C. 3.1.3.26) plant origin

Phytic acid phosphorus represents 50-85% of the total phosphorus present in plant seeds. The localisation of phytic acid in the seeds varies. In grain, PA is mainly in the bran (aleurone layer, testa and pericarp). In the case of maize it is found mainly in the germ. With legume seeds, PA accumulates in the cotyledon and, in linseed, in the endosperm. Detailed information on the PA

content of various foods and feedstuffs can be found in several reviews (Oberleas and Harland 1981;Lantzsch 1990;Eckhout and De Paepe 1994;Ravindran, et al. 1995;Schröder, et al. 1996).

Phytase, on the other hand, is the enzyme which catalyses the hydrolytic phosphate splitting of phytic acid. The International Enzyme Commission's enzyme code is given in brackets (IUPAC-IUB (Recommendations of the Nomenclature Committee of the International Union of Biochemistry) 1984). Phytase of microbial origin (E.C. 3.1.3.8) splits the phosphate group at the C3 atom of the inosit ring, whereas plant-source (E.C. 3.1.3.26) phytase acts at the C6 atom. By the splitting of a phosphate group the lower inositol phosphate esters pentakis, tetrakis, tris, bis and mono phosphate are produced. Figure 2 presents a schematic representation of the hydrolytic action of a microbial phytase on inositol hexaphosphate.

FIGURE 2. Hydrolysis of phytic acid (myo-inositol 1,2,3,4,5,6 hexakis dihydrogen phosphate) by microbial phytase (E.C.3.1.3.8.) generating D-myo-inositol 1,2,4,5,6 pentakis dihydrogen phosphate and inorganic phosphate (Pi)



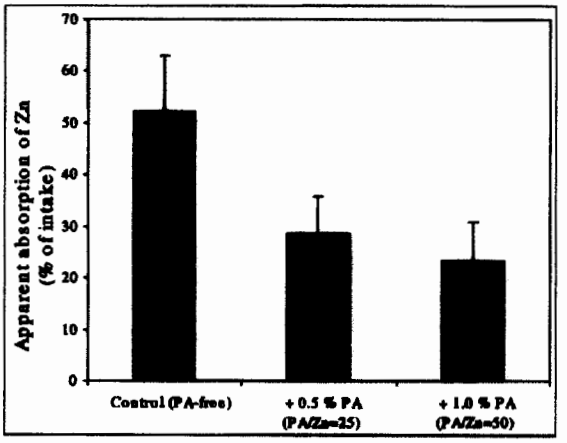
The pH optimum for plant phytase was determined to be in the range of 4.0-6.6. The hydrolysis of phytic acid by phytase from Aspergillus ficuum shows two distinct pH optima at pH 2.5 and 5.5, but the enzyme is 40% less active at pH 2.5 than at pH 5.5 (Gibson and Ullah 1990).

Antinutritive effects of phytic acid

The antinutritive effect of phytic acid is based on its molecular structure. At complete dissociation, the six phosphate groups of phytic acid carry twelve negative charges which, in weak acidic to neutral pH conditions, bind different di- and trivalent cations (e.g. Ca, Mg, Fe, Zn, Cu, Mn) into a stable complex. Interactions between phytic acid and proteins have also been reported. Under acid conditions a negative influence of the phytic acid on the solubility of proteins can be expected because of the ionic binding between the basic phosphate groups of phytic acid and protonised amino acids (Lytyl, histidyl and arginyl residue) (De Rham and Jost 1979;Fretzdorff, et al. 1995).

Zinc appears to be the trace element whose bioavailability is most influenced by PA. It has been shown in a series of in vitro and in vivo investigations that a higher molar ratio of PA to Zn results in a significant reduction in the bioavailability of zinc. A reduction of zinc bioavailability is observed with a molar PA:Zn ratio >10-15 in the diet (Davies and Olpin 1979;Morris and Ellis 1980). In Figure 3 the influence of graduated PA supplementations on the apparent Zn absorption of growing rats is demonstrated (Rimbach and Pallauf 1992).

FIGURE 3. Influence of the supplementation of phytic acid (as sodium phytate) on the apparent Zn absorption of growing rats fed a semi-synthetic diet (20 mg Zn/kg) on the basis of egg albumen and maize starch (Rimbach and Pallauf 1992)



The animals were fed a semi-synthetic diet based on egg albumin and maize starch over 21 days. The Zn concentration in all diets was 20 mg/kg. The control group (group I) received a PA-free diet. By replacing maize starch by 0.5% (group II) and 1.0% (group III) PA in the form of sodium phytate, molar PA:Zn ratios of 25 and 50 respectively were obtained. Following supplementation of 0.5% and 1.0% PA, the apparent absorption of Zn was significantly reduced, with absorption percentages of 29% (group II) and 23% (group III) compared to 52% Zn absorption in the control (group I). Comparable antinutritive effects of PA on Zn utilization of growing rats were found when PA-rich diets based on maize germs (Pallauf, et al. 1990) and soya protein isolate (Rimbach and Pallauf 1993) were fed. As investigations by Flanagan (1984) indicate, not only does phytic acid depress the bioavailability of dietary zinc, but it also substantially reduces the reabsorption of endogenous intestinal zinc. In addition, high concentrations of calcium increase the antinutritive effects of PA on zinc bioavailability due to the formation of insoluble Ca-Zn-PA-complexes. A molar PA x Ca/Zn ratio > 3.5 is regarded as a critical determinant of Zn utilization (Fordyce, et al. 1987).

As in the case of Zn, interactions between phytic acid and calcium (Taylor and Coleman 1979;Sandberg, et al. 1993), magnesium (Brink, et al. 1991;Pallauf, et al. 1992a;Pietsch, et al. 1995), iron (Thompson and Erdman 1984;Brune, et al. 1989), copper (Lee, et al. 1988) and manganese (Davidsson, et al. 1995) have also been described for laboratory animals, monogastric farm livestock and for humans.

As the lower inositol phosphate esters (IP1-IP5) can also be present in food and feed containing phytate (Sandberg and Ahderinne 1986;Phillippy and Bland 1988;Xu, et al. 1992;Harland and Morris 1995), this substance class has been

the focus of increasing attention in bioavailability studies. The antinutritive influence of different lower inositol phosphate esters was studied using rats (Lonnerdal, et al. 1989). Following intergastric application of equimolar concentrations of IP6, IP5, IP4 and IP3, 5%, 19%, 28% and 29% of the applied ^{65}Zn were recovered in the liver respectively. The ^{45}Ca absorption, however, was reduced only by IP6. Similarly, the rate of Fe- and Zn- transport by the human Caco-2 intestinal cell line declined in direct proportion to the level of inositol phosphorylation (Han, et al. 1994).

Phytic acid iron interactions

It is well recognized that in addition to the functionality of the enterocyte, the interaction of iron in the gut lumen and the chemical form in which iron is present is a major determinant of its protein-mediated uptake by the enterocyte. Non-haem iron from cereals, legumes and other plant sources is poorly absorbed because of the presence of inhibitors of iron absorption, such as certain fibres, polyphenols and phytic acid, which irreversibly bind iron in the gastrointestinal lumen, making it unavailable for absorption. Indeed such dietary components are thought to be in part responsible for the low absorption efficiency and high incidence of iron deficiency anaemia evident in most developing countries, where largely vegetarian diets are consumed. Several studies indicate an inhibition of non-haem iron absorption, at marginal dietary iron supply, due to phytic acid, in humans (Hallberg, et al. 1989) and other monogastric species (Thompson and Erdman 1984). Phytic acid and iron form insoluble complexes that are not available for absorption under the pH conditions of the small intestine.

A number of recent studies have increased our understanding of iron-phytic acid interactions and have provided a realistic means of improving mineral availability from traditionally high-phytate foods. Mendoza et al. (1998) observed that the absorption of iron from a tortilla based test meal was 49% greater when the tortillas were manufactured from genetically modified low phytate (368 mg per 100 g) maize relative to the native maize (847 mg phytate per 100g). Inhibition of iron absorption as a result of dietary phytate can also be partially counteracted by activation of native or the addition of extrinsic phytase to phytate-rich diets or by chemical hydrolyses of the phytate present (Sandberg et al. 1996; Biehl et al. 1997; Pallauf et al. 1999). Various food processes such as malting, fermenting and soaking result in the hydrolyses of phytate (*myo*-inositol hexaphosphate-IP6) into intermediate *myo*-inositol phosphates: inositol mono-, bi-, tri-, tetra- and pentaphosphates (IP1, IP2, IP3, IP4 and IP5 respectively). In a study by Sandberg *et al.* (1996) the absorption of iron from a simple bread roll test meal in the presence or absence of various phytate derivates was investigated. The authors concluded that ≥ 5 of the six possible sites on inositol need to be phosphorylated before an inhibitory effect on iron absorption was observed and that IP6 is more inhibitory than IP5. The addition of IP3 and IP4 had little effect on Fe-absorption when fed individually. However these isoforms may contribute to the inhibitory effect by interacting with the higher phosphorylated molecules. Therefore reduction of the phytate present in plant products of less phosphorylated derivates appears to provide a viable means of improving mineral absorption from phytate-rich foods. However not all studies have demonstrated negative effects of phytic acid on iron availability (House and Welch 1987). Differences in experimental data may be attributed to factors such as body iron status, the methodology used to study iron absorption, the chemical form and the concentration of iron in the diets, other components of the test meal, and species differences when comparing human and animal studies (Schricker, et al. 1983;Derman, et al. 1987). Moreover the source of phytic acid seems to be important. As mentioned previously phytate occurs naturally as a mixed potassium, magnesium and calcium salt in complex diets (Zhou and Erdman 1995). It therefore has probably less potential for binding iron compared with added sodium phytate because the phosphate groups may complex other components in the diet preparation. Therefore results obtained with sodium phytate should not be regarded as generally transferable to other phytate-rich matrices with a different source of phytic acid. However, overall it appears that phytate is a significant inhibitor of iron absorption, with the higher phosphorylated derivates (IP5 and IP6) being more potent than the less phosphorylated forms.

Microbial Phytase

Many bacteria, yeasts and fungi contain the enzyme phytase. Nelson, et al. (1968) were the first to study the effect of a mould phytase on the availability of phytate phosphorus from a liquid soybean meal in chickens. In a subsequent experiment it could be shown that the direct addition of phytase from *Aspergillus ficuum* to a maize/soya diet produced an increase in percentage bone ash indicating hydrolysis of phytate by the enzyme (Nelson, et al. (1971). Chickens utilized the hydrolysed phytate phosphorus as well as supplemental inorganic phosphorus. In recent years DNA technology has been successfully employed to produce high levels of extracellular microbial Aspergillus-phytase for poultry and pig diets (Ravindran et al. 1995). When purified microbial phytase was added to low P-diets of broilers the availability of P increased to over 60% and the amount of P in the droppings decreased by 50% (Simons, et al. 1990). Increasing Ca-content has resulted in a negative influence on phytase efficiency when rations were P-deficient, but not in the case of normal commercial poultry rations (Schöner, et al. 1993). The level of 0.9% Ca in broiler rations should not be exceeded. From regression equations it can be calculated that 500-1000 U microbial phytase was equivalent to 1 g P in broilers fed maize-soybean meal diets without any negative effect on performance (Schöner et al. 1991; Schöner et al. 1993; Vogt 1992a; Vogt 1992b; Simons and Versteegh 1993; Jeroch 1994; Kornegay et al. 1996; Yi et al. 1996a; Windisch and Kirchgessner 1996). Chymus analyses of broilers (Liebert et al. 1993) have shown that the primary sites for added microbial phytase activity are the crop and the stomach. No measurable indications of phytase activity were evident in the small intestine.

Role of microbial phytase for the bioavailability of calcium and magnesium

Table 1 summarizes the effect of microbial phytase on Ca

bioavailability in rats and pigs. Most studies show consistently that phytase supplementation significantly improves both apparent absorption and retention of calcium when phytate-rich diets were fed. The improvement of calcium utilization due to microbial phytase is also reflected by a higher bone mineralization and bone strength in rats (Porres et al. 2006) and pigs (Young, et al. 1993; Schone et al. 1995). Overall microbial phytase has been shown to be effective in releasing phytate-bound Ca from phytate-rich diets.

In a study by Porres et al. (2006) young albino rats were fed a diet based on raw lupin flour with a dietary Mg-content of 713 mg/kg diet and a microbial phytase addition of 750 phytase units/kg diet (Table 2). In this study phytase supplementation resulted in an increased level of magnesium in the femur and sternum. Furthermore in a study by Rimbach et al. (1995) with growing rats, phytase addition (2000 phytase units/kg diet) to a semisynthetic diet based on egg white and cornstarch (750 mg Mg per kg diet) significantly improved apparent magnesium absorption. An improvement of magnesium bioavailability due to microbial phytase was also found in several studies in pigs where phytate-rich diets based on maize, soybean extract and soybean oil (1500 mg Mg; 500 or 1000 phytase units/kg diet) (Pallauf et al. 1992a; Rimbach et al. 1997), field beans, wheat, peas and barley (1000 mg Mg per kg diet; 350 or 750 phytase units/kg diet) (Pallauf et al. 1994a), maize and soybean meal (1320 mg Mg per kg diet; 1500 phytase units/kg diet) (Adeola, et al. 1995), maize and soybean meal (2200 mg Mg per kg diet; 400 phytase units/kg diet) (Kemme et al. 1997b), barley-maize-soybean meal diet (1200 or 1500 mg Mg; 800 phytase units/kg diet) (Walz and Pallauf 2003), cornstarch, barley, soybean meal and sunflower seed meal (1600 mg Mg per kg diet; 100, 250, 500, 750, 1500 or 15,000 phytase units/kg diet) (Kies et al. 2006) supplemented with microbial phytase were fed. However, although most studies report an improved dietary magnesium utilisation, following phytase supplementation, in some studies there was no improvement in apparent absorption (Pallauf et al. 1994b). Differences in the literature regarding the effect of microbial phytase on magnesium bioavailability may be partly related to differences in dietary magnesium levels.

TABLE 1. The effect of microbial phytase on Ca bioavailability (P = pig, R = rat)

Species (n per group)	Live weight	Element	Diet	Ca content of diet (mg/kg)	Phytase added (IU/kg diet)	Experimental duration (days)	Finding	Reference
P (n = 8)	9-25 kg	Ca	Maize, Soybean meal	6300	500 1000	55	Ca Digestibility↑	(Pallauf et al. 1992b)
R (n = 10)	64 g	Ca	Raw lupin flour, α-Galactoside-free lupin flour	713	750	10	Ca Digestibility ↓ Femur Ca ↑ Sternum Ca ↑	(Porres et al. 2006)
P (n = 4)	10 kg	Ca	Maize, Soybean meal, Canola meal	5900-7300	500 1000	21	Ca Digestibility ↑ Bone Ca ↑	(Young et al. 1993)
P (n = 6)	8 kg	Ca	Maize, Soybean meal	5800	250 500 750	28	Ca Digestibility ↑ Fecal Ca ↓ Serum Ca ↑ Ca Utilization↑	(Lei et al. 1993b)
P (n = 8)	9-12 kg	Ca	Wheat, Barley, Soybean meal	5500-5600	350 700	14	Ca Digestibility ↑ Ca Excretion ↓ Apparent Ca Retention↑	(Pallauf et al. 1994b)
P (n = 8)	12-16 kg	Ca	Field beans, Wheat, Peas, Barley	4600-4800	350 700	14	Ca Digestibility ↑ Apparent Ca Retention↑	(Pallauf et al. 1994a)
P (n = 5)	45-110 kg	Ca	Maize, Tapioca, Soybean meal	7300	800	22	ATTD ↑ Ca Retention ↑	(Mroz et al. 1994)
P (n = 12)	9-18 kg	Ca	Maize, Soybean meal	8000	1500	21	Ca Absorption↑ Ca Retention ↑	(Adeola et al. 1995)
P (n = 12)	10-30 kg	Ca	Barley, Wheat, Soybean meal	8000	1000	28	Bone Ca↑	(Schone et al. 1995)
P (n = 8)	7.5 kg	Ca	Maize, Soybean meal	4300; 6400; 7800	350 700 1050 1400	35	Ca Absorption ↑ (16% P)	(Yi et al. 1996b)
P (n = 8)	7.8 kg	Ca	Maize, Soybean meal	7800; 7200; 9800	350 700 1050 1400	35	Ca Digestibility ↑	(Kornegay and Qian 1996)
P (n = 8-13)	187-259 kg	Ca	Maize, Soybean meal	4200	400	d 107 of gestation to d 21 of lactation	ATTD ↑	(Kemme, et al. 1997b)
P (n = 6)	40-95 kg 95-120 kg	Ca	Barley, Tapioca meal, Soybean meal	4200	400	n. s.	ATTD↑	(Kemme et al. 1997c)
P (n = 7) Piglets	10-40 kg	Ca	Wheat middlings, Maize, Tapioca meal	6220; 6300	500	n. s.	Ca Digestibility↑	(Kemme et al. 1997a)
P (n = 4) Growing-Finishing Pigs	50-100 kg	Ca	Wheat middlings, Maize, Tapioca meal	6220; 6300	500	n. s.	Ca Digestibility↑ (BW 40 kg)	(Kemme et al. 1997a)
P (n = 2) Multiparous sows	n. s.	Ca	Wheat middlings, Maize, Tapioca meal	6220; 6300	500	n. s.	n. s.	(Kemme et al. 1997a)
P (n = 9)	19 kg	Ca	Maize, Soybean meal, low in P	5500	250 500	42	Ca Absorption ↑	(Liu et al. 1997)
P (n = 3)	10-50 kg	Ca	Pearl millet-soybean meal-based	1030; 1050	700 1000	35	Apparent Ca Absorption↑ Ca Retention↑ Ca Utilization↑	(Maury et al. 1997)

P (n = 2)	50-118 kg	Ca	Sorghum, Soybean meal	3900-4000	300 500	30	Ca Digestibility↑ ATTD ↑ Ca DLI↑	(O'Quinn et al. 1997)
P (n = 4)	25-30 kg	Ca	Soybean meal, Rapeseed meal	5900	750	14	Ca Excretion ↓	(Rodehutscord et al. 1997)
P (n = 8)	7-10 kg	Ca	Maize, Soybean meal	1270; 4280	250 500 700	28	Ca Digestibility↑	(Radcliffe et al. 1998)
P (n = 4-5)	15-35 kg	Ca	Maize, Barley, Soybean meal	5800	400	14	Ca net Absorption ↑ Renal excretion ↑ (BW 59.1 kg)	(Rodehutscord et al. 1999)
P (n = 7)	25 kg	Ca	Cornstarch, Dextrose	5000	500 1000 1500	67	Ca Digestibility ↑ Ca Utilisation ↑	(Traylor et al. 2001)
P (n = 8)	70-110 kg	Ca	Barley, Maize, Soybean meal	5600; 6200	800	47-53	n. e.	(Walk and Pallauf 2003)
P (n = 8)	11 kg	Ca	Maize, Soybean meal, Rice bran	10,500	1500	21	AFD ↑ Ca Digestibility ↑	(Kies et al. 2005)
P (n = 2)	48 kg	Ca	Maize, Soybean meal	4400	250 500	14	AID ↑	(Radcliffe et al. 2006)
P (n = 7)	7-28 kg	Ca	Maize, Barley, Soybean meal, Sunflower seed meal	3500	100 250 500 750 1500 15,000	43	Ca Digestibility ↑	(Kies et al. 2006)
P (n = 6)	10-25 kg	Ca	Maize, Soybean meal	5300	16,500 33,000 49,500	28	Ca Digestibility ↑ Ca Retention ↑	(Nyannor et al. 2007)

AFD = Apparent Faecal Digestibility; AID = Apparent Ileal Digestibility; ATTP = Apparent Total Tract Digestibility; Ca %R/A = percentage of retention in relation to absorption; DLI = Disappearance in large intestine; n. e. = no effect on Ca bioavailability; n. s. = not specified; UE = Urinary excretion

TABLE 2. The effect of microbial phytase on Mg bioavailability (P = pig, R = rat)

Species (n per group)	Live weight	Element	Diet	Mg content of diet (mg/kg)	Phytase added (FU/Kg diet)	Experimental duration (days)	Finding	Reference
R (n = 6)	47 g	Mg	Egg white, Cornstarch	750	2000	28	Mg Digestibility ↑	(Rimbach et al. 1995b)
R (n = 7)	47 g	Mg	Maize, Soybean meal, Cornstarch, Soybean oil	1200	250 500 1000 2000	28	n. e.	(Rimbach et al. 1997)
R (n = 10)	64 g	Mg	Raw lupin flour, α-Galactoside-free lupin flour	713	750	10	Plasma Mg ↓ Femur Mg ↑ Sternum Mg ↑	(Porres et al. 2006)
P (n = 8)	9-25 kg	Mg	Maize, Soybean extract, Soybean oil	1500	500 1000	35	Mg Digestibility ↑	(Pallauf et al. 1992a)
P (n = 8)	9-12 kg	Mg	Wheat, Barley, Soybean meal	1200	350 700	14	n. e.	(Pallauf et al. 1994b)
P (n = 8)	12-16 kg	Mg	Field beans, Wheat, Peas, Barley	1000	350 700	14	Mg Digestibility ↑ Mg Utilisation ↑	(Pallauf et al. 1994a)
P (n = 12)	9-18 kg	Mg	Maize, Soybean meal	1320	1500	21	Plasma Mg ↑	(Adeola et al. 1995)
P (n = 8-13)	187-259 kg	Mg	Maize, Soybean meal	2200	400	d 107 of gestation to d 21 of lactation	Mg Digestibility ↑	(Kemme et al. 1997b)
P (n = 8)	70-110 kg	Mg	Barley, Maize, Soybean meal	1200; 1500	800	47-53	Mg Digestibility ↑	(Walk and Pallauf 2003)
P (n = 7)	7-28 kg	Mg	Maize, Barley, Soybean meal, Sunflower seed meal	1600	100 250 500 750 1500 15,000	43	Mg Digestibility ↑	(Kies et al. 2006)

n. e. = no effect on Mg bioavailability

Role of microbial phytase for the bioavailability of the essential trace elements iron, zinc, copper and manganese

Iron

The effect of microbial phytase on iron absorption and iron status has been evaluated in several animal studies and in a few studies with humans (Table 3). In an animal experiment five groups of weaned male Wistar rats were fed a semisynthetic diet composed of cornstarch and casein over 4 weeks [Pallauf et al. 1999]. The diets were designed to provide 35 mg Fe per kg diet and two levels (7.5g and 15g/kg diet) of phytic acid from sodium phytate were added. The supplementation of 1000 phytase units from Aspergillus niger per kg diet partly counteracted the antinutritive effect of phytic acid on iron digestibility and significantly improved several parameters of iron status such as haematocrit as well as

total and latent iron binding capacity of the plasma. Furthermore the transferrin saturation and femur iron content were significantly increased in the phytase group, as compared to the control groups.

In a study by Stahl et al. (1999) the supplementation of 1200 phytase units/kg diet was associated with improved iron status (as measured by haemoglobin concentration) in pigs fed diets based on maize, soy protein concentrate and soybean (Fe content 52 or 111 mg/kg diet). In a study of Walz and Pallauf (2003) four groups of eight finishing pigs were fed a basal diet of barley, maize and soybean meal. The dietary iron content ranged from 78 to 94 mg/kg diet. In this trial the addition of 800 phytase units/kg diet had no effect on apparent iron digestibility in pigs. These data are in accordance with previous findings where the supplementation of 500 and 1000 phytase units/kg diet did not change iron digestibility in pigs (Pallauf, et al. 1992a). Kies et al. (2005) studied the effect of phytase supplementation (1500 units/kg diet) on iron absorption in male pigs receiving a diet based on maize, soybean meal and rice bran (Fe content 40 mg/kg diet). Phytase supplementation resulted in a significant increase in Fe digestibility. In another study by Shelton et al. (2005) pigs were fed a maize-soybean meal diet (Fe content 276-303 mg kg diet). Phytase supplementation of 500 units/kg diet significantly improved bone iron levels.

The effects on iron absorption of two kinds of phytase, cereal phytase and microbial phytase from Aspergillus niger were evaluated by Sandberg et al. (1996) in two studies with 10 healthy human volunteers each. In study 1 iron absorption was measured from single meals containing wheat rolls supplemented with wheat bran with or without phytase activity, whereas in experiment 2 phytase-deactivated wheat bran with or without addition of microbial phytase was investigated. The test meal contained 52 mg Fe per kg and 100,000 phytase units per kg diet were added. Phytase addition to the phytate-containing meal just before consumption was found to markedly increase the iron absorption from 14 to 26 %. The influence of phytic acid degradation on iron absorption from cereal porridges in humans was systematically investigated by Hurrell and coworkers. The dephytinized cereal porridges were prepared by adding phytase to an aqueous slurry adjusted to pH 5.0-5.5, and kept at 40°C for approximately 2 hours until all the phytate was degraded.. The test meals were based on rice, wheat, maize, oat, sorghum and a wheat-soy blend. Iron absorption from the test meal was measured by the extrinsic-label radio-iron technique. It could be convincingly shown that dephytinization significantly enhanced iron absorption from: rice porridge from 1.7% to 5.3%, oats from 0.3% to 2.8%, maize from 1.8% to 8.9%, wheat from 1% to 11.5%, wheat-soy blend without ascorbic acid from 1.1% to 3.7%, and from the wheat-soy blend with ascorbic acid from 2.4% to 8.5% (Hurrell, et al. 2003). The bioavailability of iron in infants fed infant cereals with relatively low phytic acid content was studied by Davidsson and coworkers. The infant cereal was dephytinized by adding commercial phytase during manufacture resulting in degradation of 88% of the native phytic acid content. The iron absorption was similar between test meals treated with and without microbial phytase. The authors concluded that dephytinization of infant cereals containing a relatively low native phytic acid content is unnecessary to ensure adequate bioavailability of iron (Davidsson, et al. 1997).

Overall current data indicate that microbial phytase improved iron absorption and iron status in several studies with pigs, rats and in a number of human trials. In humans the effect was particularly evident when the food/diet had a high natural phytic acid content. However, in some pig studies no improvement of iron bioavailability due to microbial phytase was evident which may be related to the relatively high dietary iron concentrations in these studies. An improvement of iron bioavailability in humans might be evident in phytate-rich diets but not in diets with a low content of phytic acid.

TABLE 3. The effect of microbial phytase on Fe bioavailability (H = human, P = pig, R = rat)

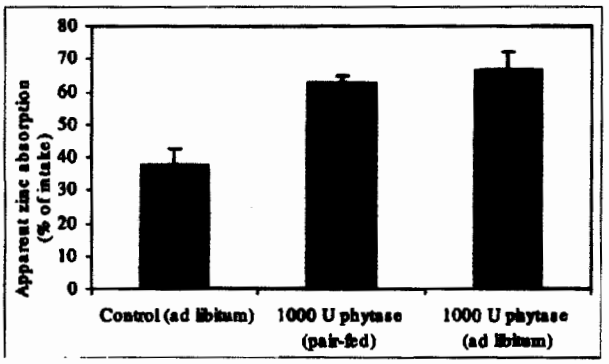
Species (n per group)	Live weight	Element	Diet	Fe content of diet (mg/kg)	Phytase added (FTU/Kg diet)	Experimental duration (days)	Finding	Reference
R (n = 7)	42 g	Fe	Cornstarch, Casein	35	1000	28	Fe Digestibility ↑ Haematocrit ↑ PBC (total) ↑ PBC (latent) ↑ Transferrin saturation ↑ Femur Fe↑	(Pallauf et al. 1999)
P (n = 8)	9-25 kg	Fe	Maize, Soybean extract, Soybean oil	113; 109	500 1000	35	n. e.	(Pallauf et al. 1992a)
P (n = 2)	5-12 kg 7-27 kg	Fe	Maize, Soy protein concentrate	52; 111	1200	28-35	Fe • Hemoglobin concentration ↑	(Stahl et al. 1999)
P (n = 8)	70-110 kg	Fe	Barley, Maize, Soybean meal	78-94	800	47-53	n. e.	(Walz and Pallauf 2003)
P (n = 8)	11 kg	Fe	Maize, Soybean meal, Rice bran	40	1500	21	Fe Digestibility ↑ UE ↑	(Kies et al. 2005)
P (n = 6-7)	5-111 kg	Fe	Maize, Soybean meal	276 - 303	500	24	Bone Fe ↑	(Shelton et al. 2005)
H (n = 78)	n. s.	Fe	Rice, Wheat, Maize, Oat, Sorghum, Wheat-soy blend	2.5 mg or 5 mg/meal	n. s.	32	Fe absorption ↑	(Hurrell et al. 2003)
H (n = 12)	8.4 kg	Fe	White wheat flour, Skim milk powder	2.5 mg/meal	n. s.	19	n. e.	(Davidsson et al. 1997)
H (n = 10)	n. s.	Fe	White wheat rolls, Wheat bran	52	100,000	n. s.	Fe Absorption ↑	(Sandberg et al. 1996)

n. e. = no effect on Fe bioavailability n. s. = not specified PBC = Plasma binding capacity UE = Urinary excretion

Zinc, copper, and manganese

Although the effect of dietary phytate and microbial phytase on zinc bioavailability has been studied both in rats and pigs (Table 4), systematic studies regarding the effect of microbial phytase on zinc bioavailability in humans are lacking. The addition of microbial phytase (1000 phytase units/kg diet) significantly improved zinc status (as measured by plasma zinc concentration, plasma alkaline phosphatase activity and femur zinc) in growing rats fed phytate-enriched semisynthetic diets based on cornstarch and casein (20 mg Zn per kg diet) for 3 weeks (Rimbach and Pallauf 1992). These data are in accordance with a study of Rimbach and Pallauf (1993) where growing rats were fed diets based on soyprotein isolate and cornstarch (Zn content 15-16 mg/kg diet). Following the addition of 1000 phytase units/kg diet of microbial phytase from Aspergillus niger the digestibility of zinc (Figure 4) as well as the zinc content in plasma, femur and testes significantly increased.

Figure 4: Influence of an addition of microbial phytase to a phytate-rich diet of soy protein isolate and cornstarch on the apparent absorption of zinc in growing rats [Rimbach and Pallauf 1993]



In two other experimental trials the effect of increasing levels of microbial phytase on zinc bioavailability was studied in growing rats. The basal diets which consisted of maize, soybean meal, cornstarch and soybean oil were designed to contain 0, 250, 500, 1000 or 2000 phytase units/kg diet and the zinc content was set to be 24-25 mg/kg diet. Phytase supplementation resulted in a dose dependent increase in zinc digestibility which was accompanied by improved zinc status (Rimbach, et al. 1997;Rimbach, et al. 1998).

Over the last fifteen years numerous studies have reported on the effect of dietary microbial phytase on zinc bioavailability in pigs. In a study of Pallauf et al. (1992a) pigs were fed basal diets composed of maize, soybean extract and soybean oil with 58 mg zinc per kg of feed. The addition of 500 or 1000 phytase units/kg diet improved Zn-digestibility, plasma-Zn levels as well as the percentage unsaturated zinc binding capacity. Two experimental trials with weanling pigs were conducted by Lei et al. (1993b) to determine the optimal dietary supplementation of Aspergillus niger phytase activity to a maize-soybean meal based diet. In the first experiment pigs were fed the basal diet containing 61 mg zinc with or without phytase supplementation of 750, 1050, 1250 or 1350 phytase units/kg diet. This treatment resulted in higher plasma-Zn concentrations in all groups. Similar effects were found in the second experiment (73 mg zinc per kg with an addition of 1200 phytase units/kg diet). In another study of Lei et al. (1993c) the addition of 1350 phytase units/kg diet to a maize-soybean meal diet with a zinc content of 25-32 mg/kg diet resulted in an improved activity of the zinc metalloenzyme alkaline phosphatase and higher plasma-Zn levels. Pallauf et al. (1994b) studied the effect of phytase supplementation of 350 and 700 units/kg diet to a phytate-rich diet based on field beans, wheat, peas and barley in male castrated piglets. All diets contained 60 mg Zn per kg diet. Zinc digestibility tended to be higher in the phytase groups, and plasma zinc concentration was also significantly increased by the addition of microbial phytase. Similar effects were found when diets based on wheat, barley and soybean meal (61-67 mg zinc kg diet) supplemented with 350 or 700 phytase units/kg diet were fed to male piglets (Pallauf, et al. 1994b). In a study by Adeola et al. (1995) pig diets based on maize and soybean meal with zinc levels of 23 and 123 mg per kg diet were fed. The supplementation of microbial phytase (1500 units per kg diet) significantly increased zinc digestibility and zinc retention as well as plasma zinc levels. Murry et al. (1997) examined the effect of dietary microbial phytase in gilts. Three levels of phytase (0, 700, 1000 phytase units per kg diet) were added to a soybean meal based diet with a zinc content of 98 mg/kg diet. In this study phytase addition tended to increase serum Zn concentration. Walz and Pallauf (2003) evaluated the effect of microbial phytase on mineral and trace element digestibility in pigs with 25-100 kg live weight. The pigs were fed a basal diet composed of barley, maize and soybean meal containing 64-68 mg zinc and 800 phytase units per kg diet. Phytase supplementation led to significantly increased zinc concentrations in bones indicating an improved zinc bioavailability. In a study of Revy et al. (2006) piglets were fed a maize, soybean meal, wheat bran diet with a zinc content of 33 to 73 mg/kg diet which was supplemented with 700 phytase units/kg diet. Zinc concentration in plasma and femur as well as plasma alkaline phosphatase activity increased due to dietary phytase supplementation. Similar findings have been reported by Shelton et al. (2004 and 2005) who found enhanced bone zinc levels in pigs receiving maize-soybean meal diets (Zn content of 158-160 mg/kg diet) supplemented with 500 phytase units/kg diet. However in other studies no positive effect of dietary microbial phytase on zinc bioavailability in rats (Rimbach, et al. 1995b) and in pigs (Shelton, et al. 2005) was reported.

In a study by Kies et al. (2005) phytase supplementation (1500 phytase units/kg diet) of a diet based on maize, soybean meal and rice bran (Cu content 187 mg/kg diet) increased copper digestibility in pigs and was accompanied by an increased urinary Cu excretion (Table 5). In another study by Kies et al. (2006) the effect of increasing levels of microbial phytase (0, 100, 250, 500, 750, 1500 or 15,000 phytase units/kg diet) added to a pig diet composed of maize, barley, soybean meal and sunflower seed meal (Cu content 113 mg/kg diet) was studied. Phytase supplementation with 750 phytase units/kg diet resulted in a dose dependent increase in Cu digestibility. These studies are in accordance with other studies where an improved digestibility due to microbial phytase was shown (Pallauf, et al. 1992a;Adeola, et al. 1995;Shelton, et al. 2004).

There are only few studies in the literature regarding the effect of dietary microbial phytase on manganese bioavailability in monogastric animals (Table 6). In some experiments no positive effect of phytase

supplementation on the apparent absorption of manganese and the manganese status in rats (Rimbach, et al. 1995b) and pigs (Pallauf, et al. 1992a;Shelton, et al. 2004;Adeola, et al. 1995) was evident. However in a study by Shelton et al. (2005) phytase supplementation (500 phytase units/kg diet) to a maize-soybean meal diet (39-41 mg Mn per kg diet) resulted in an increase in bone manganese concentration in pigs. Further studies are required to fully elucidate the impact of microbial phytase on manganese bioavailability.

TABLE 4. The effect of microbial phytase on Zn bioavailability (P = pig, R = rat)

Species (n per group)	Live weight	Element	Diet	Zn content of diet (mg/kg)	Phytase added (IU/Kg diet)	Experimental duration (days)	Finding	Reference
R (n = 7)	50 g	Zn	Egg white, Cornstarch	20	1000	21	Zn Digestibility ↑ Zn Retention ↑ Femur Zn ↑ Testes Zn ↑ Plasma Zn ↑ Plasma ALP activity ↑	(Rimbach and Pallauf 1992)
R (n = 11)	50 g	Zn	Soy protein isolate, Cornstarch	15 - 16	1000	21	Zn Digestibility ↑ Plasma Zn ↑ Femur Zn ↑ Testes Zn ↑	(Rimbach and Pallauf 1993)
R (n = 6)	47 g	Zn	Egg white, Cornstarch	103	2000	28	n. e.	(Rimbach et al. 1995b)
R (n = 6)	47 g	Zn	Egg white, Cornstarch	16	2000	28	Zn Digestibility ↑ Plasma Zn ↑ Femur Zn ↑ Plasma ALP activity ↑	(Rimbach et al. 1995a)
R (n = 7)	47 g	Zn	Maize, Soybean meal, Cornstarch, Soybean oil	24 - 25	250 500 1000 2000	28	ZnDigestibility ↑	(Rimbach et al. 1997)
R (n = 7)	47 g	Zn	Maize, Soybean meal, Cornstarch, Soybean oil	24 - 25	250 500 1000 2000	28	(≥ 500 IU) Zn Digestibility ↑ Plasma Zn ↑ Femur Zn ↑ Testes Zn ↑ Plasma ALP activity ↑	(Rimbach et al. 1998)
P (n = 8)	9-25 kg	Zn	Maize, Soybean extract, Soybean oil	58	500 1000	35	Zn Digestibility ↑ Plasma Zn ↑ P-ZBC ↑	(Pallauf et al. 1992a)
P (n = 5)	7 kg	Zn	Maize, Soybean meal	61	750 1050 1250 1350	14	Zn Plasma ↑	(Lei et al. 1993c)
P (n = 4)	7 kg	Zn	Maize, Soybean meal	73	1200	14	Plasma Zn ↑	(Lei et al. 1993c)
P (n = 4)	7 kg	Zn	Maize, Soybean meal	25; 32	1350	28	Plasma Zn ↑ Plasma ALP activity ↑	(Lei et al. 1993a)
P (n = 8)	9-12 kg	Zn	Wheat, Barley, Soybean meal	61; 67	350 700	14	Zn Plasma ↑	(Pallauf et al. 1994b)
P (n = 8)	12-16 kg	Zn	Field beans,	60	350	14	Zn Digestibility ↑	(Pallauf et al. 1994a)
P (n = 12)	9-18 kg	Zn	Wheat, Peas, Barley Maize, Soybean meal	23; 123	700 1500	21	Plasma Zn ↑ Zn Digestibility ↑ Plasma Zn ↑ Zn Retention ↑	(Adeola et al. 1995)
P (n = 3)	10-30 kg	Zn	Pearl millet, Soybean meal	98	700 1000	35	Zn Serum ↑	(Murry et al. 1997)
P (n = 8)	70-110 kg	Zn	Barley, Maize, Soybean meal	64; 68	800	47-53	Zn Digestibility ↑ Zn retention per kg BWG Zn in Phalanx prima IV ↑ Zn Availability ↑	(Walz and Pallauf 2003)
P (n = 4)	22-109 kg	Zn	Maize, Soybean meal	154	500	26	Liver Zn ↑ Bone Zn ↑	(Shelton et al. 2004)
P (n = 6-7)	5-111 kg	Zn	Maize, Soybean meal	158 -160	500	24	Bone Zn • Pancreas Zn- ↑	(Shelton et al. 2005)
P (n = 6)	7-16 kg	Zn	Maize, Soybean meal, Wheat bran	33; 43; 58; 73	700	26	Plasma ALP activity ↑ Plasma Zn ↑ Bone Zn ↑	(Revy et al. 2006)

ALP = Zinc metalloenzyme alkaline phosphatase; n. e. = no effect on Zn bioavailability; P-ZBC = percentage unsaturated zinc binding capacity

TABLE 5. The effect of microbial phytase on Cu bioavailability (P = pig, R = rat)

Species (n per group)	Liveweight	Element	Diet	Cu content of diet (mg/kg)	Phytase added (IU/Kg diet)	Experimental duration (days)	Finding	Reference
R (n = 6)	47 g	Cu	Egg white, Cornstarch	7	2000	28	n. e.	(Rimbach et al. 1995b)
P (n = 8)	9-25 kg	Cu	Maize, Soybean extract, Soybean oil	12	500 1000	35	Cu Digestibility ↑	(Pallauf et al. 1992a)
P (n = 12)	9-18 kg	Cu	Maize, Soybean meal	14	1500	21	Plasma Cu ↑ Cu Absorption ↑ Cu Retention ↑	(Adeola et al. 1995)
P (n = 4)	22-109 kg	Cu	Maize, Soybean-meal	20	500	26	Muscle Cu ↑ Bone Cu ↓ Liver Cu ↓ Cu Absorption ↑ Cu Retention ↑	(Shelton et al. 2004)
P (n = 6-7)	5-111 kg	Cu	Maize, Soybean-meal	20 – 23	500	24	Bile Cu ↓ Loin muscle Cu ↓ Pancreas Cu ↓	(Shelton et al. 2005)
P (n = 8)	11 kg	Cu	Maize, Soybean meal, Rice bran	187	1500	21	Cu Digestibility ↑ UE ↑	(Kies et al. 2005)
P (n = 7)	7-28 kg	Cu	Maize, Barley, Soybean meal, sunflower seed meal	113	100 250 500 750 1500 15,000	43	AFD ↑ (dose-dependent, but negative below 750) IU/kg)	(Kies et al. 2006)

AFD = Apparent faecal digestibility n. e. = no effect on Cu bioavailability UE = Urinary excretion

TABLE 6. The effect of microbial phytase on Mn bioavailability (P = pig, R = rat)

Species (n per group)	Liveweight	Element	Diet	Mn content of diet (mg/kg)	Phytase added (IU/Kg diet)	Experimental duration (days)	Finding	Reference
R (n = 6)	47 g	Mn	Egg white, Cornstarch	46	2000	28	n. e.	(Rimbach et al. 1995b)
P (n = 8)	9-25 kg	Mn	Maize, Soybean extract, Soybean oil	21 - 22	500 1000	35	n. e.	(Pallauf et al. 1992a)
P (n = 12)	9-18 kg	Mn	Maize, Soybean meal	46	1500	21	n. e.	(Adeola et al. 1995)
P (n = 4)	22-109 kg	Mn	Maize, Soybean meal	37	500	26	Bile Mn ↓ Liver Mn ↓	(Shelton et al. 2004)
P (n = 6-7)	5-111 kg	Mn	Maize, Soybean meal	39 - 41	500	24	Bile Mn ↑ Bone Mn ↑	(Shelton et al. 2005)

n. e. = no effect on Mn bioavailability

Interactions between phytic acid, microbial phytase and cadmium

The effect of dietary microbial phytase on the accumulation of cadmium in growing rats was evaluated in two different studies by Rimbach et al. (1995a and b). In the first study albino rats were fed phytate-rich diets based on maize, soybean meal, cornstarch and soybean oil. The experimental diets were designed to contain 0, 250, 500, 1000 or 2000 phytase units/kg from Aspergillus niger (Table 7). All diets were supplemented with 5 mg Cd per kg diet. Cadmium concentration, total cadmium content and fractional cadmium accumulation in liver and kidneys were not significantly altered in response to different dietary treatments (Rimbach, et al. 1998). In the second study albino rats were fed semisynthetic diets composed of egg white and cornstarch enriched with sodium phytate and supplemented with 5 mg cadmium/kg diet from cadmium chloride ($CdCl_2$). One diet was supplemented with 2000 phytase units of microbial phytase per kg diet. Phytase supplementation resulted in lowered liver and kidney cadmium accumulation in this study (Rimbach, et al. 1995b). These data are in accordance with previous studies with growing rats (Rimbach, et al. 1995a). The effect of dietary microbial phytase (800 phytase units/kg) on the accumulation of cadmium has also been studied in pigs that were fed diets based on barley, maize and extracted soybean meal. The dietary cadmium levels were between 13 and 21 µg/kg diet. Contrary to findings for rats fed semisynthetic diets enriched with high $CdCl_2$ levels, phytase supplementation to the P- and Ca-reduced pig diet with a low Cd concentration significantly enhanced liver and kidney cadmium accumulation. Differences in dietary Cd levels, the binding form of Cd in the diets and the duration of the experimental trials may partially explain the differences found between rats and pigs. Complex interactions between cadmium and various elements, especially calcium, might also have influenced the carry over of cadmium in the pig study. Irrespective of the dietary treatment, liver

and kidney cadmium concentrations in the pigs were considerably lower than maximal permitted values (Rimbach, et al. 1996). In the study by Zacherias et al. (2001) the addition of 800 phytase units/kg to a barley-soybean meal diet caused an increase of Cd retention in kidney and liver of pigs at 30 and 50 kg body weight. Interestingly, this effect was counteracted by the addition of calcium.

TABLE 7. The effect of microbial phytase on Cd accumulation in liver and kidney (P = pig, R = rat)

Species (n per group)	Liveweight	Element	Diet	Cd content of diet (mg/kg)	Phytase added (IU/Kg diet)	Experimental duration (days)	Finding	Reference
R (n = 6)	47 g	Cd	Egg white, Cornstarch	5	2000	28	Liver Cd ↓ Kidney Cd ↓	(Rimbach et al. 1995a)
R (n = 6)	47 g	Cd	Egg white, Cornstarch	5	2000	28	Liver Cd ↓ Kidney Cd↓	(Rimbach et al. 1995b)
R (n = 7)	47 g	Cd	Maize, Soybean meal, Cornstarch, Soybean oil	5	250 500 1000 2000	28	n. e.	(Rimbach et al. 1998)
P (n = 6)	25-100 kg	Cd	Barley, Maize, Soybean meal	21; 15; 13	800	93-107	Liver Cd ↑ Kidney Cd↑	(Rimbach et al. 1996)
P (n = 8; 16)	16.7-50 kg	Cd	Barley, Soybean meal	0.78	800	n. s.	Cd retention in liver↑ Cd retention in kidney↑	(Zacharias et al. 2001)

n. e. = no effect on Cd accumulation
n. s. = not specified

CONCLUSION

Due to its chemical structure, at weak acidic to neutral pH conditions, phytic acid binds essential minerals and trace elements including calcium, magnesium, iron, zinc, copper and manganese thereby decreasing their bioavailability to man and monogastric animals. Overall microbial Aspergillus phytases have the potential to increase mineral and trace element bioavailability from phytate-rich food. Importantly, the FDA has approved Aspergillus niger phytase enzyme preparation as generally recognized as safe (Wodzinski and Ullah 1996).However, so far there have been only a few human studies regarding microbial phytase trace element interactions. Therefore, further systematic investigations in humans regarding the effect of microbial phytase on iron and zinc bioavailability are necessary.

REFERENCES

Adeola, O., Lawrence, B.V., Sutton, A.L., Cline, T.R. (1995) Phytase-induced changes in mineral utilization in zinc-supplemented diets for pigs. *Journal of Animal Science* 73, 3384-91.

Biehl, R.R., Emmert, J.L., Baker, D.H. (1997) Iron bioavailability in soybean meal as affected by supplemental phytase and 1 alpha-hydroxycholecalciferol. *Poultry Science* 76, 1424-7.

Brink, E.J., Dekker, P.R., Van Beresteijn, E.C., Beynen, A.C. (1991) Inhibitory effect of dietary soybean protein vs. casein on magnesium absorption in rats. *The Journal of Nutrition* 121, 1374-81.

Brune, M., Rossander, L., Hallberg, L. (1989) Iron absorption: no intestinal adaptation to a high-phytate diet. *American Journal of Clinical Nutrition* 49, 542-5.

Davidsson, L., Almgren, A., Juillerat, M.A., Hurrell, R.F. (1995) Manganese absorption in humans: the effect of phytic acid and ascorbic acid in soy formula. *American Journal of Clinical Nutrition* 62, 984-7.

Davidsson, L., Galan, P., Cherouvrier, F., Kastenmayer, P., Juillerat, M.A., Hercberg, S., Hurrell, R.F. (1997) Bioavailability in infants of iron from infant cereals: effect of dephytinization. *American Journal of Clinical Nutrition* 65, 916-20.

Davies, N.T., Olpin, S.E. (1979) Studies on the phytate: zinc molar contents in diets as a determinant of Zn availability to young rats. *The British Journal of Nutrition* 41, 590-603.

De Rham, O., Jost, T. (1979) Phytate protein interactions in soybean extracts and low-phytate soy protein products. *Journal of Food Science* 44, 596-600.

Derman, D.P., Ballot, D., Bothwell, T.H., MacFarlane, B.J., Baynes, R.D., MacPhail, A.P., Gillooly, M., Bothwell, J.E., Bezwoda, W.R., Mayet, F. (1987) Factors influencing the absorption of iron from soya-bean protein products. *The British Journal of Nutrition* 57, 345-53.

Eckhout, W., De Paepe, M. (1994) Total phosphorus, phytate-phosphorus and phytase activity in plant feedstuffs. *Animal Feed Science and Technology* 47, 19-29.

Flanagan, P.R. (1984) A model to produce pure zinc deficiency in rats and its use to demonstrate that dietary phytate increases the excretion of endogenous zinc. *The Journal of Nutrition* 114, 493-502.

Fordyce, E.J., Forbes, R.M., Robins, K.R., Erdman, J.W. (1987) Phytate x calcium/zinc molarratios: are they predictive of zinc bioavailibility? *Journal of Food Science* 52, 440-444.

Fretzdorff, B., Brümmer, J.M., Röcken, W., Greiner, R., Konietzny, U., Jany, K.D. (1995) Reduktion des Phytinsäure-Gehaltes bei der Herstellung von Backwaren und

Getreidenährmitteln. *AID-Verbraucherdienst* 40, 12-20.

Gibson, D.M., Ullah, A.B.J. (1990) Phytases and their action on phytic acid. *In: Morre, D. M. (Ed.) Inositol metabolism in plants.* Wiley-Liss Inc.,New York, 77-92.

Hallberg, L., Brune, M., Rossander, L. (1989) Iron absorption in man: ascorbic acid and dose-dependent inhibition by phytate. *American Journal of Clinical Nutrition* 49, 140-4.

Han, O., Failla, M.L., Hill, A.D., Morris, E.R., Smith, J.C., Jr. (1994) Inositol phosphates inhibit uptake and transport of iron and zinc by a human intestinal cell line. *The Journal of Nutrition* 124, 580-7.

Harland, B.F., Morris, E.R. (1995) Phytate: a good or bad component? *Nutrition Research (New York, N.Y.)* 15, 733-754.

House, W.A., Welch, R.M. (1987) Bioavailability to rats of iron in six varieties of wheat grain intrinsically labelled with radio iron. *The Journal of Nutrition* 117, 476-80.

Hurrell, R.F., Reddy, M.B., Juillerat, M.A., Cook, J.D. (2003) Degradation of phytic acid in cereal porridges improves iron absorption by human subjects. *American Journal of Clinical Nutrition* 77, 1213-9.

IUPAC-IUB. (1978) Nomenclature of phosphorus-containing compounds of biochemical importance. (Recommendations 1976). IUPAC-IUB Commission on Biochemical Nomenclature. *The Biochemical Journal* 171, 1-19.

IUPAC-IUB. ((Recommendations of the Nomenclature Committee of the International Union of Biochemistry) 1984) Enzyme nomenclature. *Academic Press Inc., Orlando, San Diego, New York, London, Toronto, Montreal, Sydney, Tokyo.*

Jeroch, H. (1994) Bisherige Erkenntnisse zum Phytaseeinsatz beim Geflügel. *Archiv Geflügelkunde* 58, 1-7.

Kemme, P.A., Jongbloed, A.W., Mroz, Z., Beynen, A.C. (1997a) The efficacy of Aspergillus niger phytase in rendering phytate phosphorus available for absorption in pigs is influenced by pig physiological status. *Journal of Animal Science* 75, 2129-38.

Kemme, P.A., Radcliffe, J.S., Jongbloed, A.W., Mroz, Z. (1997b) The effects of sow parity on digestibility of proximate components and minerals during lactation as influenced by diet and microbial phytase supplementation. *Journal of Animal Science* 75, 2147-53.

Kemme, P.A., Radcliffe, J.S., Jongbloed, A.W., Mroz, Z. (1997c) Factors affecting phosphorus and calcium digestibility in diets for growing-finishing pigs. *Journal of Animal Science* 75, 2139-46.

Kies, A.K., Gerrits, W.J., Schrama, J.W., Heetkamp, M.J., van der Linden, K.L., Zandstra, T., Verstegen, M.W. (2005) Mineral absorption and excretion as affected by microbial phytase, and their effect on energy metabolism in young piglets. *The Journal of Nutrition* 135, 1131-8.

Kies, A.K., Kemme, P.A., Sebek, L.B., van Diepen, J.T., Jongbloed, A.W. (2006) Effect of graded doses and a high dose of microbial phytase on the digestibility of various minerals in weaner pigs. *Journal of Animal Science* 84, 1169-75.

Kornegay, E.T., Denbow, D.M., Yi, Z., Ravindran, V. (1996) Response of broilers to graded levels of microbial phytase added to maize-soyabean-meal-based diets containing three levels of non-phytate phosphorus. *The British Journal of Nutrition* 75, 839-52.

Kornegay, E.T., Qian, H. (1996) Replacement of inorganic phosphorus by microbial phytase for young pigs fed on a maize-soyabean-meal diet. *The British Journal of Nutrition* 76, 563-78.

Lantzsch, H. (1990) Untersuchungen über ernährungsphysiologische Effekte des Phytats bei Monogastriern (Ratte, Schwein):. *Übersichten Tierernährung* 18, 839-852.

Lee, D.Y., Schroeder, J., 3rd, Gordon, D.T. (1988) Enhancement of Cu bioavailability in the rat by phytic acid. *The Journal of Nutrition* 118, 712-7.

Lei, X., Ku, P.K., Miller, E.R., Ullrey, D.E., Yokoyama, M.T. (1993a) Supplemental microbial phytase improves bioavailability of dietary zinc to weanling pigs. *The Journal of Nutrition* 123, 1117-23.

Lei, X.G., Ku, P.K., Miller, E.R., Yokoyama, M.T. (1993b) Supplementing corn-soybean meal diets with microbial phytase linearly improves phytate phosphorus utilization by weanling pigs. *Journal of Animal Science* 71, 3359-67.

Lei, X.G., Ku, P.K., Miller, E.R., Yokoyama, M.T., Ullrey, D.E. (1993c) Supplementing corn-soybean meal diets with microbial phytase maximizes phytate phosphorus utilization by weanling pigs. *Journal of Animal Science* 71, 3368-75.

Liebert, F., Wecke, C., Schöner, F.J. (1993) Phytase activities in different gut contents of chickens as dependent of level of phosphorus and phytase supplementations. *In: Wenk, C., Boessinger, M. (Eds.) Enzymes in Animal Nutrition. Institut für Nutztierwissenschaften ETH Zürich* 202-205.

Liu, J., Bollinger, D.W., Ledoux, D.R., Ellersieck, M.R., Veum, T.L. (1997) Soaking increases the efficacy of supplemental microbial phytase in a low-phosphorus corn-soybean meal diet for growing pigs. *Journal of Animal Science* 75, 1292-8.

Lonnerdal, B., Sandberg, A.S., Sandstrom, B., Kunz, C. (1989) Inhibitory effects of phytic acid and other inositol phosphates on zinc and calcium absorption in suckling rats. *The Journal of Nutrition* 119, 211-4.

Mendoza, C., Viteri, F.E., Lonnerdal, B., Young, K.A., Raboy, V., Brown, K.H. (1998) Effect of genetically modified, low-phytic acid maize on absorption of iron from tortillas. *American Journal of Clinical Nutrition* 68, 1123-7.

Morris, E.R., Ellis, R. (1980) Effect of dietary phytate/zinc molar ratio on growth and bone zinc response of rats fed semipurified diets. *The Journal of Nutrition* 110, 1037-45.

Mroz, Z., Jongbloed, A.W., Kemme, P.A. (1994) Apparent digestibility and retention of nutrients bound to phytate complexes as influenced by microbial phytase and feeding regimen in pigs. *Journal of Animal Science* 72, 126-32.

Murry, A.C., Lewis, R.D., Amos, H.E. (1997) The effect of microbial phytase in a pearl millet-soybean meal diet on apparent digestibility and retention of nutrients, serum mineral concentration, and bone mineral density of nursery pigs. *Journal of Animal Science* 75, 1284-91.

Nelson, T.S., Shieh, T.R., Wodzinski, R.J., Ware, J.H. (1968) The availability of phytate phosphorus in soybean meal before and after treatment with a mold phytase. *Poultry Science* 47, 1842-8.

Nelson, T.S., Shieh, T.R., Wodzinski, R.J., Ware, J.H. (1971) Effect of supplemental phytase on the utilization of phytate phosphorus by chicks. *The Journal of Nutrition* 101, 1289-93.

Nyannor, E.K., Williams, P., Bedford, M.R., Adeola, O. (2007) Corn expressing an Escherichia coli-derived phytase gene: a proof-of-concept nutritional study in pigs. *Journal of Animal Science* 85, 1946-52.

O'Quinn, P.R., Knabe, D.A., Gregg, E.J. (1997) Efficacy of Natuphos in sorghum-based diets of finishing swine. *Journal of Animal Science* 75, 1299-307.

Oberleas, D., Harland, B.F. (1981) Phytate content of foods: effect on dietary zinc bioavailability. *Journal of the American Dietetic Association* 79, 433-6.

Pallauf, J., Höhler, D., Rimbach, G. (1992a) Effekt einer Zulage an mikrobieller Phytase zu einer Mais-Soja-Diät auf die scheinbare Absorption von Mg, Fe, Cu, Mn und Zn sowie auf Parameter des Zinkstatus beim Ferkel. *Journal of Animal Physiology and Animal Nutrition.* 68, 1-9.

Pallauf, J., Höhler, D., Rimbach, G., Neusser, H. (1992b) Einfluß einer Zulage an mikrobieller Phytase zu einer Mais-Soja-Diät auf die scheinbare Absorption von Phosphor und Calcium beim Ferkel. *Journal of animal physiology and animal nutrition.* 67, 30-40.

Pallauf, J., Kramer, K., Markwitan, A., Ebel, D. (1990) [The effect of a supplement of citric acid on the bioavailability of zinc from corn germ]. *Zeitschrift für Ernährungswissenschaft* 29, 27-38.

Pallauf, J., Pippig, S., Most, E., Rimbach, G. (1999) Supplemental sodium phytate and microbial phytase influence iron availability in growing rats. *Journal of Trace Elements in Medicine and Biology* 13, 134-40.

Pallauf, J., Rimbach, G., Pippig, S., Schindler, B., Hohler, D., Most, E. (1994a) Dietary effect of phytogenic phytase and an addition of microbial phytase to a diet based on field beans, wheat, peas and barley on the utilization of phosphorus, calcium, magnesium, zinc and protein in piglets. *Zeitschrift für Ernährungswissenschaft* 33, 128-35.

Pallauf, J., Rimbach, G., Pippig, S., Schindler, B., Most, E. (1994b) Effect of Phytase Supplementation to a Phytate-Rich Diet Based on Wheat, Barley and Soya on the Bioavailability of Dietary Phosphorus, Calcium, Magnesium, Zinc and Protein in Piglets. *Agribiological Research* 47, 39-48.

Phillippy, B.Q., Bland, J.M. (1988) Gradient ion chromatography of inositol phosphates. *Analytical Biochemistry* 175, 162-6.

Pietsch, M., Rimbach, G., Pallauf, J. (1995) Zur Auswirkung einer abgestuften alimentären Phytinsäurezufuhr auf den Magnesiumstatus wachsender Ratten. *Proceedings of the Society of Nutrition Physiology* 3, 93.

Porres, J.M., Aranda, P., Lopez-Jurado, M., Urbano, G. (2006) Nutritional evaluation of protein, phosphorus, calcium and magnesium bioavailability from lupin (Lupinus albus var. multolupa)-based diets in growing rats: effect of alpha-galactoside oligosaccharide extraction and phytase supplementation. *The British Journal of Nutrition* 95, 1102-11.

Radcliffe, J.S., Pleasant, R.S., Kornegay, E.T. (2006) Estimating equivalency values of microbial phytase for amino acids in growing and finishing pigs fitted with steered ileo-cecal valve cannulas. *Journal of Animal Science* 84, 1119-29.

Radcliffe, J.S., Zhang, Z., Kornegay, E.T. (1998) The effects of microbial phytase, citric acid, and their interaction in a corn-soybean meal-based diet for weanling pigs. *Journal of Animal Science* 76, 1880-6.

Ravindran, V., Bryden, W.W., Kornegay, E.T. (1995) Phytates: occurrence, bioavailability, and implication in poultry nutrition. *Poultry and Avian Biology Reviews* 6, 125-143.

Revy, P.S., Jondreville, C., Dourmad, J.Y., Nys, Y. (2006) Assessment of dietary zinc requirement of weaned piglets fed diets with or without microbial phytase. *Journal of Animal Physiology and Animal Nutrition* 90, 50-9.

Rimbach, G., Brandt, K., Most, E., Pallauf, J. (1995a) Supplemental phytic acid and microbial phytase change zinc bioavailability and cadmium accumulation in growing rats. *Journal of Trace Elements in Medicine and Biology* 9, 117-22.

Rimbach, G., Pallauf, J. (1992) [The effect of a supplement of microbial phytase on zinc availability]. *Zeitschrift für Ernährungswissenschaft* 31, 269-77.

Rimbach, G., Pallauf, J. (1993) Enhancement of zinc utilization from phytate-rich soy protein isolate by microbial phytase. *Zeitschrift für Ernährungswissenschaft* 32, 308-15.

Rimbach, G., Pallauf, J., Brandt, K., Most, E. (1995b) Effect of phytic acid and microbial phytase on Cd accumulation, Zn status, and apparent absorption of Ca, P, Mg, Fe, Zn, Cu, and Mn in growing rats. *Annals of Nutrition and Metabolism* 39, 361-70.

Rimbach, G., Pallauf, J., Walz, O.P. (1996) Effect of microbial phytase on cadmium accumulation in pigs. *Archiv für Tierernährung* 49, 279-86.

Rimbach, G., Walter, A., Most, E., Pallauf, J. (1997) Effect of supplementary microbial phytase to a maize-soya-diet on the availability of calcium, phosphorus, magnesium and zinc: in vitro dialysability in comparison with apparent absorption in growing rats. *Journal of Animal Physiology and Animal Nutrition* 77, 1-9.

Rimbach, G., Walter, A., Most, E., Pallauf, J. (1998) Effect of microbial phytase on zinc bioavailability and cadmium and lead accumulation in growing rats. *Food and Chemical Toxicology* 36, 7-12.

Rodehutscord, M., Faust, M., Hof, C. (1997) Digestibility of phosphorus in protein-rich ingredients for pig diets. *Archiv für Tierernährung* 50, 201-11.

Rodehutscord, M., Krause, G., Pfeffer, E. (1999) Effect of body weight on phosphorus digestibility and efficacy of a microbial phytase in young pigs. *Archiv für Tierernährung* 52, 139-53.

Sandberg, A.S., Ahderinne, R. (1986) HPLC Method for determination of inositol tri-, terta-, penta, and hexaphosphates in foods and intestinal contents. . *The Journal of Nutrition* 51, 547-550.

Sandberg, A.S., Hulthen, L.R., Turk, M. (1996) Dietary Aspergillus niger phytase increases iron absorption in humans. *The Journal of Nutrition* 126, 476-80.

Sandberg, A.S., Larsen, T., Sandstrom, B. (1993) High dietary calcium level decreases colonic phytate degradation in pigs fed a rapeseed diet. *The Journal of Nutrition* 123, 559-66.

Schone, F., Ludke, H., Kirchheim, U. (1995) [Evaluation of Aspergillus niger phytase and phosphate in weaned piglets. 2. Content and gain of fat, energy, ash, Ca and P in the animal body]. *Archiv für Tierernährung* 47, 219-28.

Schöner, F.J., Hoppe, P.P., Schwarz, G. (1991) Vergleich der Effekte von mikrobieller Phytase und anorganischem Phosphat auf die Leistung und die Retention von Phosphor, Calcium und Rohasche bei Masthühnerküken in der Anfangsmast. *Journal of Animal Physiology and Animal Nutrition* 66, 248-255.

Schöner, F.J., Hoppe, P.P., Schwarz, G., Wiesche, H. (1993) Vergleich von mikrobieller Phytase und anorganischen Phosphat bei Masthahnenküken: Wirkung auf die Mastleitung und die Mineralstoffretention bei variierter Calciumversorgung. *Journal of Animal Physiology and Animal Nutrition* 69, 235-244.

Schricker, B.R., Miller, D.D., Van Campen, D. (1983) Effects of iron status and soy protein on iron absorption by rats. *The Journal of Nutrition* 113, 996-1001.

Schröder, B., Breves, G., Rodehutscord, M. (1996) Mechanisms of intestinal phosphorus absorption and availability of dietary phosphorus in pigs. *Deutsche Tierärztliche Wochenschrift* 103, 209-214.

Shelton, J.L., LeMieux, F.M., Southern, L.L., Bidner, T.D. (2005) Effect of microbial phytase addition with or without the trace mineral premix in nursery, growing, and finishing pig diets. *Journal of Animal Science* 83, 376-85.

Shelton, J.L., Southern, L.L., LeMieux, F.M., Bidner, T.D., Page, T.G. (2004) Effects of microbial phytase, low calcium and phosphorus, and removing the dietary trace mineral premix on carcass traits, pork quality, plasma metabolites, and tissue mineral content in growing-finishing pigs. *Journal of Animal Science* 82, 2630-9.

Simons, P.C., Versteegh, H.A., Jongbloed, A.W., Kemme, P.A., Slump, P., Bos, K.D., Wolters, M.G., Beudeker, R.F., Verschoor, G.J. (1990) Improvement of phosphorus availability by microbial phytase in broilers and pigs. *The British Journal of Nutrition* 64, 525-40.

Simons, P.C., Versteegh, H.A.J. (1993) Role of Phytase in poultry nutrition. *In: Wenk, C., Boessinger, M. (Eds.) Enzymes in Animal Nutrition. Institut für Nutztierwissenschaften ETH Zürich* 181-186.

Stahl, C.H., Han, Y.M., Roneker, K.R., House, W.A., Lei, X.G. (1999) Phytase improves iron bioavailability for hemoglobin synthesis in young pigs. *Journal of Animal Science* 77, 2135-42.

Taylor, T.G., Coleman, J.W. (1979) A comparative study of the absorption of calcium and the availability of phytate-phosphorus in the golden hamster (Mesocricetus auratus) and the laboratory rat. *The British Journal of Nutrition* 42, 113-9.

Thompson, D.B., Erdman, J.W., Jr. (1984) The effect of soy protein isolate in the diet on retention by the rat of iron from radiolabeled test meals. *The Journal of Nutrition* 114, 307-11.

Traylor, S.L., Cromwell, G.L., Lindemann, M.D., Knabe, D.A. (2001) Effects of level of supplemental phytase on ileal digestibility of amino acids, calcium, and phosphorus in dehulled soybean meal for growing pigs. *Journal of Animal Science* 79, 2634-42.

Vogt, H. (1992a) Einsatz von Phytase im Broilermastfutter mit unterschiedlichem Phosphatgehalt. *Archiv Geflügelkunde* 56, 93-98.

Vogt, H. (1992b) Einsatz von Phytase im Broilermastfutter mit unterschiedlichem Phosphorgehalt: 2. Versuch. *Archiv Geflügelkunde* 56, 222-226.

Walz, O.P., Pallauf, J. (2003) The effect of the combination of microbial phytase and amino acid supplementation of diets for finishing pigs on P and N excretion and carcass quality. *Archiv für Tierernährung* 57, 413-28.

Windisch, W., Kirchgessner, M. (1996) Zum Einfluss von mikrobieller Phytase auf Mastleistung und den Stoffwechsel von Phosphor, Calcium und Stickstoff bei abgestufter Calcium-Versorgung in der Broilermast. *Archiv Geflügelkunde* 60, 42-47.

Xu, P., Price, J., Aggett, P.J. (1992) Recent advances in methodology for analysis of phytate and inositol phosphates in foods. *Progress in Food & Nutrition Science* 16, 245-62.

Yi, Z., Kornegay, E.T., Ravindran, V., Denbow, D.M. (1996a) Improving phytate phosphorus availability in corn and soybean meal for broilers using microbial phytase and calculation of phosphorus equivalency values for phytase. *Poultry Science* 75, 240-9.

Yi, Z., Kornegay, E.T., Ravindran, V., Lindemann, M.D., Wilson, J.H. (1996b) Effectiveness of Natuphos phytase in improving the bioavailabilities of phosphorus and other nutrients in soybean meal-based semipurified diets for young pigs. *Journal of Animal Science* 74, 1601-11.

Young, L.G., Leunissen, M., Atkinson, J.L. (1993) Addition of microbial phytase to diets of young pigs. *Journal of Animal Science* 71, 2147-50.

Zacharias, B., Lantzsch, H.J., Drochner, W. (2001) The influence of dietary microbial phytase and calcium on the accumulation of cadmium in different organs of pigs. *Journal of Trace Elements in Medicine and Biology* 15, 109-14.

Zhou, J.R., Erdman, J.W., Jr. (1995) Phytic acid in health and disease. *Critical Reviews in Food Science and Nutrition* 35, 495-508.

Schuster, E.

Appl Microbiol Biotechnol (2002) 59:426–435
DOI 10.1007/s00253-002-1032-6

E. Schuster · N. Dunn-Coleman · J. C. Frisvad
P. W. M. van Dijck

On the safety of *Aspergillus niger* – a review

Received: 6 March 2002 / Revised: 17 April 2002 / Accepted: 19 April 2002 / Published online: 25 June 2002

Abstract *Aspergillus niger* is one of the most important microorganisms used in biotechnology. It has been in use already for many decades to produce extracellular (food) enzymes and citric acid. In fact, citric acid and many *A. niger* enzymes are considered GRAS by the United States Food and Drug Administration. In addition, *A. niger* is used for biotransformations and waste treatment. In the last two decades, *A. niger* has been developed as an important transformation host to over-express food enzymes. Being pre-dated by older names, the name *A. niger* has been conserved for economical and information retrieval reasons and there is a taxonomical consensus based on molecular data that the only other common species closely related to *A. niger* in the *Aspergillus* series Nigri is *A. tubingensis*. *A. niger*, like other filamentous fungi, should be treated carefully to avoid the formation of spore dust. However, compared with other filamentous fungi, it does not stand out as a particular problem concerning allergy or mycopathology. A few medical cases, e.g. lung infections, have been reported, but always in severely immunocompromised patients. In tropical areas, ear infections (otomycosis) do occur due to *A. niger* invasion of the outer ear canal but this may be caused by mechanical damage of the skin barrier. *A. niger* strains produce a series of secondary metabolites, but it is only ochratoxin A that can be regarded as a mycotoxin in the strict sense of the word. Only 3–10% of the strains examined for ochratoxin A production have tested positive under favourable conditions. New and unknown isolates should be checked for ochratoxin A production before they are developed as production organisms. It is concluded, with these restrictions, that *A. niger* is a safe production organism.

Dr. Schuster (presently retired from Röhm GmbH – Röhm is now AB Enzymes GmbH) prepared the first draft of this manuscript in 1993 on behalf of the AD HOC 5 working group of AMFEP [Association of Manufacturers of Fermentation Enzyme Products, Brussels]; later on, contributions from Dr. Dunn-Coleman and Dr. Frisvad were included. The present manuscript is a revised and updated version prepared by Drs. Frisvad and Van Dijck.

E. Schuster
Enzyme Technology, Röhm GmbH, Kirschenallee 45,
64293 Darmstadt, Germany

N. Dunn-Coleman
Genencor International Inc., 925 Page Mill Road,
Palo Alto, CA 94304, USA

J.C. Frisvad
BioCentrum-DTU, Building 221,
Technical University of Denmark,
2800 Kgs. Lyngby, Denmark

P.W.M. van Dijck (✉)
DSM Food Specialties, Department of Regulatory Affairs,
600–0245, P.O. Box 1, 2600MA, Delft, The Netherlands
e-mail: piet.dijck-van@dsm.com

Introduction

Aspergillus niger has been the subject of research and industrial use for several decades. It first acquired practical importance in 1919, when its ability to produce citric acid was industrially exploited. Gluconic and fumaric acids have been produced with *A. niger*, although they are of less economic importance. However, since the 1960s, *A. niger* has become a source of a variety of enzymes that are well established as technical aids in fruit processing, baking, and in the starch and food industries. Gene technology has been successfully applied to improve production processes and to make use of *A. niger* as an expression system for foreign proteins. The intense research over the past decade has resulted in a range of new processes and products.

Ecology

Many black *Aspergilli* have been isolated from all over the world. *A. niger* is a filamentous fungus growing aerobically on organic matter. In nature, it is found in soil and litter, in compost and on decaying plant material. Reiss (1986) collected data on the influence of temperature, water activity and pH on the growth of various

Aspergilli. *A. niger* is able to grow in the wide temperature range of 6–47°C with a relatively high temperature optimum at 35–37°C. The water activity limit for growth is 0.88, which is relatively high compared with other *Aspergillus* species. *A. niger* is able to grow over an extremely wide pH range: 1.4–9.8. These abilities and the profuse production of conidiospores, which are distributed via the air, secure the ubiquitous occurrence of the species, with a higher frequency in warm and humid places (Rippel-Baldes 1955).

Taxonomy

Raper and Fennel (1965) divided the genus *Aspergillus* into groups according to the colour of the conidiospores. *Aspergilli* with brown to black-shaded spores constitute the *A. niger* group. Although the members of this group vary considerably, only a few differ so clearly from the majority that they can easily be classified as separate species (e.g. *A. carbonarius*, *A. japonicus*, *A. ellipticus*, *A. heteromorphus* and *A. aculeatus*). Most of the brown to black *Aspergilli* belong to the other group of species, which are difficult to distinguish: *A. ficuum*, *A. phoenicis*, *A. niger* and *A. awamori* being the most prominent. In practice, this group of species is often together called *A. niger* van Tieghem.

The apparently insignificant differences between members of the *A. niger* group were the decisive reasons for Al-Musallam (1980) to classify some species as varieties of *A. niger*, while Raper and Fennell considered them to be separate species.

Introducing restriction fragment length polymorphism (RFLP) analysis to *Aspergillus* taxonomy, Kusters-van Someren et al. (1990, 1991) analysed the ribosomal banding patterns and the hybridisation patterns of genomic digests from strains in the *A. niger* group, using pectin lyase genes as probes for hybridisation. They proposed a different, but in their opinion more reliable, classification of the *A. niger* group, reducing the number of species from 13 (Raper and Fennel 1965) to 6: *A. carbonarius*, *A. japonicus*, *A. ellipticus*, *A. heteromorphus*, *A. niger* and *A. tubingensis*, the latter of which consists of strains formerly called *A. niger*. The difference between *A. niger* and *A. tubingensis* has been repeatedly confirmed in further studies using RFLP of mitochondrial DNAs and ribosomal repeat units (Varga et al. 1993, 1994; Parenicová et al. 1997), but also in studies using randomly amplified polymorphic DNA (Megnegneau et al. 1993); internal transcribed spacer sequence data (Accensi et al. 1999; Parenicová et al. 2001), and nuclear genes encoding polygalacturonases, arabinoxylan-arabinofuranohydrolase and xylanases (Bussink et al. 1991; de Graaf et al. 1994; Gielkens et al. 1997). Despite this agreement on the molecular separation of *A. niger* and *A. tubingensis*, no phenotypic differences have yet been found between the two species (Varga et al. 2000; Parenicová et al. 2000). This may be the reason that *A. tubingensis* is not yet listed among species of *Aspergillus* in current use (Pitt and Samson 2000; Pitt et al. 2000).

The name *A. niger* is predated by the names *A. phoenicis* and *A. ficuum* and, provided it is accepted that these three taxa are all conspecific, as implied by most molecular studies (Parenicová et al. 2000; Varga et al. 2000), the latter two taxa would have nomenclatural priority. Since these latter two names are nowadays rarely used, it was proposed at the Second International Workshop on *Penicillium* and *Aspergillus* that the name *A. niger* was to be conserved and *A. phoenicis* and *A. ficuum* to be rejected (Frisvad et al. 1990; Kozakiewicz et al. 1992). *A. niger* is a species of major economic importance and the name is now conserved for practical, information retrieval and economical reasons, and in the interest of continuity in legal affairs and approval procedures. Despite this, *A. phoenicis*, *A. awamori* and *A. foetidus* are still mentioned in the list of types of species in current use (Pitt and Samson 2000), whereas the species *A. tubingensis*, *A. acidus*, and *A. citricus* are not listed (Kozakiewicz 1989). A case can be made for keeping the name *A. awamori* for the domesticated form of *A. niger* in parallel with keeping the name *A. oryzae* for the domesticated form of *A. flavus*, *A. sojae* for the domesticated form of *A. parasiticus*, and *Penicillium camemberti* for the domesticated form of *P. commune* (Pitt et al. 2000). The appearance on this list of *A. phoenicis* and *A. foetidus* is dubious, however.

Today's practice, that the designation *A. niger* van Tieghem includes strains which could be named *A. awamori*, *A. ficuum*, *A. foetidus*, *A. phoenicis*, *A. pulverulentus*, *A. tubingensis*, *A. inuii* and *A. usamii* should be continued. If, however, phenotypic differences are found between *A. niger* and *A. tubingensis* this may lead to acceptance of the latter taxon. Some molecular data have also indicated that *A. foetidus* (Parenicová et al. 2000) and a *nomen nudum A. brasiliensis* (Varga et al. 2000) are distinct species, but phenotypic data backing this up are meagre at best. The species currently acceptable in section *Nigri* are listed in Table 1.

The most recent supraspecific scheme for the genus *Aspergillus* was suggested by Gams et al. (1985), placing all species with dark brown to black-shaded conidia into the section *Nigri* of a proposed subgenus *Circumdati*. Their proposal has been accepted by the International Commission on *Penicillium* and *Aspergillus* (Samson 1992).

Industrial use

A. niger became an industrially used organism when citric acid was first produced by fermentation in 1919. Citric acid is widely used in a variety of industries and, by sales volume, greatly exceeds other metabolites such as gluconic acid (Roukas 2000). Citric acid is the primary acidulant in the food and beverage industries. It is used in foods such as soft drinks, fruit juices, desserts, jams, jellies, candy and wine. In the pharmaceutical in-

Table 1 Currently accepted species in *Aspergillus* subgenus *Circumdati* section *Nigri* (Kusters-van Someren et al. 1991; Parenicová et al. 2000, 2001)

Species generally accepted	Ochratoxin A production potential
A. niger	+ (low frequency)
A. carbonarius	++ (high frequency)
A. ellipticus	–
A. heteromorphus	–
A. aculeatus	–
A. japonicus	–
Species distinguishable with molecular data only:	
A. tubingensis (= *A. acidus* = *A. acidus* var. *pallidus*)	–
Species accepted by some authors (but = *A. niger* based on molecular data)	
A. foetidus	–
A. citricus	++ (high frequency)

dustry, iron citrate is used as a source of iron and citric acid as a preservative for stored blood; in the cosmetics and toiletries industries it is used as a buffer, for pH adjustment and as an anti-oxidant. It is also used in industrial applications including detergents, leather tanning, in electroplating and other applications where sequestering agent activity in the neutral to low pH range is required. Citric acid is produced almost exclusively by fermentation of *A. niger* and *A. wentii* because yields of these organisms are economic and formation of undesired side products is minimal. The Food and Drug Administration (FDA) has listed *A. niger* as a source of citric acid (21 Code of Federal Regulations §173.280).

In addition to citric acid, *A. niger* is a rich source of enzymes. Pectinase, protease and amyloglucosidase were the first to be exploited, and were originally produced in surface culture (Frost and Moss 1987). Although it had been shown by Kluyver's group in Delft as early as 1932 that it was possible to cultivate a filamentous fungus like *A. niger* in submerged culture (Kluyver and Perquin 1932), the technology was first applied to the production process of penicillin G by *Penicillium chrysogenum* in 1942. After 1950, production technology for fungal products gradually changed from surface culture to stirred-tank processes, but up until the mid 1960s companies used surface culture processes (Barbesgaard et al. 1992). Several additional enzymes like cellulase and hemicellulase were manufactured using black *Aspergillus* strains in stirred tank processes.

For the manufacture of many products, starch – one of the most abundant carbohydrates – must be hydrolyzed to syrups, which contain glucose, maltose and low molecular weight dextrins. Amyloglucosidase, also referred to as glucoamylase, is an exo-amylase catalysing the release of successive glucose units from the non-reducing ends of starch by hydrolysing α-1,4-D-glucosidic linkages. The glucose syrup and the alcohol industries are the principal users of amyloglucosidase produced by *A. niger*.

Pectin, a heteropolysaccharide, is a principal component in commercially important fruits and vegetables. Several enzymes, including pectin esterases, endo- and exo-polygalacturonidases and pectin lyases, produced from *A. niger* degrade pectin; they are used in wine and fruit juice production to reduce juice viscosity before pressing and improve clarification (Grassin and Fauguenbergue 1999).

It is established practice to improve the baking process by adding hemicellulases from *A. niger* when mixing the dough. The enzymes modify the rheological properties of the dough and give higher loaf volume and better crumb structure of bread and pastry.

A. niger glucose oxidase and catalase are used for determination of glucose mainly in diagnostic enzyme kits, for the removal of either glucose or oxygen from foods and beverages and for the production of gluconic acid from glucose (Berka et al. 1992).

FAO/WHO experts have repeatedly reviewed and accepted enzyme preparations from *A. niger* including the organism itself (FAO/WHO 1972, 1978, 1981, 1987, 1990), listing them with an Acceptable Daily Intake of 'not specified'. The FDA in the United States has accepted numerous enzymes for food use: in the early 1960s the FDA issued opinion letters recognizing that α-amylase, cellulase, amyloglucosidase, catalase, glucose oxidase, lipase and pectinase from *A. niger* can be 'generally regarded as safe' (GRAS) under the condition that non-pathogenic and non-toxigenic strains and current good manufacturing practices be used in production. In addition to these enzymes, Godfrey and Reichelt (1983) claimed GRAS status for β-galactosidase and protease from *A. niger*. Carbohydrase and cellulase from *A. niger* are also approved as a secondary direct food additive by the FDA as an aid in clam and shrimp processing (21 Code of Federal Regulations §173.120).

Until the 1980s, *A. niger* industrial production strains were isolated through the use of classical mutagenesis followed by screening and/or selection. Parasexual crossing has also been used in strain improvement efforts in *Aspergilli*, which lack a sexual cycle. For example, Das and Roy (1978) have reported improved production of citric acid by a diploid strain of *A. niger* generated by parasexual crossing.

With the development of DNA-mediated transformation of *Aspergilli*, initially in *A. nidulans* (Ballance et al. 1983; Tilburn et al. 1983), and subsequently in *A. niger*

(Buxton et al. 1985; Kelly and Hynes 1985; Van Hartingsveldt et al. 1987; Ward et al. 1988; Campbell et al. 1989), this very useful technology was applied to using *A. niger* as a host for gene expression. For example, the production of native *A. niger* catalase has been increased using recombinant techniques (Berka et al. 1994a, b), whereas a 1,000-fold improvement in the expression level for *A. niger* phytase was achieved by using recombinant technology (Van Gorcom et al. 1991; Van Hartingsveldt et al. 1993; Selten 1994).

The long history of safe use on an industrial scale makes *A. niger* exceptionally well suited to be used as a host for heterologous expression. A number of genes of commercial importance and their regulatory sequences that could be used as components in industrial expression systems have been cloned (Nunberg et al. 1983, 1984; Bussink et al. 1990; Harmsen et al. 1990; Nguyen et al. 1991).

The strategy of employing the promoter of a highly expressed fungal gene for the expression of a heterologous gene (Cullen et al. 1987) proved successful when Dunn-Coleman et al. (1991) obtained expression by *A. niger* var. *awamori* of commercially viable levels of calf chymosin under the control of the glucoamylase promoter. This enzyme has been accorded GRAS status by the FDA (Federal Register 1993).

After the cloning of the phytase gene (Mullaney et al. 1991; Van Gorcom et al. 1991; Van Hartingsveldt et al. 1993; Piddington et al. 1993) the gene cloned from an *A. niger* strain was inserted in an expression cassette under the control of the strong glucoamylase promoter. This expression cassette was randomly integrated in multiple copies in the genome of an industrial *A. niger* glucoamylase production strain (Selten 1994; Van Dijck 1999). One of the reasons for the high production level of glucoamylase in this particular strain is the multiplication of a region in the DNA containing, among other things, the promoter and coding sequence of the glucoamylase gene, the *gla*A-locus. Using advanced proprietary genetic modification techniques (Selten et al. 1995, 1998), this locus was "emptied" and subsequently "filled" with "genes of interest" in expression cassettes under the control of the host *gla*-promoter. Compared to the original glucoamylase overproducing strain they are completely identical except for the fact that the "gene of interest" replaces the glucoamylase gene (Groot et al. 2000). This has several advantages. New production strains can now be designed and built in a predictable manner. In addition, and this is important from a regulatory point of view, this technique of targeted integration by definition cannot cause any pleiotropic efforts by perturbing the rest of the genome. This is often raised as a (hypothetical) possibility by regulatory bodies in case of product approvals where the production strain was obtained by random integration of genes.

To date, many enzyme products are available on the market from recombinant strains of *A. niger*. In a recent listing prepared by the Enzyme Technical Association, the enzymes α-amylase, arabinofuranosidase, catalase, chymosin, glucoamylase, glucose oxidase, pectin esterase, phospholipase A2, phytase, and xylanase are mentioned as being produced by recombinant strains of *A. niger* (Pariza and Johnson 2001).

Safety aspects

A. niger is generally regarded as a safe organism. This is documented in lists of the organisations responsible for occupational health and safety [e.g. Berufsgenossenschaft der Chemischen Industrie (1998)]. In rare cases when persons are exposed to intense spore dust, hypersensitivity reactions have been observed.

Pathogenicity

A. niger is generally regarded as a non-pathogenic fungus widely distributed in nature. Humans are exposed to its spores every day without disease becoming apparent. Only in few cases has *A. niger* been able to colonise the human body as an opportunistic invader and in almost all these cases the patients have a history of severe illness or immunosuppressive treatment.

Animal studies

Several experimental studies to demonstrate the pathogenic potential of *A. niger* have come to the conclusion that neither ingestion of large doses of spores (Nyiredy et al. 1975) nor inhalation of spores (Bhatia and Mohapatra 1969) induces mycosis in experimental animals. One day after ingestion, *A. niger* was no longer detected in the digestive tract, although ingested *A. nidulans* was isolated from the intestine of the animals. In contrast to *A. fumigatus*, which is known to be pathogenic, *A. niger* showed no significant effect on the animals in the inhalation study.

Compromising the immune system by steroid hormones seems to promote the spreading of the fungus in the body after an infection. Jacob et al. (1984) conducted a study with mice infected intravenously with high doses of *A. niger* isolated from sputum. They found evidence of pathogenic action only in groups that had been treated with a hydrocortisone drug. Addition of Decadron, a steroid hormone, to the culture medium of *A. niger* induced more vigorous corneal ulceration in rabbit eyes infected with spores compared to animals inoculated with spores from medium without the steroid (Hasany et al. 1973). The authors conclude that exposure of the ordinarily harmless fungus to the steroid made them behave like a pathogen.

Medical case reports

Washburn et al. (1986) found in their immunological study using human sera that *A. fumigatus* produces substances inhibiting complement, which induces phagocy-

tosis of fungal cells by leukocytes. This defence mechanism against infection was not impaired by liquid from an *A. niger* culture.

Few cases of primary cutaneous aspergillosis caused by *A. niger* are given in the literature. Cahill et al. (1967) reported a severe infection, which had long been wrongly been diagnosed as leprosy, and its successful treatment with nystatin.

Mycosis of the ear is one of the frequent health problems in the tropics. *A. niger* has been isolated from 5% of cases of chronic otitis media in Nigeria (Ibekwe and Okafor 1983). However, the authors consider the fungus to be a secondary invader rather than the causative organism because in most cases the patients had been treated with antibiotics before *A. niger* was isolated from their ears. Paldrok (1965) identified 61 fungal isolates from ear lesions in Sweden, 22 of which were of the *A. niger* group. The high incidence of *A. niger* in the outer ear, they speculate, could possibly be due to the fact that *Aspergilli* are resistant to the fungistatic action of ear wax (cerumen).

These ear infections, called otomycosis, cause local inflammation and mycelial growth on cerumen on the skin of the external ear canal. Whilst relatively common in the tropics, this is not a serious condition and can be treated easily with topical antifungal ointment (Mugliston and O'Donoghue 1985; Paulose et al. 1989). Loh et al. (1998) point to self-cleaning of the ears, leading to mechanical damage of the skin barrier, as an important factor in the occurrence of otomycosis.

A. niger can cause pulmonary infection (Binder et al. 1982; Denning 1998). In rare cases it will invade existing pulmonary cavities and create a ball of matted hyphae known as aspergilloma. This aspergilloma may be present for years and may produce oxalic acid in situ, which may lead to renal problems caused by oxalosis (Nime and Hutchins 1973; Severo et al. 1997).

A number of reports on secondary aspergillosis (reviewed in Abramson et al. 1986; Rippon 1982; Saravia-Gomez 1979), caused by often unidentified *Aspergilli*, describe infection in patients suffering from diabetes, drug abuse, alcoholism, severe diseases such as pneumonia, tuberculosis, enterocolitis or patients receiving antibiotic, steroid, cytotoxin or radiation therapy. These groups of debilitated patients, whose immune systems are in many cases weakened, are characterised by an increased susceptibility to opportunistic micro-organisms that do not pose any risk to healthy people.

While abnormal ports of entry like wounds, burns and lesions of the mucosa can facilitate infection in patients, the only entrances in the healthy person are the digestive and respiratory tracts. Thus, reports on health problems of people extensively exposed to *Aspergillus* spores are of special relevance for risk assessment.

Hypersensitivity

Tomsikova et al. (1981) investigated the hypersensitivity pneumonitis of workers in a citric acid plant. They were exposed to spore dust from the production organism *A. niger* and from contaminating fungi. Although both *Aspergillus* and *Penicillium* have been isolated from the respiratory tracts of this group, the concentration of antibodies against *Penicillium* was significantly higher and more frequent than that against *Aspergillus*. This led the authors to the conclusion that hypersensitivity pneumonitis has developed mainly as a result of inhaled *Penicillium* spores and not *Aspergillus* spores.

In another citric acid plant, Topping et al. (1985) showed that only one-half of the workers suffering from bronchospasm were sensitive to *A. niger* spores, while the other half was sensitive neither to spores nor to other substances collected by filtration from the air inside the plant. Now that the spores have been recognised as the most frequent source of hypersensitivity, their dispersal is minimised by technical means and by turning from surface culture methods to submerged production processes which reduce sporulation of *A. niger*. In an 8-year follow-up study, Seaton and Wales (1994) conclude that *A. niger* is a weak antigen and that simple hygiene measures effectively protect the workforce. If such measures are taken, exclusion of recruits with positive skin tests is then not necessary.

Toxins

Despite the long history and intensive nature of *Aspergillus* research, only few cases of toxin formation by *A. niger* have been reported. However, in no case has *A. niger* been proven to produce aflatoxins or trichotecenes.

Two reports (Kulik and Holaday 1966; Hanssen 1969) that *A. niger* cultures produced aflatoxin B1 have been disproved. The evidence was mainly based on an assay by thin layer chromatography. Chances are that fluorescing substances with similar mobilities (Murakami et al. 1967) have been erroneously interpreted as aflatoxin B1. Later on, more detailed studies (Parrish et al. 1966), including those strains which Kulik and Holaday classified as positive (Mislivec et al. 1968; Wilson et al. 1968), clearly showed that none of the *A. niger* strains produced any aflatoxin. Bullerman and Ayres (1968) were also unable to demonstrate aflatoxin production in *A. niger* they had isolated from cured meats. From the numerous investigations it becomes very clear that *A. niger* does not have the ability to produce aflatoxins.

Several more incidental findings indicate that metabolic products may be toxic (Moreau 1979; Cole and Cox 1981; Reiss 1981). Reports on poisoning of animals after they were fed mouldy feed (Moreau 1979, page 178) are difficult to interpret because toxin formation took place under uncontrolled conditions in storage where various contaminating organisms grow as a mixture. The author suspects oxalic acid, a metabolite of *A. niger*, to be the compound responsible for the toxic effect. Jahn (1977) presents results showing coincidence of the toxic effect of fodder and the presence of an *A. niger* strain producing unusually high amounts of oxalic acid.

The nephrotoxic and carcinogenic mycotoxin ochratoxin A was first reported for the black *Aspergillus* species by Ueno et al. (1991) in the species *A. foetidus*. This was later confirmed by Téren et al. (1996) for another isolate of *A. foetidus*. Abarca et al. (1994) first reported ochratoxin A production in *A. niger* (var. *niger*) and this was later confirmed by Téren et al. (1996), Nakajima et al. (1997), Téren et al. (1997), Heenan et al. (1998) and Taniwaki et al. (1999). Téren et al. (1996) also reported that *A. awamori* produced ochratoxin A, and 1 year previously Ono et al. (1995) reported ochratoxin A production in *A. awamori* var. *fumeus*, *A. amamori* var. *minimus*, *A. usamii* and *A. usamii* mut. shiro-usamii. As all these names are synonyms of *A. niger*, it seems to be confirmed by several authors that some isolates of *A. niger* produces ochratoxin A. However, as mentioned by Varga et al. (2000) only about 6% (1.7% to 18.5% as listed by Abarca et al. 2001) of *A. niger* isolates appear to produce ochratoxin A. More research is needed in order to find out which conditions are optimal for ochratoxin A production by *A. niger* (Frisvad and Samson 2000).

Strain CBS 618.78, listed as *A. foetidus*, when studied under optimal laboratory conditions, has the potential to produce ochratoxin A. CBS 618.78 was regarded as an *A. niger* by Kusters-van Someren et al. (1991). From comparing the several isolates in the culture collections it appears that CBS 618.78 is related to strain CBS 126.48, listed by CBS as *A. niger*, available also as ATCC 10254, NRRL 337, IMI 015954, DSM 734, and IFO 6428. When checked, both strains *A. niger* CBS 126.48 and *A. foetidus* IMI 041871 – identical to *A. foetidus* CBS 618.78 – also produce large amounts of ochratoxin A and B when studied under optimal mycotoxin-inducing conditions. On the other hand, *A. niger* IMI 015954 produced rather small amounts of ochratoxin A. Strain NRRL 337 has been used extensively for production of enzymes (Le Mense et al. 1947; Elmayergi and Scharer 1973; Iwai et al. 1983; Okomura et al. 1983) and organic acids (Shu and Johnson 1947; Bercovitz et al. 1990), and for treatment of baked bean processing wastewater (Hang and Woodams 1979), alcoholic fermentation from alkaline potato peel waste (Bloch et al. 1973), utilisation of brewery spent grain (Hang et al. 1975, 1977) and fungal treatment of beet waste (Hang 1976). In the latter paper the fungus (NRRL 337) is called a "food fungus" and named *A. niger*.

This should be taken as a warning that all *A. niger* van Tieghem isolates, either from nature or obtained from a culture collection should be carefully checked for their potential to produce ochratoxin A at the start of the development of a production process for an enzyme used in the food industry. If a strain exhibits the potential to produce this compound, a control system should be in place to assure that it does not end up in the product at levels that induce a toxic effect. When above-threshold levels are found in a product, the use of the strain in the process should be discontinued (Pariza and Johnson 2001).

A. carbonarius is a more efficient producer of ochratoxin A and a much higher percentage of the isolates tested have been found positive (Horie 1995; Téren et al. 1996; Wicklow et al. 1996; Heenan et al. 1998; Varga et al. 2000). No other species in *Aspergillus* section *Nigri* has been reported to produce ochratoxin A (Varga et al. 2000).

A summary of the ochratoxin A production potential of the species in the *Aspergillus* subgenus *Circumdati* section *Nigri* is given in Table 1.

Kojic acid, though mentioned by Wilson (1971) to be a metabolite of *A. awamori*, is not produced by the *A. niger* strains, as Parrish et al. (1966) clearly stated. This is confirmed by the industrial experience that, under the conditions of enzyme production using *A. niger*, kojic acid has not been demonstrated in the culture liquid.

In a comprehensive screening for toxins from *Aspergilli*, Semeniuk et al. (1971) did not detect any markedly toxigenic strain in the *A. niger* group after they had cultivated 392 *Aspergillus* strains on wheat and soybean feed and fed it to chickens or mice. The authors classified 15 strains of the *A. niger* group (of 34 in the test) as moderately to mildly toxigenic. In *A. awamori*, *A. ellipticus*, *A. heteromorphus* and *A. pulverulentus* they did not find any toxigenicity at all. Unfortunately, the study did not include known toxic substances as controls, which would have been helpful in judging the significance of the results.

In a few cases only, suspected toxins have been purified from cultures and tested in animal studies. Nigragillin has been isolated from cultures of *A. niger* and *A. phoenicis* (Caesar et al. 1969; Cole and Cox 1981, p. 798). The LD_{50} was found to be approximately 250 mg/kg bodyweight when fed to 1-day-old cockerels. Malformins, a group of closely related cyclic peptides, generate deformations in plants and have been isolated from cultures of *A. niger*, *A. ficuum*, *A. awamori* and *A. phoenicis* (Steyn 1977). Two malformins were checked for toxic action on rodents: malformin A1 showed an LD_{50} of 3.1 mg/kg when applied intraperitoneally but there was no evidence of acute toxicity when up to 50 mg/kg were given orally to male mice (Yoshizawa et al. 1975). Anderegg et al. (1976) found the LD_{50} for malformin C in both newborn and 28-day-old rats to be 0.9 mg/kg when it was given intraperitoneally.

A series of naphto-γ-pyrones, produced by some strains of *A. niger*, have been reported to be vertebrate central nervous system toxins (Ghosal et al. 1979; DeLucca et al. 1983; Ehrlich et al. 1984). However, these secondary metabolites cannot be regarded as mycotoxins (Bennett 1989), as they were not shown to be toxic when administered by a natural route but rather after intraperitoneal injection. Furthermore, they accumulate only in the mycelium (Ehrlich et al. 1984). Thus these naphto-γ-pyrones do not appear to be a cause for concern in biotechnological products.

The whole body of knowledge from the literature is carefully taken into consideration when testing industrial strains for any possible risk during the development of a fermentation process. Whenever possible the production

organism is chosen from strains that have been in use for many years and that are examined for their ability to produce known toxins under the fermentation conditions used. Finally, the products are regularly checked to ensure that they meet the requirements of the health authorities as given in the Food Chemical Codex (1996) or in the FAO specifications (FAO/WHO 1992).

Summary

The *A. niger* group is composed of black-spored *Aspergillus* species, several of which have a long history of safe use in the fermentation industry. These species have never been identified to be the primary cause of any disease in man. The risk of allergic hypersensitivity to inhaled spores can be handled in an industrial environment by minimising the exposure of the workers to spore dust. Sporadic toxin formation under undefined conditions has not been observed under controlled fermentation conditions. Thus it is concluded that *A. niger* is a safe production organism for industrial use provided the rules of good manufacturing practice are observed. The relatively new discovery that a low percentage of *A. niger* strains have the potential to produce ochratoxin A under optimal laboratory conditions requires, however, that all *A. niger* isolates, (over)expressing a specific gene of interest, be evaluated for their potential to produce ochratoxin prior to being further developed into a new accepted production strain. The use of strains of established and proven safe industrial strain lineages as hosts to over-express these genes of interest is a good and fast alternative to avoid this.

References

Abarca ML, Bragulat MR, Castellá G, Cabanes FJ (1994) Ochratoxin A production by strains of *Aspergillus niger* var. *niger*. Appl Environ Microbiol 60:2650–2652

Abarca ML, Accensi F, Bragulat MR, Cabanes FJ (2001) Current importance of ochratoxin A producing *Aspergillus* spp. J Food Prot 64:903–906

Abramson C, McCarthy DJ, Reed TM (1986) Morphologic and immunologic implications of pedal cutaneous aspergillosis. J Am Podiatr Med Assoc 76:168–178

Accensi F, Cano J, Figuera L, Abarca ML, Cabanes FJ (1999) New PCR method to differentiate species in the *Aspergillus niger* aggregate. FEMS Microbiol Lett 180:191–196

Al-Mussalam A (1980) Revision of the black *Aspergillus* species. PhD Thesis, Rijksuniversiteit, Utrecht

Anderegg RJ, Biemann K, Büchi G, Cushman M (1976) Malformin C, a new metabolite of *Aspergillus niger*. J Am Chem Soc 98:3365–3370

Ballance DJ, Buxton FP, Turner G (1983) Transformation of *Aspergillus nidulans* by the orotidine-5′-phosphate decarboxylase gene of *Neurospora crassa*. Biochem Biophys Res Commun 112:284–289

Barbesgaard P, Heldt-Hansen HP, Diderichsen B (1992) On the safety of *Aspergillus oryzae*: a review. Appl Microbiol Biotechnol 36:569–572

Bennett JW (1989) Mycotoxins, mycotoxincosis, mycotoxicology and mycopathologica. Mycopathologia 100:3–5

Bercovitz A, Peleg Y, Battat E, Rokem JS, Goldberg I (1990) Localization of pyruvate carboxylase in organic acid-producing *Aspergillus* strains. Appl Environ Microbiol 56:1594–1597

Berka RM, Dunn-Coleman NS, Ward M (1992) Industrial enzymes from *Aspergilli*. In: Bennett JW, Klich MA (eds) *Aspergillus*, biology and industrial applications. Butterworth-Heinemann, London, pp 155–202

Berka RM, Fowler T, Rey MW (1994a) Gene sequence encoding *Aspergillus niger* catalase-R. United States Patent 5,360,901

Berka RM, Fowler T, Rey MW (1994b) Production of *Aspergillus niger* catalase-R. United States Patent 5,360,732

Berufsgenossenschaft der Chemischen Industrie (1998) Merkblatt B 007. Jedermann, Heidelberg

Bhatia YN, Mohapatra LN (1969) Experimental aspergillosis in mice. Part I: Pathogenic potential of *Aspergillus fumigatus, Aspergillus flavus and Aspergillus niger*. Mykosen 12:651–654

Binder RE, Faling FJ, Pugatch RD, Mahasaen C, Snider GL (1982) Chronic necrotizing pulmonary aspergillosis: a discrete clinical entity. Medicine 61:109–124

Bloch F, Brown GE, Farkas DF (1973) Utilization of alkaline potato peel waste by fermentation. Amylase production by *Aspergillus foetidus* NRRL 337, and alcoholic fermentation. Am Potato J 50:357–364

Bullerman LB, Ayres JC (1968) Aflatoxin-producing potential of fungi isolated from cured and aged meats. Appl Microbiol 16:1945–1946

Bussink HJD, Kester HCM, Visser J (1990) Molecular cloning, nucleotide sequence and expression of the gene encoding prepro-polygalacturonase II of *Aspergillus niger*. FEBS Lett 273:127–130

Bussink HJD, Buxton FP, Visser J (1991) Expression and sequence comparison of the *Aspergillus niger* and *A. tubingensis* genes encoding polygalacturonase II. Curr Genet 19:467–474

Buxton FP, Gwynne DI, Davies RW (1985) Transformation of *Aspergillus niger* using the *arg*B gene of *Aspergillus nidulans*. Gene 37:207–214

Caesar F, Jansson K, Mutschler E (1969) Nigragilline, a new alkaloid from the *Aspergillus niger* group. I. Isolation and structural elucidation of nigragilline and dioxopiperazine. Helv Physiol Pharmacol Acta 44:676–690

Cahill KM, El Mofty AM, Kawaguchi TP (1967) Primary cutaneous aspergillosis. Arch Dermatol 96:545–547

Campbell EI, Unkles SE, Macro JA, Van den Hondel C, Contreras R, Kinghorn JR (1989) Improved transformation efficiency of *Aspergillus niger* based on the *pyr*G gene. Mol Gen Genet 206:71–75

Cole RJ, Cox RH (1981) Handbook of toxic fungal metabolites. Academic Press, New York

Cullen D, Gray GL, Wilson LJ, Hayenga KJ, Lamsa MH, Rey MW, Norton S, Berka RM (1987) Controlled expression and secretion of bovine chymosin in *Aspergillus nidulans*. Biotechnology 5:369–376

Das A, Roy P (1978) Improved production of citric acid by a diploid strain of *Aspergillus niger*. Can J Microbiol 24:622–625

DeLucca AJ, Ehrlich KB, Ciegler A (1983) Toxicity of extracts of *Aspergillus niger* isolated from stored cotton seed. J Food Safety 5:95–101

Denning DW (1998) Invasive aspergillosis. Clin Infect Dis 26:781–805

Dunn-Coleman NS, Bloebaum P, Berka RM, Bodie E, Robinson N, Armstrong G, Ward M, Przetak M, Carter G, LaCost R, Wilson LJ, Kodama KH, Baliu EF, Bower B, Lamsa M, Heinsohn H (1991) Commercial levels of chymosin production by *Aspergillus*. Biotechnology 9:976–981

Ehrlich KB, DeLucca AJ, Ciegler A (1984) Naphtho-γ-pyrone production by *Aspergillus niger* isolated from stored cottonseed. Appl Environ Microbiol 48:1–4

Elmayergi H, Scharer JM (1973) Physiological studies on *Aspergillus niger* fermentation with polymer additive. J Gen Appl Microbiol 19:385–392

FAO/WHO (1972) Specifications for the identity and purity of some enzymes and certain other substances. 15th report of the Joint FAO/WHO Expert Committee on Food Additives, Food and Agricultural Organization of the United Nations, World Health Organization, Rome

FAO/WHO (1978) Specifications for identity and purity. The 22nd meeting of the Joint FAO/WHO Expert Committee on Food Additives, Food and Agricultural Organization of the United Nations, World Health Organization, Rome

FAO/WHO (1981) Specifications for identity and purity. The 25th meeting of the Joint FAO/WHO Expert Committee on Food Additives, Food and Agricultural Organization of the United Nations, World Health Organization, Rome

FAO/WHO (1987) Toxicological evaluation of certain food additives. The 31st meeting of the Joint FAO/WHO Expert Committee on Food Additives, World Health Organization, Geneva

FAO/WHO (1990) Toxicological evaluation of certain food additives. The 35th meeting of the Joint FAO/WHO Expert Committee on Food Additives, World Health Organization, Geneva

FAO/WHO (1992) Compendium of food additive specifications, vol 1 and 2. Food and Nutrition Paper no. 51/1 and 51/2. Food and Agricultural Organization of the United Nations, World Health Organization, Rome

Federal Register (1993) Direct food substances affirmed as generally recognized as safe; chymosin enzyme preparation derived from *Aspergillus niger* van Tieghem variety *awamori* (Nakazawa) Al-Mussalam. 58:27197

Food Chemical Codex (1996) 4th edn. National Academy Press, Washington

Frisvad JC, Samson RA (2000) *Neopetromyces* gen. nov. and an overview of teleomorphs of *Aspergillus* subgenus *Circumdati*. Stud Mycol (Baarn) 45:201–207

Frisvad JC, Hawksworth DL, Kozakiewicz Z, Pitt JI, Samson RA, Stolk AS (1990) Proposals to conserve important species names in *Aspergillus* and *Penicillium*. In: Samson RA, Pitt JI (eds) Modern concepts in *Penicillium* and *Aspergillus classification*. Plenum Press, New York, pp 83–88

Frost GM, Moss DA (1987) Production of enzymes by fermentation. In: Rehm HJ, Reed G (eds) Biotechnology, vol 7a. VCH, Weinheim, pp 65–102

Gams W, Christensen M, Onions AH, Pitt JI, Samson RA (1985) Intrageneric taxa of *Aspergillus*. In: Samson RA, Pitt JI (eds) Advances in *Penicillium* and *Aspergillus* systematics. Plenum Press, pp 55–62

Ghosal S, Biswas K, Chakrabarti DK (1979) Toxic naphtho-γ-pyrones from *Aspergillus niger*. J Agric Food Chem 27:1347–1351

Gielkens MMC, Visser J, de Graaf LH (1997) Arabinoxylan degradation by fungi: characterization of the arabinoxylan-arabinofuranohydrolase encoding genes of *Aspergillus niger* and *A. tubingensis*. Curr Genet 31:22–29

Godfrey T, Reichelt J (1983) Industrial enzymology. The application of enzymes in industry. Nature Press, New York

Graaf LH de, van den Broek HC, van Ooijen AJJ, Visser J (1994) Regulation of the xylanase-encoding *xlnA* gene of *Aspergillus tubingensis*. Mol Microbiol 12:479–490

Grassin C, Fauguenbergue P (1999) Enzymes, fruit juice processing. In: Flickinger MC, Drew SW (eds) Encyclopedia of bioprocess technology: fermentation, biocatalysis, and bioseparation. Wiley & Sons, Inc, New York

Groot GSP, Herweijer MA, Simonetti ALM, Selten GCM, Misset O (2000) Enzymes in food and feed: past, present and future. In: Bielecki S, Tramper J, Polak J (eds) Food Biotechnology – progress in biotechnology, vol. 17. Elsevier, Amsterdam, pp 95–99

Hang YD (1976) Fungal treatment of beet waste. Prog Water Technol 8:325–327

Hang YD, Woodams EE (1979) Characterization of baked bean processing wastewater and its assimilation by *Aspergillus foetidus*. J Food Sci 44:1548–1549

Hang YD, Splittstoesser DF, Woodams EE (1975) Utilization of brewery spent grain liquor by *Aspergillus niger*. Appl Microbiol 30:879–880

Hang YD, Splittstoesser DF, Woodams EE, Sherman RM (1977) Citric acid fermentation of brewery waste. J Food Sci 42:383–384

Hanssen E (1969) Schädigung von Lebensmitteln durch Aflatoxin B1. Naturwissenschaften 56:90

Harmsen JAM, Kusters-van Someren MA, Visser J (1990) Cloning and expression of a second *Aspergillus niger* pectin lyase gene (pelA): indications of a pectin-lyase gene family in *A. niger*. Curr Genet 18:161–166

Hasany SM, Basu MB, Kazdan MD (1973) Production of corneal ulcer by opportunistic and saprophytic fungi. 1. The effect of pre-treatment of fungi with steroid. Can J Ophthalmol 8:119–131

Heenan CN, Shaw KJ, Pitt JI (1998) Ochratoxin A production by *Aspergillus carbonarius* and *A. niger* isolates and detection using coconut cream agar. J Food Mycol 1:67–72

Horie Y (1995) Productivity of ochratoxin of *Aspergillus carbonarius* in *Aspergillus* section *Nigri*. Nippon Kingakkai Kaiho 36:73–76

Ibekwe AC, Okafor JI (1983) Pathogenic organisms in chronic suppurative otitis media in Enugu, Nigeria. Trop Geogr Med 35:389–391

Iwai M, Okumura S, de Leal EL, Tsujisaka Y (1983) Purification of four esterases from *Aspergillus niger* NRRL 337. Agric Biol Chem 47:1865–1868

Jacob Z, Shukla PK, Wahab S, Srivastava OP, Srivastava GN (1984) *Aspergillus terreus, A. fumigatus* and *A. niger* from the sputum of a human patient, their growth, drug sensitivity and pathogenicity. Biol Mem 9:87–94

Jahn F (1977) Oxalsäurebildungsvermögen und Toxizität eines Stammes von *Aspergillus niger* aus Verschimmeltem Heu. Wien Tierärztl Monatsschr 64:79–82

Kelly JM, Hynes MJ (1985) Transformation of *Aspergillus niger* by the amdS gene of *Aspergillus nidulans*. EMBO J 4:475–479

Kluyver AJ, Perquin LHC (1932) Zur Methodik der Schimmelstoffwechseluntersuchung. Biochem Z 266:68–81

Kozakiewicz Z (1989) *Aspergillus* species on stored products. Mycol Pap 161:1–188

Kozakiewicz Z, Frisvad JC, Hawksworth DL, Pitt JI, Samson RA, Stolk AC (1992) Proposals for nomina specifica conservanda and rejicienda in *Aspergillus* and *Penicillium* (Fungi). Taxon 41:109–113

Kulik MM, Holaday CE (1966) Aflatoxin: a metabolic product of several fungi. Mycopathol Mycol Appl 30:137–140

Kusters-van Someren MA, Kester HMC, Samson RA, Visser J (1990) In: Samson RA, Pitt JI (eds) Modern concepts in *Penicillium* and *Aspergillus* classification. Plenum Press, New York, pp 83–88

Kusters-van Someren MA, Samson RA, Visser J (1991) The use of RFLP analysis in classification of the black *Aspergilli*: reinterpretation of the *Aspergillus niger* aggregate. Curr Genet 19:21–26

Le Mense EH, Corman J, van Lanen JM, Langlykke AF (1947) Production of mold amylases in submerged culture. J Bacteriol 54:149–159

Loh KS, Tan KK, Kumarasinghe G, Leong HK, Yeoh KH (1998) Otitus externa – the clinical pattern in a tertiary institution in Singapore. Ann Acad Med Singapore 27:215–218

Megnegneau B, Debets F, Hoekstra RF (1993) Genetic variability and relatedness in the complex group of black Aspergilli based on random amplification of polymorphic DNA. Curr Genet 23:323–329

Mislivec PB, Hunter JH, Tuite J (1968) Assay for aflatoxin production by the genera *Aspergillus* and *Penicillium*. Appl Microbiol 16:1053–1055

Moreau C (1979) Moulds, toxins and food. Wiley, New York

Mugliston T, O'Donoghue G (1985) Otomycosis – a continuing problem. J Laryngol Otol 99:327–333

Mullaney EJ, Gibson DM, Ullah AHJ (1991) Positive identification of a lambda gtll clone containing a region of fungal phytase gene by immunoprobe and sequence verification. Appl Microbiol Biotechnol 35:611–614

Murakami H, Takase S, Ishii T (1967) Non-productivity of aflatoxin by Japanese industrial strains of *Aspergillus*. I. Production of fluorescent substances in agar slant and shaking cultures. J Gen Appl Microbiol 13:323–334

Nakajima M, Tsubouchi H, Miyabe M, Ueno Y (1997) Survey of aflatoxin B1 and ochratoxin A in commercial green coffee beans by high-performance liquid chromatography linked with immunoaffinity chromatography. Food Agric Immunol 9:77–83

Nguyen QK, Ruttkowski E, Leidinger K, Albrecht H, Gottschalk M (1991) Characterization and expression of a genomic pectin methyl esterase-encoding gene in *Aspergillus niger*. Gene 106:71–77

Nime FA, Hutchins GM (1973) Oxalosis caused by *Aspergillus* infection. Johns Hopkins Med J 133:183–194

Nunberg J, Schweikart V, Lawyer F, Innis M, Flatgaar J, Wittman W, Meade J (1983) Cloning of the *Aspergillus awamori* glucoamylase gene. Proceedings of the ASM conference on gene manipulations in the exploitation and study of fungi. Elkhart, Indiana

Nunberg JH, Meade JH, Cole G, Lawyer FC, McCabe P, Schweickart V, Tal R, Wittman VP, Flatgaard JE, Innes MA (1984) Molecular cloning and characterization of the glucoamylase gene of *Aspergillus awamori*. Mol Cell Biol 4:2306–2315

Nyiredy I, Etter L, Fesüs I, Mayer G (1975) The fate of mould "spores" in the digestive tract of chicks. Acta Vet Acad Sci Hung 25:123–128

Okumura S, Iwai M, Tsujisaka Y (1983) Properties and substrate specificities of four esterases from *Aspergillus niger* NRRL 337. Agric Biol Chem 47:1869–1872

Ono H, Kaqtaoka A, Koakutsu M, Tanaka K, Kawasugi S, Wakazawa M, Ueno Y, Manabe M (1995) Ochratoxin A producibility by strains of *Aspergillus niger* group stored in IFO culture collection. Mycotoxins 41:47–51

Paldrok H (1965) Report on a case of subcutaneous dissemination of *Aspergillus niger*, type *awamori*. Acta Derm Venereol 45:275–282

Parenicová L, Suykerbuyk MEG, Samson RA, Visser J (1997) Evaluation of RFLP analysis for the classification of selected black Aspergilli. Mycol Res 101:810–814

Parenicová L, Skouboe P, Samson RA, Rossen L, Visser J (2000) Molecular tools for the classification of black aspergilli. In: Samson RA, Pitt JI (eds) Integration of modern taxonomic methods for *Penicillium* and *Aspergillus* classification. Harwood, Amsterdam, pp 413–424

Parenicová L, Skouboe P, Frisvad JC, Samson RA, Rossen L, ten Hoor-Suykerbuyk M, Visser J (2001) Combined molecular and biochemical approach identifies *Aspergillus japonicus* and *Aspergillus aculeatus* as two species. Appl Environ Microbiol 67:521–527

Pariza MW, Johnson EA (2001) Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. Regul Toxicol Pharmacol 33:1–14

Parrish FW, Wile BJ, Simmons EG, Long L Jr (1966) Production of aflatoxins and kojic acid by species of *Aspergillus* and *Penicillium*. Appl Microbiol 14:139

Paulose KO, Alkhalifa S, Shenoy P, Sharma RK (1989) Mycotic infection of the ear (otomycosis): a prospective study. J Laryngol Otol 103:30–35

Piddington CS, Houston CS, Paloheimo M, Cantrell M, Miettinen-Oinonen A, Nevalainen H, Rambosek J (1993) The cloning and sequencing of the genes encoding phytase (phy) and pH 2.5 optimum acid phosphatase (aph) from *Aspergillus niger* var. *awamori*. Gene 133:55–62

Pitt JI, Samson RA (2000) Types of *Aspergillus* and *Penicillium* and their teleomorphs in current use. In: Samson RA, Pitt JI (eds) Integration of modern taxonomic methods for *Penicillium* and *Aspergillus* classification. Harwood, Amsterdam, pp 51–72

Pitt JI, Swamson RA, Frisvad JC (2000) List of accepted species and their synonyms in the family Trichocomaceae. In: Samson RA, Pitt JI (eds) Integration of modern taxonomic methods for *Penicillium* and *Aspergillus* classification. Harwood, Amsterdam, pp 9–49

Raper KB, Fennell DI (1965) The genus *Aspergillus*. Williams and Wilkins, Baltimore

Reiss J (1981) Mycotoxine in Lebensmitteln. Fischer, Stuttgart

Reiss J (1986) Schimmelpilze. Lebensweise, Nutzen, Schaden, Bekämpfung. Springer, Berlin Heidelberg New York, pp 33–41

Rippel-Baldes A (1955) Grundzüge der Mikrobiologie, 3rd edn. Springer, Berlin Heidelberg New York

Rippon JW (1982) Medical mycology: the pathogenic fungi and the pathogenic actinomycetes, 2nd edn. Saunders, Philadelphia, pp 565–594

Roukas T (2000) Citric and gluconic acid production from fig by *Aspergillus niger* using solid state fermentation. J Ind Microbiol Biotechnol 25:298–304

Samson RA (1992) Current taxonomic schemes of the genus *Aspergillus* and its teleomorphs. In: Bennett JW, Klich MA (eds) *Aspergillus*, biology and industrial applications. Butterworth-Heinemann, London, pp 355–390

Saravia-Gomez J (1979) Aspergillosis of the central nervous system. In: Vinken PJ, Bruyen GW (eds) Handbook of clinical neurology, vol 35. Infections of nervous systems, part III. GW North Holland, pp 395–400

Seaton A, Wales D (1994) Clinical reactions to *Aspergillus niger* in a biotechnology plant: an eight year follow up. Occup Environ Med 51:54–56

Selten G (1994) The versatile *Aspergillus niger*. Gist 60:5–7

Selten GCM, van Gorcom RFM, Swinkels BW (1995) Selection marker gene free recombinant strains: a method for obtaining them and the use of these strains. European Patent Application EPO0635574 A1

Selten GCM, Swinkels BW, Bovenberg RAL (1998) Gene conversion as a tool for the construction of recombinant industrial filamentous fungi. International Patent Application WO 98/46772

Semeniuk G, Harshfield GS, Carlson CW, Hesseltine CW, Kwolek WF (1971) Mycotoxins in *Aspergillus*. Mycopathol Mycol Appl 43:137–152

Severo LC, Geyer GR, Da Silva Porto N, Wagner MB, Londero AT (1997) Pulmonary *Aspergillus niger* colonisation. Report of 23 cases and a review of the literature. Rev Iberoam Micol 14:104-110

Shu P, Johnson MJ (1947) Effect of the composition of the sporulation medium on citric acid production *by Aspergillus niger* in submerged culture. J Bacteriol 54:161–167

Steyn PS (1977) Some new mycotoxins. Pure Appl Chem 49:1771–1778

Taniwaki MH, Pitt JI, Urbano GR, Teixeira AA, Leitao MFF (1999) Fungi producing ochratoxin A in coffee. 18th International Scientific Colloquium on Coffee. ASIC, Helsinki, pp 239–247

Téren J, Varga J, Hamari Z, Rinyu E, Kevei F (1996) Immunochemical detection of ochratoxin A in black *Aspergillus* strains. Mycopathologia 134:171–176

Téren J, Palágyi A, Varga J (1997) Isolation of ochratoxin producing aspergilli from green coffee beans of different origin. Cereal Res Commun 25:303–304

Tilburn J, Scazzocchio C, Taylor GG, Zabicky-Zissman JH, Lockington RA, Davis RW (1983) Transformation by integration in *Aspergillus nidulans*. Gene 26:205–221

Tomsikova A, Zavazal V, Sach J, Novackova D (1981) Ein der Farmerlunge Ähnliches Syndrom. Mykosen 24:354–366

Topping MD, Scarisbrick DS, Luczynska CM, Clarke EC, Seaton A (1985) Clinical and immunological reactions to *Aspergillus niger* among workers at a biotechnology plant. Brit J Ind Med 42:312–318

Ueno Y, Kawamura O, Sugiura Y, Horiguchi K, Nakajima M, Yamamoto K, Sato S (1991) Use of monoclonal antibodies, enzyme-linked immunosorbent assay and immunoaffinity column chromatography to determine ochratoxin A in porcine sera, coffee products and toxin-producing fungi. In: Castagnero M, Plestina R, Dirheimer G, Chernozemsky IN, Bartsch H (eds) Mycotoxins, endemic nephropathy and urinary tract tumors. IARC Scientific Publication No. 115. International Agency for Research on Cancer, Lyon, pp 71–75

Van Dijck PWM (1999) Chymosin and phytase. Made by genetic engineering. J Biotechnol 67:77–80

Van Gorcom RFM, van Hartingsveldt W, van Paridon PA, Veenstra AE, Luiten RGM, Selten GCM (1991) Cloning and expression of microbial phytase. European Patent Application 0.420.358

Van Hartingsveldt W, Mattern IE, Van Zeijl CMJ, Pouwels PH, van den Hondel CAMJJ (1987) Development of a homologous transformation system for *Aspergillus niger* based on the *pyr*G gene. Mol Gen Genet 206:1–75

Van Hartingsveldt W, van Zeijl CMJ, Harteveld GM, Gouka RJ, Suykerbuyk MEG, Luiten RGM, van Paridon PA, Selten GCM, Veenstra AE, van Gorcom RFM, van den Hondel CAMJJ (1993) Cloning, characterization and overexpression of the phytase-encoding gene (*phy*A) of *Aspergillus niger*. Gene 127:87–94

Varga J, Kevei F, Fekete C, Coenen A, Kozakiewicz Z, Croft JH (1993) Restriction fragment length polymorphisms in the mitochondrial DNAs of the *Aspergillus niger* aggregate. Mycol Res 97:1207–1212

Varga J, Kevei F, Vriesma A, Debets F, Kozakiewicz Z, Croft JH (1994) Mitochondrial DNA restriction fragment length polymorphisms in field isolates of the *Aspergillus niger* aggregate. Can J Microbiol 40:612–621

Varga J, Kevei F, Hamari Z, Tóth B, Téren J, Croft JH, Kozakiewicz Z (2000) Genotypic and phenotypic variability among black aspergilli. In: Samson RA, Pitt JI (eds) Integration of modern taxonomic methods for *Penicillium* and *Aspergillus* classification. Harwood, Amsterdam, pp 397–411

Ward M, Wilson LJ, Carmonia CL, Turner G (1988) The *olic*3 gene of *Aspergillus niger* – isolation, sequence and use as a selective marker for transformation. Curr Genet 14:37–42

Washburn RG, Hammer CH, Bennett JE (1986) Inhibition of complement by culture supernatants of *Aspergillus fumigatus*. J Infect Dis 154:944–951

Wicklow DT, Dowd PF, Alfatafta AA, Gloer JB (1996) Ochratoxin A: an anti-insectan metabolite from the sclerotia of *Aspergillus carbonarius* NRRL 369. Can J Microbiol 42:1100–1103

Wilson BJ (1971) Miscellaneous *Aspergillus* toxins. In: Ciegler A, Kadis S, Ajl SJ (eds) Microbial toxins, vol 6. Academic Press, New York, pp 207–295

Wilson BJ, Campbell TC, Hayes AW, Hanlin RT (1968) Investigation of reported aflatoxin production by fungi outside the *Aspergillus flavus* group. Appl Microbiol 16:819–821

Yoshizawa T, Tsuchiya Y, Morooka N, Sawada Y (1975) Malformin A as a mammalian toxicant from *Aspergillus niger*. Agric Biol Chem 39:1325–1326

Selle, P.H. and
Ravindran. V.

Available online at www.sciencedirect.com
ScienceDirect
Animal Feed Science and Technology
135 (2007) 1–41
ELSEVIER
ANIMAL FEED SCIENCE AND TECHNOLOGY
www.elsevier.com/locate/anifeedsci

Review

Microbial phytase in poultry nutrition

Peter H. Selle [a], Velmurugu Ravindran [b,*]

[a] *Faculty of Veterinary Science, The University of Sydney, Camden, NSW 2750, Australia*
[b] *Institute for Food, Nutrition and Human Health, Massey University, Palmerston North, New Zealand*

Received 13 March 2006; received in revised form 14 June 2006; accepted 19 June 2006

Abstract

During the past decade, the inclusion of microbial phytase in poultry diets has increased remarkably, mainly in response to heightened concerns over phosphorus (P) pollution of the environment. The capacity of this feed enzyme to release phytate-bound P and reduce P excretion is now well documented. Effectively, phytase is an alternative, economical P source and, as global phosphate reserves are not renewable, this is beneficial to their preservation. Based on limited studies, it appears that exogenous phytase hydrolyses less than 0.35 of dietary phytate in broilers at the ileal level. If so, there is considerable scope to enhance phytate degradation by the introduction of more effective phytate-degrading enzymes or enzyme combinations, and facilitative nutritional and management strategies. Alternatively, dietary phytate concentrations may be reduced by the inclusion of selected, low-phytate feedstuffs or dephytinised feed ingredients. There is a distinct possibility that phytate negatively influences protein and energy utilisation in poultry and, as these influences would be ameliorated by phytase, there are substantial, practical implications. Nevertheless, there is still no consensus as to the extent that phytase enhances protein and energy utilisation. Responses in amino acid digestibilities following phytase supplementation are variable and the underlying mechanisms have not been completely understood; consequently, these two aspects are considered in detail in this review. The impact of phytase on protein and energy utilisation may be more positive than generally realised, but this should become increasingly evident if greater phytate degradation rates can be achieved. The experimental use of dephytinised feed ingredients may define the negative impact of phytate on protein and energy utilisation and facilitate the identification of the contributing factors, particularly in relation to energy utilisation. Some recent studies suggest that phytate increases, and phytase decreases, endogenous sodium losses. Although the basis for this phytate-induced shift

* Corresponding author. Tel.: +64 6 350 5528; fax: +64 6 350 5684.
E-mail address: V.Ravindran@massey.ac.nz (V. Ravindran).

doi:10.1016/j.anifeedsci.2006.06.010

of sodium into the gut lumen is not clear, it may have implications for acid–base homeostasis and intestinal uptakes of glucose and amino acids. If the momentum in the practical acceptance of microbial phytase in poultry diets continues, it is likely that phytase feed enzymes will re-define nutrient requirements, particularly in relation to P and calcium, and increasingly contribute to ecologically sustainable poultry production in the future. This would be facilitated by a more fundamental research focus, which, arguably, has been lacking in the past.

Keywords: Microbial phytase; Phytate; Poultry

Contents

1. Introduction

The inclusion of feed enzymes in poultry diets to enhance nutrient utilisation and performance by counteracting the negative influence of targeted substrates has become commonplace within the last two decades. Exogenous enzymes capable of degrading non-starch polysaccharides (NSP) in broiler diets based on 'viscous' grains, including wheat

and barley (Campbell and Bedford, 1992; Bedford and Schulze, 1998), initiated this practice. However, phytate and phytate-bound phosphorus (P) is present in all poultry diets and the partial availability of phytate-P has long been recognised (Lowe et al., 1939). Possibly, Warden and Schaible (1962) were the first to show that exogenous phytase enhances phytate-P utilisation and bone mineralisation in broiler chicks. Nevertheless, three decades elapsed before an *Aspergillus niger*-derived phytase feed enzyme, with the capacity to liberate phytate-bound P and reduce P excretion, was commercially introduced in 1991. It was then considered that the use of microbial phytases would be confined to areas where financial penalties are imposed on excessive P levels in effluent from intensive pig and poultry units (Chesson, 1993). Contrary to this forecast, the inclusion of phytase feed enzymes in monogastric diets has been far more widely accepted and now exceeds that of NSP-degrading enzymes (Bedford, 2003). Phytase feed enzymes have more general application as their substrate is invariably present in pig and poultry diets and their dietary inclusion economically generates bioavailable P and reduces the P load on the environment. The prohibition of protein meals of animal origin, which also provide P, has accelerated the acceptance of phytase feed enzymes in certain countries. Also, in recognition of the so-called 'extra-phosphoric effects' of phytase, some nutritionists elect to place matrix values on phytase feed enzymes for protein/amino acids and energy, in addition to calcium (Ca) and P (Shelton et al., 2004) as this approach facilitates their incorporation into least-cost formulations.

Because of its multi-faceted properties, phytate is also a topic of great interest in human nutrition, medical science, food and feed technology, plant physiology and plant breeding (Feil, 2001). This has been fortuitous for poultry researchers, as this interest has generated a wealth of relevant information. In particular, the negative influence of phytate on the availability of Ca and trace minerals, particularly zinc, in human foodstuffs has been extensively investigated. However, the presence of phytate in human diets is also claimed to have potential benefits, including anti-carcinogenic properties, as reviewed by Harland and Morris (1995). Animal nutritionists have long regarded phytate as both indigestible and an anti-nutritional factor for non-ruminant animals (Swick and Ivey, 1992). Phytate is ubiquitous in plant-sourced feed ingredients as it serves as a P reservoir during seed germination. Because poultry possess insufficient inherent phytase activity, phytate-P is only partially available, and this availability is also variable. Phytate is a polyanionic molecule with the potential to chelate positively charged nutrients, which is almost certainly fundamental to the anti-nutritive properties of phytate. These anti-nutritive properties require further investigation, but phytate probably compromises the utilisation of protein/amino acids, energy, calcium and trace minerals. Phytase, which occurs widely throughout nature, is the requisite enzyme to degrade phytate and notionally, phytase has the capacity to hydrolyse phytate and release inorganic P. The implications of phytates in poultry nutrition were considered by Ravindran et al. (1995a), which provides background to the present review.

During the last 10 years there has been an escalating usage of microbial phytase in pig and poultry diets, cascades of scientific publications, increasing field experience, and the introduction of new phytase feed enzymes. Nevertheless, many fundamental issues relating to phytate and phytase remain to be elucidated. This reflects both the inherent complexity of the subject, coupled with the development and evaluation of exogenous phytases that has not always been well directed. The main focus herein is to review the available literature

on the use of microbial phytase in poultry nutrition in relation to P utilisation and the extra-phosphoric effects of phytase, with an emphasis on broiler chickens. The specific objective of the current review is to clarify the current understanding to the extent possible and, identify gaps in our knowledge and topics for more instructive research.

2. The substrate: phytate

Three terminologies, namely phytate, phytin and phytic acid, are used in the literature to describe the substrate for phytase enzymes. The most commonly used term, phytate, refers to the mixed salt of phytic acid (*myo*-inositol hexaphosphate; IP_6). The term, phytin, specifically refers to the deposited complex of IP_6 with potassium, magnesium and calcium as it occurs in plants, whereas phytic acid is the free form of IP_6.

Phytate was first identified more than a century ago (Hartig, 1855). The partial availability of the P component ($282\,g\,kg^{-1}$) of phytate to simple-stomached species assumes importance as the world's rock phosphate reserves are not renewable, which could lead to a P supply crisis in the future (Abelson, 1999; Mullaney et al., 2000). The global harvest of crop seeds and fruits contains an estimated 14.4 million tonnes of phytate-P, which is equivalent to 65% of annual sales of P as fertilisers (Lott et al., 2000). Typically, poultry diets contain from 2.5 to $4.0\,g\,kg^{-1}$ phytate-P (Ravindran, 1995) and, in 2002, global feed consumption by broilers and layers was estimated to be approximately 321 million tonnes (Farrell, 1997); therefore, poultry consume in the order of one million tonnes of phytate-P annually. Clearly, preservation of global P reserves would be facilitated by more efficient phytate-P utilisation by poultry.

Initially, Averill and King (1926) recorded phytate concentrations in human foods but a number of surveys of total P and phytate-P concentrations in feedstuffs are now available (Nelson et al., 1968a; Kirby and Nelson, 1988; Eeckhout and de Paepe, 1994; Ravindran et al., 1994; Viveros et al., 2000; Selle et al., 2003d; Godoy et al., 2005). A summary of these surveys for major feed ingredients is shown in Table 1, where phytate determinations were based on variants of the 'ferric chloride-precipitation' method (discussed below). The tabulated values indicate that a typical maize–soy broiler diet contains in the order of $2.5\,g\,kg^{-1}$ phytate-P (or $8.9\,g\,kg^{-1}$ phytic acid); but, clearly, this concentration may vary. In plant-sourced feed ingredients the majority of total P is present as phytate-P and the proportion is reasonably constant. Therefore, it is possible to calculate broad estimates of phytate-P from total P concentrations from linear regression equations derived for given feedstuffs (Viveros et al., 2000). In feed ingredients it is likely that IP_6 exists as mineral-bound complexes involving magnesium, calcium and potassium (Reddy et al., 1982), known as phytin. As reported by Kasim and Edwards (1998), the IP_6 ester is the dominant form of phytate in maize (0.972), rice bran (0.878), sorghum (0.972), soybean meal (0.844) and wheat (1.00).

Logically, dietary concentrations of phytate are crucial to its anti-nutritive properties and its negative impact on P availability. For example, the addition of $15\,g\,kg^{-1}$ sodium phytate into aquacultural diets has been shown to cause substantial reductions in weight gain, feed efficiency, and protein efficiency ratios in Hamilton fry (Usmani and Jafri, 2003). In broilers, the addition of sodium phytate to glucose-based diets has been shown to increase

Table 1
Weighted mean (and range) of total P and phytate-P concentrations, and proportion of phytate-P of total P, in key poultry feed ingredients

Feed ingredient	Number of data-sets/samples	Total P ($g\,kg^{-1}$)	Phytate-P ($g\,kg^{-1}$)	Proportion (%)
Cereals				
Barley	4/41	3.21 (2.73–3.70)[a]	1.96 (1.86–2.20)[a]	61.0 (59–68)[a]
Maize	7/45	2.62 (2.30–2.90)	1.88 (1.70–2.20)	71.6 (66–85)
Sorghum	5/41	3.01 (2.60–3.09)	2.18 (1.70–2.46)	72.6 (65–83)
Wheat	6/97	3.07 (2.90–4.09)	2.19 (1.80–2.89)	71.6 (55–79)
Oilseed meals				
Canola meal	4/28	9.72 (8.79–11.50)	6.45 (4.00–7.78)	66.4 (36–76)
Cottonseed meal	3/21	10.02 (6.40–11.36)	7.72(4.9–9.11)	77.1 (70–80)
Soyabean meal	6/89	6.49 (5.70–6.94)	3.88 (3.54–4.53)	59.9 (53–68)
By-products				
Rice bran	6/37	17.82 (13.40–27.19)	14.17 (7.90–24.20)	79.5 (42–90)
Wheat bran	6/25	10.96 (8.02–13.71)	8.36 (7.00–9.60)	76.3 (50–87)

Derived from studies by Nelson et al. (1968a), Kirby and Nelson (1988), Eeckhout and de Paepe (1994), Ravindran et al. (1994), Viveros et al. (2000), Selle et al. (2003d) and Godoy et al. (2005).
[a] Range of values.

endogenous losses of amino acids and minerals (Cowieson et al., 2004). It follows that responses to phytase will be more pronounced with increasing dietary phytate levels and indications of this have been recorded in poultry (Cabahug et al., 1999; Ravindran et al., 2000, 2006) and pigs (Selle et al., 2003a). Therefore, the capacity to analyse total P and phytate-P in complete diets rapidly and accurately would be very beneficial in practice, from both nutritional and ecological standpoints. However, as discussed below, this is not yet feasible, although near-infrared reflectance spectroscopy (NIR) determinations may hold promise.

2.1. Analytical methods to determine phytate concentrations

Because phytate does not have a characteristic absorption spectrum nor a specific identifying reagent, phytate analyses are 'fraught with difficulties' (Lasztity and Lasztity, 1990). Undoubtedly, problems associated with the determination of phytate concentrations in feed ingredients, complete diets, ileal digesta and excreta have impeded research and the practical application of phytase. Standard analytical methods are based on the principle that phytate and ferric chloride will form insoluble Fe^{3+}–phytate precipitates at acidic pH (Heubner and Stadler, 1914). Various methods of phytate determination, based on the 'ferric chloride-precipitation' principle, have been described (Wheeler and Ferrel, 1971; Miller et al., 1980; Haug and Lantzsch, 1983). Standard ferric chloride-precipitation methods are suitable to assay the majority of feed ingredients, but on an individual basis. Importantly, ferric chloride-precipitation methods appear unsuitable for more complex samples, including complete diets (Selle et al., 2003d). Exaggerated phytate-P readings with the ferric chloride-precipitation method in feed samples containing meat-and-bone meal, fishmeal and dicalcium phosphate have been recorded, presumably because P from

these ingredients co-precipitates with Fe^{3+}–phytate complexes (Ellis et al., 1977) and, possibly, other contaminants. Frolich et al. (1986) determined phytate concentrations in wheat flour by both a ferric chloride-precipitation method and ^{31}P nuclear magnetic resonance (NMR) spectroscopy and concluded that co-precipitation with ferric chloride-precipitation methods overestimates phytate contents.

However, Harland and Oberleas (1977) eliminated ferric chloride-precipitation by directly extracting phytate with hydrochloric acid and separating inorganic P via elution through an ion-exchange resin. The eluted phytate was then digested with nitric and sulphuric acids and liberated P measured colourimetrically. This approach was subsequently modified by Latta and Eskin (1980), and their method has been used in several recent poultry experiments.

An important scientific limitation is that these methods determine phytate-P but they do not have the capacity to identify the various *myo*-inositol phosphate esters of phytate. However, it is possible to differentiate phytate esters with high performance liquid chromatography (Rounds and Nielsen, 1993) and anion exchange chromatography (Phillippy and Johnston, 1985). Skoglund et al. (1997a,b, 1998) have documented refinements to these analytical methods, although they may be expensive and time-consuming (Kwanyuen and Burton, 2005).

Additional approaches include NIR analyses of total P and phytate-P in feed ingredients and complete diets, which has been considered by de Boever et al. (1994). More recently, however, Smith et al. (2001) used NIR to determine total P and phytate-P in broiler excreta, which suggests this approach holds promise. Also, methods based on phytase incubation, where the quantity of P liberated by exogenous phytase is used to assess phytate concentrations are alternatives (Shen et al., 2005).

3. The enzyme: phytase

Although phytase activity was first detected in rice bran nearly a century ago (Suzuki et al., 1907), attempts to develop a phytase feed enzyme did not commence until 1962 in North America (Wodzinski and Ullah, 1996). This interest is reflected in contemporary publications by Nelson (1967) and Nelson et al. (1968a,b,c, 1971), who were concerned with the negative effects of phytate on both Ca and P availability in broiler chicks. Nevertheless, it was not until 1991 that the first phytase feed enzyme became commercially available, which was largely in response to legislation designed to limit P pollution of the environment in the Netherlands. The complex identification, production and characteristics of phytate-degrading enzymes or phytases (*myo*-inositol hexaphosphate phosphohydrolases) has been the subject of several review papers, including Wodzinski and Ullah (1996), Mullaney et al. (2000), Konietzny and Greiner (2002), Simon and Igbasan (2002), Vohra and Satanarayana (2003) and Haefner et al. (2005).

An international standard unit does not exist for the measurement of phytase activity, which has created considerable confusion in the commercial feed industry and in efficacy comparisons of different phytase sources. The defined measurement unit of phytase activity depends on assay conditions including concentration of substrate (sodium phytate) used, assay temperature and pH. In the *A. niger* phytase introduced in 1991, phytase activity

is defined as *fytase* units (FTU), where one FTU is the amount of enzyme that liberates 1 μmol inorganic orthophosphate/min from 0.0051 mol L^{-1} sodium phytate at pH 5.5 and a temperature of 37 °C (Engelen et al., 1994). This definition provides a useful measure of quantity of phytase activity and represents a simple bench mark measurement under well-defined assay conditions. Several other abbreviations, including FYT, U and PU, have been used to denote phytase activity of different commercial microbial phytases, although these activities appear to be determined under reasonably similar *in vitro* conditions. It must be cautioned, however, that the efficacy of utilisation of a given quantity of enzyme will vary with assay conditions such as pH, temperature, duration, mineral content, agitation, *etc.* Also it is likely that 'natural' phytate may not be as readily hydrolysed as sodium phytate. For example, calcium phytate is not utilised as well as sodium phytate *in vivo*, which indicates that it is more resistant to degradation (Maddaiah et al., 1963). The acceptance of a standard assay, possibly based on a substrate other than sodium phytate, would therefore be beneficial.

Phytase feed enzymes fall into two categories depending on the site where the hydrolysis of the phytate molecule is initiated. 3-Phytase (EC 3.1.3.8) preferentially liberates the P moiety at position C_3, whereas 6-phytase (EC 3.1.3.26) commences at position C_6 of the *myo*-inositol hexaphosphate ring. In theory, enzymic hydrolysis of phytate generates a series of lower *myo*-inositol phosphates esters ($IP_6 \Rightarrow IP_5 \Rightarrow IP_4 \Rightarrow IP_3 \Rightarrow IP_2 \Rightarrow IP_1$), via a progression of step-wise dephosphorylation reactions, to yield inositol and six inorganic P moieties. It is important to note, however, that the axial P residue at the C_2 position is relatively refractory to hydrolysis (Wodzinski and Ullah, 1996). Consequently, hydrolysis of phytate by phytase is more likely to yield *myo*-inositol monophosphate (IP_1) and five inorganic P moieties. However, as discussed below, hydrolysis of dietary phytate by exogenous phytase in broilers does not usually progress to this extent.

Several distinct microbial phytase products are now commercially available. The three commonly used phytase feed enzymes are derived from *A. niger*, which is a 3-phytase and *Peniophora lycii* and *Escherichia coli*, which are 6-phytases. Phytase feed enzymes may be included in poultry rations as granulates or as liquids, via post-pelleting application systems, to avoid thermostability problems at high pelleting temperatures (>80 °C). There are, however, perceived advantages in inherently heat stable phytase feed enzymes that can withstand steam-pelleting, as illustrated by the investigations of Wyss et al. (1998) and Garrett et al. (2004).

The site of phytase activity in the gastrointestinal tract of poultry has received little attention. However, it is likely that phytate hydrolysis mainly takes place in the fore-stomach (crop, proventriculus, gizzard) where the pH is more conducive to phytase activity. The crop is probably the primary site of phytate degradation by exogenous phytase (Liebert et al., 1993; Takemasa et al., 1996; Kerr et al., 2000). However, there is evidence that *E. coli*-derived phytase is more active in the small intestine than phytase derived from *P. lycii* (Onyango et al., 2005b), which may be attributable to the greater resistance of *E. coli*-derived phytase to endogenous, proteolytic enzymes (Igbasan et al., 2000).

The presence of phytase activity at various segments of the gastrointestinal tract is not necessarily indicative of potential hydrolysis of the substrate, which is facilitated by relatively low gut pH at which phytate is more soluble (Campbell and Bedford, 1992). Few credible studies have determined phytate degradation in poultry either at the level

of the ileum or on the basis of total tract assessments. However, phytate hydrolysis by 500 FTU kg^{-1} *A. niger* phytase at the ileal level was determined in two separate studies in which broilers were offered maize–soy diets containing 2.8 g kg^{-1} phytate-P. Camden et al. (2001) found that phytase degraded 0.293 and Tamim et al. (2004) reported that 0.335 of dietary phytate was degraded. Thus, these two studies suggest that microbial phytase degrades less than 0.35 of dietary phytate at the ileal level in broilers at recommended inclusion rates.

Phytase derived from caecal microflora will influence total tract assessments of phytate degradation (Ravindran et al., 1995a). Nevertheless, Leske and Coon (1999) determined the extent of phytate hydrolysis in a range of individual feed ingredients induced by *A. niger* phytase on the basis of total tract assessments. In broilers, 600 FTU kg^{-1} phytase degraded, on average, 0.254 of phytate in seven feed ingredients and, in layers, 300 FTU kg^{-1} degraded 0.377 phytate in three feed ingredients. Shirley and Edwards (2003) supplemented maize–soy diets with up to 12,000 FTU kg^{-1} *A. niger* phytase. However, at 750 FTU kg^{-1}, phytase degraded 0.181 of dietary phytate on the basis of total tract disappearance. Clearly, the ileal and total tract assessments indicate that degradation of phytate is incomplete in poultry, particularly in broiler chicks, following phytase supplementation at standard inclusion rates. Therefore, the form in which the residual phytate is present in the gut is of interest. From data generated *in vitro* (Frolich et al., 1986) and in pigs (Rapp et al., 2001), it appears that residual phytate remains largely intact, as *myo*-inositol hexapahosphate (IP_6), with relatively small amounts of lower *myo*-inositol phosphate esters. This is important because the chelating capacity of lower phytate esters is disproportionately less than IP_6, as demonstrated by their impact on zinc absorption in rats (Lonnerdal et al., 1989). It has been shown *in vitro* that, in relation to zinc and copper, the binding strengths of IP_4 and IP_3 are less than IP_6 and IP_5 (Persson et al., 1998). Also, the *in vitro* capacity of phytate to inhibit pepsin digestion of casein and bovine serum albumen is most pronounced for IP_5 and IP_6, whereas IP_1 and IP_2 did not inhibit pepsin activity (Knuckles et al., 1989).

3.1. Plant, mucosal and microfloral phytases

In poultry, in addition to phytase feed enzymes, phytate degradation may be influenced by plant phytase derived from certain feed ingredients, and phytases generated by the small intestinal mucosa (Maenz and Classen, 1998) and gut microflora (Kerr et al., 2000). Plant phytase activity is negligible in the majority of feed ingredients, but significant activity is known to be present in barley, rye, triticale, wheat and wheat by-products (Weremko et al., 1997) and some feed ingredients also possess acid phosphatase activity (Viveros et al., 2000). However, intact plant phytase activity is less effective than microbial phytase in the gut because of a narrower pH spectrum of activity (Eeckhout and de Paepe, 1991). Also, very low pH may destroy wheat phytase and it is more susceptible to proteolytic digestion than *A. niger* phytase (Phillippy, 1999). Nevertheless, wheat phytase has been shown to enhance P utilisation in broilers and layers (Olaffs et al., 2000). Importantly, however, plant phytase is more heat labile and its activity is reduced or even eliminated in steam-pelleted diets (Jongbloed and Kemme, 1990).

Mucosal phytase (and phosphatase) activity in the digestive tract of poultry is often dismissed as having little importance. Nevertheless, mucosal phytase has the capacity to

hydrolyse phytate as Tamim et al. (2004) found that mucosal phytase activity degraded 69.2% of phytate at the ileal level in maize–soy broiler diets containing 2 g kg^{-1} Ca. However, the dietary addition of 5 g kg^{-1} Ca, as limestone, reduced phytate degradation to 0.254. These findings are consistent with an earlier study (Tamim and Angel, 2003) and illustrate the importance of Ca on phytate degradation at intestinal pH; presumably, this is largely a consequence of insoluble Ca–phytate complex formation (Wise, 1983). Also, when offered P inadequate diets, broilers have the adaptive capacity to increase intestinal phytase and phosphatase activities (McCuaig et al., 1972; McCuaig and Motzok, 1972). For example, reducing non-phytate-P in broiler diets from 6.0 to 1.2 g kg^{-1} increased total tract phytate hydrolysis from 0.098 to 0.245 (Ballam et al., 1985). Interestingly, the capacity of chicks to degrade phytate is reported to be inheritable, which raises the possibility of selecting broilers for this trait to enhance phytate-P utilisation (Aggrey et al., 2002; Zhang et al., 2003, 2005).

Very few studies have considered the influence of phytase generated by gut microflora in poultry. However, Kerr et al. (2000) concluded that microfloral phytase has an important role on phytate degradation. These workers found very low levels of IP_6 and lower phytate esters in the caeca, and suggested that this was consistent with near complete IP_6 hydrolysis by phytase derived from hindgut fermentation. However, Ballam et al. (1985) found that total tract degradation of phytate by mucosal and microfloral phytases ranged from 0.06 to 0.57, depending on dietary levels of Ca and non-phytate-P.

4. Phosphorus, phytase and the environment

The inclusion of microbial phytases in pig and poultry diets was prompted by the need to reduce P excretion and its loss into the environment, where P pollution is a hazard to water quality. Excessive P concentrations are the most common cause of eutrophication of rivers, lakes and reservoirs (Correll, 1999). Surface runoff from soils with accumulated P accelerates eutrophication, which may result in toxic algal blooms and fish kills (Sharpley, 1999). Consequently any reduction in P excreted by (pigs and) poultry is of benefit to both the environment and sustainable production.

In the most frequently cited paper on microbial phytase research, Simons et al. (1990) demonstrated the potential of microbial phytase to reduce P excretion. These workers found that 1500 FTU kg^{-1} phytase activity, coupled with reductions in dietary P (7.5–4.5 g kg^{-1}) and Ca (9.0–6.0 g kg^{-1}), reduced P excretion by an average of 61% in two broiler experiments. More recently, Zyla et al. (2001) totally eliminated dicalcium phosphate from wheat–soy broiler diets, which reduced non-phytate-P (4.1–1.7 g kg^{-1}) and Ca (9.8–5.9 g kg^{-1}) levels. A combination of phytase and acid phosphatase was included in this modified diet. In a 43-day feeding study, this regime generated a 45% reduction of P in litter (14.8 g/bird *versus* 26.8 g/bird). Additionally, exogenous enzymes significantly enhanced toe ash (164 g kg^{-1} *versus* 150 g kg^{-1}), carcass yield (71.3% *versus* 69.1%) and feed efficiency (1.86 *versus* 1.97), although there was a numerical reduction in weight gain (2124 g/bird *versus* 2215 g/bird).

In a more straightforward assessment, Paik (2003) reduced non-phytate-P levels in starter (4.5–3.5 g kg^{-1}) and finisher broiler diets (3.5–2.5 g kg^{-1}) without, and with, the addition

of 500 FTU kg^{-1} phytase. Decreasing non-phytate-P reduced P excretion by 14.8%, but the weight gain was depressed. Phytase inclusion further increased the reduction in P excretion by 29.6% but, in contrast, growth performance was not compromised.

The impact of phytase on P excretion was based on total P assessments in the above studies. From a limited number of investigations, the phytate-P proportion of total P excreted by poultry offered non-supplemented diets is probably less than anticipated. This proportion ranged from 0.28 to 0.38 in excreta from maize, wheat, triticale and barley-based broiler diets (Pintar et al., 2005) and, in turkeys, a range from 0.16 to 0.32 has been reported (Toor et al., 2005). However, using NIR, Smith et al. (2001) found phytate-P represented 0.56 of total P in excreta from broilers offered maize–soy diets containing 2.6 g kg^{-1} phytate-P. Using NMR, McGrath et al. (2005) reported that phytate-P represented 0.57 of total P in the excreta of broilers fed non-supplemented diets and 0.50 in those fed phytase-supplemented diets. These relative levels of phytate-P excretion may be indicative of excessive dietary P levels, undigested inorganic P, endogenous P losses and inherent phytase activity in the gut.

Of ecological concern is the possibility that phytase supplementation may increase P solubility in excreta and litter as soluble P in run-off is more likely to exacerbate eutrophication. For example, Miles et al. (2003) reported that while phytase supplementation of maize–soy diets reduced total P in broiler litter, levels of soluble P were increased (2.85 g kg^{-1} *versus* 2.17 g kg^{-1}). In contrast, however, Applegate et al. (2003b) compared standard maize–soy broiler diet with three different phytase-supplemented diets. Overall, phytase reduced total P in fresh litter by 32.2% (7.55 g kg^{-1} DM *versus* 11.14 g kg^{-1} DM) and soluble P by 43.1% (1.23 g kg^{-1} *versus* 2.16 g kg^{-1}). It is noteworthy that dietary total P concentrations were lower in the study of Applegate et al. (2003b). The ecological benefits of phytase-supplemented broiler diets, formulated to reduced non-phytate-P specifications, coupled with minimal increases in litter moisture during storage were emphasised by McGrath et al. (2005).

4.1. Phosphorus requirements for poultry

Because P is crucial for skeletal integrity and growth performance, nutritionists tend to incorporate moderately excessive P levels into poultry diets to guarantee a safety margin (Waldroup, 1999). However, as excreted P is a function of total P, current poultry diets should contain the minimum amount of P that will properly sustain production, in recognition of ecological issues. Consequently, the validity of standard recommendations for dietary P levels has become the subject of scrutiny. For example, NRC (1994) requirements for non-phytate-P range from 4.5 g kg^{-1} (0–3 weeks), 3.5 g kg^{-1} (3–6 weeks) to 3.0 g kg^{-1} (6–8 weeks) in broiler diets.

From a detailed study with starter broilers (0–21 days), Waldroup et al. (2000) contend that, on the basis of tibia ash, the non-phytate-P requirement is 3.9 g kg^{-1} for diets based on normal maize, which is reduced to 2.9 g kg^{-1} by 800 FTU kg^{-1} phytase supplementation. For diets based on high available phosphate maize the corresponding requirements are 3.7 and 3.2 g non-phytate-P kg^{-1}. Moreover, these recommendations, in comparison to 4.5 g non-phytate-P kg^{-1} in a non-phytate-P diet, reduced total P excretion by 9.9% (without) and 28.1% (with phytase) in diets based on normal maize and, correspondingly, by 35.5 and 47.1% in diets based on high available phosphate maize.

The accurate definition of P requirements in poultry is inherently difficult and has only been complicated by the introduction of microbial phytases. Instructively, Angel et al. (2005) evaluated phytase supplementation of diets with reduced P specifications in a four-phase feeding program in three floor pen experiments. Growth performance of broilers was not compromised following 600 FTU kg^{-1} phytase addition to starter (1–18), grower (18–32), finisher (32–42) and withdrawal (42–49 days of age) diets with non-phytate-P levels of 3.9, 2.5, 1.7 and 1.2 g kg^{-1}, respectively. Also, the researchers contend that University of Maryland recommendations for non-phytate-P levels for these age groups (4.5, 3.1, 2.3 and 1.8 g kg^{-1}, respectively) were validated in the study of Angel et al. (2005).

4.2. *Phosphorus equivalency of microbial phytase in poultry*

The capacity of phytase to increase total P digestibility in broilers has frequently been demonstrated. For example, Ravindran et al. (2000) found that phytase increased ileal P digestibility by 14.7% (0.506 *versus* 0.441) in broiler diets containing 4.5 g kg^{-1} non-phytate-P. Interestingly, a greater increase in P digestibility of 65.2% (0.664 *versus* 0.402) was recorded in 2.3 g kg^{-1} non-phytate-P diets. The essential difference was that the 4.5 g kg^{-1} non-phytate-P diet contained additional dicalcium phosphate (12 g kg^{-1}), which implies P and/or Ca exerted a negative effect on phytase efficacy. However, such studies do not define the increase in phytate-P digestibility or the quantity of P effectively generated by phytase supplementation.

Studies designed to establish the P equivalency or replacement value of microbial phytases in poultry diets are summarised in Table 2. Graded amounts of an inorganic P source or graded phytase inclusion levels are incorporated into P-deficient basal diets and P replacement values are calculated from regression equations best describing responses of selected parameters. Frequently, the parameters monitored are body weight gain and percentage toe ash, as both are considered to be sensitive indicators of P availability (Potter, 1988). The basal diets in the broiler studies were based on maize and soyabean meal (Schoner et al., 1991, 1993; Kornegay et al., 1996; Yi et al., 1996b; Yonemochi et al., 2000; Augspurger et al., 2003; Adedokun et al., 2004) or soyabean meal *per se* (Denbow et al., 1995; Yi et al., 1996b). The diets contained, on average, 4.37 g kg^{-1} total P and 2.37 g kg^{-1} phyate-P with a Ca:P ratio of 1.84. Collectively, these studies indicate that 805 FTU kg^{-1} phytase activity is equivalent to 1.050 g kg^{-1} inorganic P, which corresponds to an approximate release of phytate-bound P of 0.452. Additionally, three studies in turkeys (Ravindran et al., 1995b; Applegate et al., 2003c; Esteve-Garcia et al., 2005) and one in ducks (Orban et al., 1999) are tabulated.

The collective P equivalency value of phytase (840 FTU $kg^{-1} \equiv 1.0$ g kg^{-1} P) is somewhat lower than is suggested in practice. Also, these studies indicate that phytase hydrolyses in the order of 0.45 of phytate present in broiler diets, which is probably an over-estimate. The likely reasons for this inflated value are that phytase is exerting a positive influence on weight gain that is unrelated to the liberation of phytate-bound P (Wu et al., 2005), also phytase may be correcting Ca:P imbalances (Driver et al., 2005a) and/or the models used to calculate equivalency values are inappropriate (Rosen, 2003). Phosphorus equivalence values determined in the linear, or near-linear, segment of the P dose–response function may not be suitable quantitatively for use in more practical situations (Angel et al., 2002; Rosen,

Table 2
Summary of microbial phytase phosphorus equivalency studies in poultry

Reference	Basal diet				Inorganic P source	Response criteria	P equivalence (FTU = g P)	Phytate-P released (%)	Phytase source
	Type	Total P ($g\,kg^{-1}$)	Phytate-P ($g\,kg^{-1}$)	Ca:P ratio					
Broilers									
Schoner et al. (1991)	Maize–soy	4.5	2.3	1.33	MCP	P retention	700 = 1.000	43.5	*A. niger*
Schoner et al. (1993)	Maize–soy	3.5	2.3	1.71	MCP	Weight gain, P retention	850 = 1.000	43.5	*A. niger*
Denbow et al. (1995)	Soybean meal	3.8	1.8	2.00	DFP	Weight gain, toe ash	821 = 1.000	55.6	*A. niger*
Kornegay et al. (1996)	Maize–soy	4.4	2.4	2.00	DFP	Weight gain, toe ash	939 = 1.000	41.7	*A. niger*
Yi et al. (1996b), Expt. 1	Soybean meal	4.5	1.8	2.00	DFP	Weight gain, toe ash	1146 = 1.000	55.6	*A. niger*
Yi et al. (1996b), Expt. 2	Maize–soy	5.1	2.4	2.00	DFP	Weight gain, toe ash	785 = 1.000	41.7	*A. niger*
Yonemochi et al. (2000)	Maize–soy	6.0	3.0	1.50	DFP	Gain, intake, tibia ash and P, plasma P	500 = 1.172	39.1	*A. niger*
Augspurger et al. (2003)	Maize–soy	3.6	2.6	2.08	KH_2PO_4	Weight gain, tibia ash	500 = 1.250	48.1	*E. coli*
Adedokun et al. (2004)	Maize–soy	3.9	2.7	1.95	MSP	Gain, feed intake, toe and tibia ash,	1000 = 1.031	38.2	*E. coli*
Turkeys									
Ravindran et al. (1995b)	Soybean meal	4.9	2.2	2.00	DFP	Weight gain, toe ash	652 = 1.000	45.5	*A. niger*
Applegate et al. (2003a,b,c)	Maize–soy	7.2	2.5	1.85	MCP	Weight gain, toe ash	500 = 2.125	85.0	*E. coli*
Esteve-Garcia et al. (2005)	Maize–soy	6.1	3.6	1.97	DCP	Weight, toe and tibia ash	413 = 1.000	27.8	*H. polymorpha*
Ducks									
Orban et al. (1999)	Maize–soy	4.8	3.0	1.75	MSP	Weight gain, plasma P	750 = 0.600	20.0	*E. coli*

2003). Thus, there is the contention that it is not feasible to place a single P equivalence value on phytase feed enzymes in broiler diets (Driver et al., 2005a). In a given situation, dietary concentrations of phytate-P, in particular, Ca and non-phytate-P and phytase inclusion levels will influence P equivalency and, consequently, these factors should be taken into consideration.

5. Impact of exogenous phytase on growth performance

Since the report of Simons et al. (1990), several hundred investigations into the effects of various microbial phytases on growth performance of poultry have been completed, which precludes their individual consideration. Predictably, the addition of phytase to P-inadequate diets has been consistently shown to enhance growth performance. In the study of Simons et al. (1990), phytase addition (1500 FTU kg^{-1}) to diets containing 4.5 g kg^{-1} total P increased weight gain (733 g *versus* 338 g) and feed efficiency (1.50 *versus* 1.85) of broilers from 0 to 24 days of age. Subsequently, Cabahug et al. (1999) reported that phytase addition (400 and 800 FTU kg^{-1}) to 2.3 g kg^{-1} non-phytate-P diets increased weight gain (18.8%), feed intake (9.0%) and feed efficiency (7.9%) of broiler chicks from 7 to 25 days of age. However, responses to phytase by broilers offered 4.5 g kg^{-1} non-phytate-P diets were more modest (respective increases of 5.0, 5.0 and 0%), with a significant interaction between non-phytate-P level and phytase addition for weight gain. Generally, responses to phytase in feed intake and weight gain are more robust and consistent than feed efficiency responses. Rosen (2003), from multi-factorial analyses of phytase feeding trials, argues that feed efficiency responses to phytase have been declining with time, which he attributes to concurrent improvements in broiler strains, feeds and management techniques.

Phytase supplementation of P-adequate broiler diets has been shown to generate equivocal growth performance responses, which may be mediated by dietary nutrient specifications. For example, in a study reported by Selle et al. (1999), standard and modified sorghum-based diets were offered to broilers from 7 to 25 days of age, without and with 600 FTU kg^{-1} phytase. The modified diets contained reduced specifications in P, Ca, protein/amino acids and energy density. Phytase did not influence growth performance of broilers on standard diets but significantly increased weight gain (7.6%) and feed efficiency (4.7%) in modified diets. Moreover, there was a significant interaction between diet type and phytase addition for feed efficiency. Treatments had no effect on toe ash contents, which indicates that dietary P levels were not limiting.

It appears that nutrient specification levels, phytate concentrations and phytase inclusion rates in broiler diets are critical, interactive variables. It is likely that high nutrient specification levels may accommodate the anti-nutritive properties of dietary phytate concentrations and negate responses to phytase supplementation. Consequently, one approach is to decrease nutrient specifications appropriately and counter potential reductions in growth performance with phytase supplementation, which has been shown to be economically viable (Selle et al., 2003b). Further studies, to define optimal reductions in nutrient specifications are justified. Also, depending on nutrient specification levels, it may be that dietary phytate assumes more importance when it exceeds a threshold concentration in broiler diets. If so, it may be speculated that this occurs when dietary phytate levels approach 3.0 g kg^{-1} phytate-P.

Logically, the magnitude of responses to phytase will be more pronounced with increasing inclusion rates of the feed enzyme and, presumably, greater degradation of phytate. Shirley and Edwards (2003) investigated phytase supplementation of maize–soy broiler diets (4.60 g total P kg^{-1}; 2.72 g phytate-P kg^{-1}); responses in selected parameters to graded phytase inclusion levels to a maximum of 12,000 FTU kg^{-1} and the results are shown in Table 3. From the tabulated data it is evident that increasing phytase inclusions are associated with substantial increases in total tract phytate degradation ranging from 0.403 to 0.948. Moreover, phytate degradation was correlated with large increases in P retention, tibia ash, weight gain, feed intake, nitrogen (N) retention, feed efficiency, apparent metabolisable energy (AME) and Ca retention (ranked in descending order of significance) and these increases are numerically most pronounced at the highest phytase inclusion rate of 12,000 FTU kg^{-1}. At such extreme inclusion rates, however, the possibility arises that any minor enzymic side-activities that may be present in the phytase preparation may become significant, impacting independently on nutrient utilisation.

It is noteworthy that total P levels were low and non-phytate-P levels deficient (1.88 g kg^{-1}) in the above study. In diets with higher P levels, increasing phytase inclusion rates do not necessarily generate more pronounced responses in broilers. For example, there were remarkably little differences in responses to 400 or 800 FTU kg^{-1} phytase over a wide range of parameters in broilers as reported by Cabahug et al. (1999) and Ravindran et al. (2000). Moreover, the addition of seven levels of phytase activity (0–1000 FTU kg^{-1}) to broiler diets containing 7.5 and 3.0 g total P kg^{-1} were investigated by Ravindran et al. (2001). While increasing phytase from 750 to 1000 FTU kg^{-1} slightly benefited amino acid digestibility; in contrast, weight gain, feed efficiency and AME responses to phytase reached a plateau at 750 FTU kg^{-1}, which is quite different to the observations reported by Shirley and Edwards (2003).

Simplistically, these data suggest that increasing dietary P levels impede responses to increasing phytase inclusion levels for which there are two straightforward explanations. One is that inorganic P, the end-product of phytate hydrolysis, strongly inhibits the catalytic activity of phytase (Greiner et al., 1993; Lei and Stahl, 2000). The other is that increased liberation of P, induced by phytase, may prompt Ca and P imbalances in the gastrointestinal tract. While speculative, a third possibility, which is discussed later, is that high phytase inclusions may alter the effective dietary electrolyte balance (DEB) because phytate and phytase influence the secretion of sodium into the gut lumen (Cowieson et al., 2004; Ravindran et al., 2006). Higher than standard phytase inclusion rates should be beneficial in conventional broiler diets but it may be necessary to modify dietary P, Ca, DEB, and possibly other factors, to realise this advantage.

6. Impact of phytase on protein/amino acid availability

The extent to which phytase generates improvements in protein/amino acid digestibility in poultry is variable and the topic remains controversial. The observed variability appears to arise from a number of factors including: (i) the choice of inert marker used in digestibility assays, (ii) differences between ingredient types, (iii) dietary levels of Ca and non-phytate-P and some evidence suggests that (iv) dietary electrolyte balance may be

Table 3
The effect of phytase supplementation (0–12,000 FTU kg^{-1}) on growth performance, nutrient utilisation, bone mineralisation, energy utilisation and total tract phytate-P degradation in broilers (adapted from Shirley and Edwards, 2003)

Phytase (FTU kg^{-1})	Growth performance			Coefficient of nutrient retention			Tibia ash (g)	AMEn (MJ kg^{-1})	Phytate-P disappearance (coefficient)
	Weight gain (g/bird)	Feed intake (g/bird)	FCR (g g^{-1})	Ca	P	N			
0	287	381	1.32	0.456	0.510	0.584	26.0	13.46	0.403
375	399	490	1.23	0.423	0.538	0.689	28.9	13.97	0.495
750	424	505	1.19	0.441	0.608	0.721	29.7	14.13	0.584
1500	459	548	1.19	0.423	0.654	0.745	34.3	14.20	0.652
6000	494	580	1.17	0.495	0.777	0.769	38.6	14.28	0.849
12000	515	595	1.15	0.534	0.797	0.777	40.7	14.29	0.948

involved; these four factors are examined below in detail. Additional contributing factors probably include: age of birds, the inherent digestibility of dietary amino acids, the sources and concentrations of phytate in the diet, the amino acid specifications of the test diets and the inclusion level and type of added phytase. Studies are warranted to define the effects of these aspects.

At present, the mechanisms underlying the protein-associated responses to added phytase remain largely speculative. It has been suggested that the *de novo* formation of binary protein–phytate complexes in the gastrointestinal tract, which are refractory to pepsin activity, may be the key mechanism whereby phytate depresses the digestibility of dietary amino acids (Selle et al., 2000). The other possible mode of action is that phytate may induce increases in endogenous amino acid flows (Ravindran et al., 1999a; Cowieson et al., 2004). Both mechanisms would depress apparent ileal digestibility of amino acids in poultry diets, which should be countered, at least in part, by phytase supplementation.

Negatively charged phytate interacts with basic amino acids (lysine, histidine, arginine) to form binary protein–phytate complexes when gut pH is less than the isoelectric point of proteins (Cosgrove, 1966). Complex formation between sesame seed α-globulin and sodium phytate has been described as a bi-phasic reaction (Rajendran and Prakash, 1993). In an initial rapid step, phytate binds protein via strong electrostatic attractions; this is followed by slower protein–protein interactions culminating in precipitation when the protein–phytate complex exceeds a critical size. In poultry, the *de novo* formation of binary protein–phytate complexes is most likely to occur in the acidic conditions of the proventriculus. As exogenous phytase is mainly active in the crop, it follows that phytase partially prevents the formation of protein–phytate complexes by the prior hydrolysis of phytate. Importantly, as discussed by Selle et al. (2000), several reports have shown that phytate-bound protein is refractory to digestion by pepsin. Vaintraub and Bulmaga (1991) attributed this to reduced solubility and structural changes to protein following aggregation with phytate. Recently, Cowieson et al. (2004) reported that phytate significantly increased the excretion of total endogenous amino acids in broilers (112 mg/bird/48 h *versus* 87 mg/bird/48 h), which was ameliorated by phytase. The increased losses of amino acids were attributed to phytate stimulating secretion of gastrointestinal mucoproteins. Following gastric instillation, pepsin has been shown to increase mucin secretions in rats (Munster et al., 1987); this may be relevant as phytate renders protein refractory to pepsin activity, which could lead to an excess of 'free' pepsin in the proventriculus.

It appears likely the protein effect of phytase largely stems from phytate interfering with the dual role of pepsin in initiating and regulating the protein digestive process (Khrehbiel and Matthews, 2003). This interference with pepsin may also, indirectly, contribute to increased flows of endogenous amino acids. The possible involvement of other mechanisms should not be excluded, which, as discussed later, may include the influence of phytate and phytase on acid–base homeostasis and intestinal uptake of amino acids (Selle et al., 2005).

6.1. Microbial phytase supplementation of complete broiler diets and ileal digestibility of amino acids

A number of studies have reported improvements, although to varying extents, in the coefficient of apparent ileal digestibility (CAID) of amino acids following phytase

Table 4
Comparative summary of the effects of phytase on coefficient of apparent ileal digestibility (CAID) of essential amino acids depending on inert dietary markers

Amino acid	Acid insoluble ash or titanium oxide			Chromic oxide		
	CAID	Response (%)	Number of studies	CAID	Response (%)	Number of studies
Arginine	0.846	3.48	5	0.904	1.03	8
Histidine	0.784	4.64	5	0.856	1.63	8
Isoleucine	0.786	4.28	5	0.836	2.55	8
Leucine	0.786	4.77	5	0.867	1.44	8
Lysine	0.825	3.96	5	0.878	1.08	8
Methionine	0.899	1.75	3	0.907	0.55	8
Phenylalanine	0.798	4.62	5	0.865	1.22	8
Threonine	0.738	6.55	5	0.784	2.29	8
Tryptophan	0.783	4.57	4	0.838	0.59	3
Valine	0.775	4.97	5	0.834	1.99	8
Mean	0.802	4.36	–	0.857	1.44	–

addition to practical broiler diets. A total of 13 published studies have been identified (Table 4) where the effect of phytase on digestibility of amino acids has been determined. In these studies the inert markers selected include chromic oxide (Kornegay, 1996; Sebastian et al., 1997; Kornegay et al., 1999; Namkung and Leeson, 1999; Zhang et al., 1999; Camden et al., 2001; Dilger et al., 2004; Onyango et al., 2005a), acid insoluble ash (Ravindran et al., 2000, 2001; Selle et al., 2003c) and titanium oxide (Rutherfurd et al., 2004; Ravindran et al., 2006). The phytase responses on the CAID of essential amino acids in these 13 studies is summarised in Table 4, where it is evident that the effects are of a numerically greater magnitude when acid insoluble ash or titanium oxide were used as dietary markers in comparison to chromic oxide. It can be seen that, when averaged across studies, added phytase increased the mean digestibility coefficient of essential amino acids 4.4% in the acid insoluble ash and titanium oxide assays, whereas in the chromic oxide assays, the mean digestibility coefficient was increased by 1.4%.

6.2. *Choice of dietary inert markers*

Given this discrepancy, the recommendation by Jagger et al. (1992) that titanium oxide should be preferred to chromic oxide as a marker in nutrient digestibility studies assumes importance. Chromic oxide may lack uniform distribution in digesta during transit along the gastrointestinal tract (Sooncharernying and Edwards, 1993) and chromium chloride transcends the gut at a faster rate than other dietary constituents because of its association with the aqueous, as opposed to the solid phase, of digesta (Oberleas et al., 1990). Thus, it is possible that any phytase induced increases in feed intake may influence the passage of chromic oxide in the gut and compromise amino acid digestibility responses. Some support for this suggestion is provided in a study with turkey poults by Yi et al. (1996a) where responses in amino acid digestibility to phytase

appeared to be more pronounced in dietary treatments where phytase did not alter feed intake.

In addition, analytical methods to determine chromic oxide lack consistency (Sales and Janssens, 2003; Kozloski et al., 1998). Moreover, low recoveries and variable results with chromic oxide have been attributed to analytical problems caused by the presence of P and other minerals in digesta samples (Saha and Gilbreath, 1991; Yin et al., 2000). If so, it is possible that the release of phytate-bound P and other minerals by phytase is a confounding factor in amino acid digestibility assays involving microbial phytase and chromic oxide markers. Thus, it is tempting to speculate that the use of chromic oxide as the dietary marker in the majority of phytase amino acid digestibility assays may have generated misleading results. Based on studies using titanium oxide and acid insoluble ash assays (Table 4), it would appear that the capacity of phytase feed enzymes to enhance ileal amino acid digestibility in broilers is greater than generally appreciated.

6.3. Differences in ingredients: wheat versus maize

The magnitude of amino acid responses with supplemental phytase appears to be dependent on the ingredient used. This may be related to the concentration, structure and storage site of phytate in a particular ingredient and the examples of wheat and maize are discussed below. As shown in Table 5, both Ravindran et al. (1999a) and Rutherfurd et al. (2002) found that phytase increased the ileal digestibility of essential amino acids in wheat (9.2 and 13.4%) to a considerably greater extent than in maize (3.3 and 3.9%). Also, in another study, phytase increased the average digestibility of 14 amino acids by 5.1% (0.839 *versus* 0.800) in diets based on wheat (800 g kg^{-1}) and casein (Ravindran et al., 1999b). The interesting discrepancy between wheat and maize may be related to the propensity of proteins to be bound by phytate, which is thought to be variable (Champagne, 1988), and to the storage site of phytate in the grains (Ravindran et al., 1999b). It is possible that phytate is more likely to complex wheat protein than maize protein and, as discussed by Selle et al. (2000), there is supportive *in vitro* data for this proposition. This could explain the reported differences between the two grains following phytase supplementation and it may be that the configuration of maize protein denies phytate access to basic amino acid residues and, therefore, the initiation of protein–phytate complex formation.

6.4. Phytase and protein utilisation

Phytase has been shown to increase the CAID (Ravindran et al., 1999a) and true ileal digestibility (CTID) (Rutherfurd et al., 2002) of amino acids of individual feed ingredients in broilers to quite marked extents. Paradoxically, in several studies using purified diets, phytase was found to have no effect on protein utilisation (Peter et al., 2000; Peter and Baker, 2001; Boling-Frankenbach et al., 2001a; Augspurger and Baker, 2004). In digestibility assays, methionine is usually the least responsive amino acid to phytase supplementation and, because methionine is frequently limiting, this may negate the benefits of more pronounced increases in digestibility of the balance of amino acids in growth performance and protein efficiency ratio assessments. Also, phytase is probably less likely to generate

Table 5
Comparative effects of phytase on the coefficient of apparent ileal digestibility (CAID) and coefficient of true ileal digestibility (CTID) of amino acids of wheat and maize in broilers

Amino acid	Wheat				Maize			
	Ravindran et al. (1999a)		Rutherfurd et al. (2002)		Ravindran et al. (1999a)		Rutherfurd et al. (2002)	
	Inherent CAID	Response (%)	Inherent CTID	Response (%)	Inherent CAID	Response (%)	Inherent CTID	Response (%)
Arginine	0.76	9.3	0.79	12.7	0.82	3.7	0.88	2.3
Histidine	0.78	9.5	0.80	15.0	0.82	2.9	0.84	6.0
Isoleucine	0.82	6.6	0.79	15.2	0.78	2.7	0.87	4.6
Leucine	0.83	6.4	0.84	9.5	0.88	1.0	0.89	3.4
Lysine	0.72	10.9	0.74	17.6	0.73	3.4	0.87	2.3
Methionine			0.83	10.8			0.90	2.2
Phenylalanine	0.84	6.3	0.85	9.4	0.83	2.1	0.90	2.2
Threonine	0.66	15.7	0.77	16.9	0.66	6.7	0.82	7.3
Valine	0.78	8.8	0.79	13.9	0.76	4.1	0.86	4.7
Mean	0.774	9.2	0.800	13.4	0.785	3.3	0.870	3.9

responses in atypical diets with low (<2.0 g kg^{-1}) phytate concentrations, such as those used in these studies.

7. Impact of phytase on energy utilisation

The possibility that supplementary phytase has a positive effect on energy utilisation in poultry has considerable practical implications. Early studies involving dephytinised feed ingredients suggested that phytate negatively influences energy utilisation in broilers (Rojas and Scott, 1969; Miles and Nelson, 1974). Very few studies have been completed in layers; however, Scott et al. (2001) found that phytase increased AME in both maize (13.84 MJ kg^{-1} DM *versus* 13.36 MJ kg^{-1} DM) and wheat (14.57 MJ kg^{-1} DM *versus* 14.04 MJ kg^{-1} DM) based layer diets. Alternatively, Liebert et al. (2005) reported that phytase supplementation of maize–soy diets did not enhance N-corrected AME in layers. Thus, this discussion is confined to broilers and exogenous phytase has quite consistently increased AME of broiler diets based on wheat and/or sorghum in studies completed at The University of Sydney (Ravindran et al., 1999b, 2000, 2001; Selle et al., 1999, 2001, 2003c, 2005).

These studies and several other investigations (Driver et al., 2006; Farrell et al., 1993; Kocher et al., 2003; Namkung and Leeson, 1999; Shirley and Edwards, 2003) are summarised in Table 6. Overall, phytase supplementation increased AME by an average of 0.36 MJ kg^{-1} DM (or 2.8%) over the non-supplemented controls. The percentage responses in AME following phytase supplementation are negatively correlated ($r = -0.562$; $P<0.02$) to the energy density of the control diets. While the data indicate that phytase positively influences energy utilisation in broilers, the lack of a convincing rationale detracts from the credibility of this proposition.

In phytase experiments, wheat may be pre-pelleted separately to eliminate intrinsic phytase activity as it might compromise responses to microbial phytase. This approach was adopted in one study (Selle et al., 2001), in which phytase did not enhance energy utilisation. Interestingly, there are indications that extrusion of wheat reduces solubility of protein and phytate (Ummadi et al., 1995a,b), which may render phytate less susceptible to hydrolysis. Interestingly, Park et al. (2000) have shown that heat-treatment of rapeseed meal reduces phytate degradation by ruminal fermentation in sheep. It seems possible, therefore, that the prior steam-pelleting of wheat *per se* may have contributed to the lack of a response to phytase. However, Edwards et al. (1999) found that extruding maize–soy broiler diets reduced phytate-P retention by 13.1%, whereas steam-pelleting diets did not influence phytate-P retention.

Instructively, Camden et al. (2001) evaluated two phytase feed enzymes (*Bacillus subtilis* at 250, 500, 1000; *A. niger* at 500 FTU kg^{-1}) in broilers offered maize–soy diets. Overall, phytase increased ileal digestibility coefficients of fat by 3.5% (0.954 *versus* 0.921), protein by 2.6% (0.866 *versus* 0.844) and starch by 1.4% (0.978 *versus* 0.964). This was associated with phytase-induced increases in AME of 0.17 MJ (15.29 MJ kg^{-1} DM *versus* 15.12 MJ kg^{-1} DM) and apparent ileal digestibility of energy of 0.26 MJ (16.34 MJ kg^{-1} DM *versus* 16.08 MJ kg^{-1}). Thus, this study indicates, as suggested earlier by Baker (1998), that the positive impact of phytase on energy utilisation stems from an accumulation of increases in fat, protein and starch digestibilities.

Table 6
Effects of phytase supplementation on energy utilisation (AME or AMEn) in broiler chickens

Reference	Diet type	AME (MJ kg^{-1} DM)		Response		Phytase (FTU kg^{-1})
		Control	Phytase	MJ kg^{-1} DM	%[a]	
Driver et al. (2006)	Maize–soy (AMEn)	12.49	12.62	0.13	1.0	24,000, *Apergillus niger*
	Above plus peanut meal	12.13	12.83	0.70	5.8	24,000, *Apergillus niger*
Farrell et al. (1993)	Sorghum (AMEn)	12.80	13.10	0.30	2.3	750, *Apergillus niger*
Kocher et al. (2003)	Wheat	14.88	14.96	0.08	0.5	Mean of two phytases
	Sorghum	16.15	16.18	0.03	0.2	Mean of two phytases
Namkung and Leeson (1999)	Maize–soy (AMEn)	11.89	12.16	0.27	2.3	1200, *Apergillus niger*
Ravindran et al. (1999b)	Wheat *per se*	11.07	11.65	0.58	5.2	600, *Apergillus niger*
	Wheat *per se*	13.55	14.17	0.62	4.6	600, *Apergillus niger*
	Barley *per se*	12.36	12.69	0.33	2.7	600, *Apergillus niger*
Ravindran et al. (2000)	Wheat–sorghum 2.3 g kg^{-1}[b]	13.33	13.52	0.19	1.4	400 + 800, *A. niger*
	Wheat–sorghum 4.5 g kg^{-1}[b]	12.67	13.38	0.71	4.6	400 + 800, *A. niger*
Ravindran et al. (2001)	Wheat–sorghum blend	14.22	14.55	0.33	2.3	500, *Apergillus niger*
Selle et al. (1999)	Sorghum	12.46	12.87	0.41	3.3	600, *Apergillus niger*
Selle et al. (2001)	Wheat (pre-pelleted)	14.2	14.1	−0.1	−0.7	600, *Apergillus niger*
Selle et al. (2003c)	Wheat–sorghum blend	13.79	14.38	0.59	4.3	600, *Apergillus niger*
Selle et al. (2005)	Wheat–sorghum blend	14.22	14.56	0.34	2.4	600, *Apergillus niger*
Shirley and Edwards (2003)	Maize–soy (AMEn)	13.46	14.13	0.67	5.0	750, *Apergillus niger*
Mean		13.27	13.64	0.36	2.8	662 FTU kg^{-1}[c]

[a] Percentage improvements over non-supplemented controls.
[b] Non-phytate-P.
[c] Excluding Driver et al. (2006) and Kocher et al. (2003).

7.1. Phytase and energy derived from fat, protein and starch

As discussed by Cosgrove (1966), there is evidence of phytate interactions with lipid in maize and it was suggested that these 'lipophytins' are a complex of Ca/Mg–phytate, lipids and peptides. It seems possible therefore, that Ca–phytate and lipids may be involved in the formation of metallic soaps in the gut lumen of poultry, which are major constraints on utilisation of energy derived from lipid, particularly saturated fats (Leeson, 1993; Atteh and Leeson, 1984). Interestingly, Matyka et al. (1990) found that beef tallow reduced phytate-P utilisation in young chicks and increased the percentage of fat excreted as soap fatty acids. Ravindran et al. (2000) reported more pronounced AME responses to phytase in diets with higher Ca levels, which is consistent with the involvement of Ca–phytate complexes in the formation of insoluble metallic soaps. If Ca–phytate is a component of metallic soaps in broilers it follows that phytase would partially prevent their formation by prior hydrolysis of phytate in more proximal parts of the gut. Thus, this is one possible mechanism underlying the increased ileal digestibility of fat, reported by Camden et al. (2001), following phytase supplementation. Enhanced digestibility of amino acids would increase the utilisation of energy derived from protein, and, in this connection, the roles of phytate and phytase have been discussed in the previous section.

It has been suggested that phytate may bind with starch either directly, via hydrogen bonds, or indirectly, via proteins associated with starch (Thompson, 1988; Rickard and Thompson, 1997). This would provide a rationale for phytase increasing energy utilisation from this source; however, as discussed by Selle et al. (2000), there is a paucity of *in vitro* evidence to support the existence of starch–phytate complexes. Alternatively, phytate is a potent inhibitor of α-amylase activity. This was first demonstrated by Cawley and Mitchell (1968) and has been frequently confirmed in subsequent studies, as reviewed by Selle et al. (2000). Indeed, Desphande and Cheryan (1984) proposed that phytate inhibition of α-amylase might play a physiological role in relation to starch reserves during seed germination. While Martin et al. (1998) reported that phytase supplementation reduced amylase activity in the small intestine of ducks, it is not clear if phytate inhibition of α-amylase in the gastrointestinal tract of poultry limits starch digestion. However, responses to α-amylase supplementation have been reported in broilers (Gracia et al., 2003) and turkeys (Ritz et al., 1995). It seems possible that phytate inhibition of α-amylase may impede starch digestion, which would be countered by phytase, but it is possible that this effect is marginal.

7.2. Phytate, phytase and sodium: possible consequences

Sodium phytate and exogenous phytase have been shown to have pronounced effects on Na excretion rates in broilers offered atypical diets (Cowieson et al., 2004); phytate increased Na excretion by a four-fold factor, which phytase reduced by 44%. Importantly, similar, significant effects have also been reported in broilers offered conventional diets (Ravindran et al., 2006). Phytate, derived from rice bran, increased Na losses at the ileal level by 60% and phytase reduced these losses by 66%. Cowieson et al. (2004) suggested that the phytate-induced movement of Na into the gut lumen was to buffer this polyanionic molecule; however, in conventional diets it is conceivable that there are alternative explanations as phytate probably would bind Ca more readily than Na. Nevertheless, on the basis of these

two recent studies, phytate and phytase influence the Na status of broiler chicks. It is possible, therefore, that phytate and phytase influence acid–base homeostasis and may also compromise Na-dependant co-transport mechanisms involved in the intestinal uptake of glucose and certain amino acids in broilers (Garriga et al., 2000; Sklan and Noy, 2000; Gal-Garber et al., 2003).

Exogenous phytase has been shown to increase blood glucose concentrations in pigs (Johnston et al., 2004; Kies et al., 2005) and it is likely that phytate impedes glucose uptake in humans. For example, there were marked increases in glycaemic indices of human patients following dephytinisation of navy bean flour (Thompson et al., 1987). However, the addition of 8 g phytate kg^{-1} to a test meal of 50 g glucose significantly reduced blood glucose responses in humans (Demjen and Thompson, 1991). As discussed by Rickard and Thompson (1997), this suggests that phytate-induced reductions in glucose absorption may not involve alterations to starch digestion. Interestingly, phytate has been shown to reduce fat and protein digestibility, but not that of starch, in balance experiments with rats (Nyman and Bjork, 1989).

Phytase supplementation of lysine-deficient broiler diets increased amino acid digestibility but, surprisingly, addition of lysine monohydrochloride also increased digestibility of certain amino acids (Selle et al., 2005). Moreover, significant treatment interactions for nine amino acids (including arginine, lysine, phenylalanine, threonine and tryptophan) were observed as responses to phytase were more pronounced in lysine-deficient diets. Lysine, a basic amino acid, may contribute to the regulation of acid–base homeostasis (Austic and Calvert, 1981) and it is likely that the balance of monovalent electrolytes influences the intestinal absorption of lysine in chicks (Riley and Austic, 1989). Conceivably, the impact of phytate and phytase on Na movements into the gut may also influence acid–base homeostasis. The estimated dietary electrolyte balance (DEB) in the Selle et al. (2005) study was 155 meq kg^{-1}, which is less than the recommended DEB level of 250–300 meq kg^{-1} (Johnson and Karunajeewa, 1985). Interestingly, increasing DEB by 450 meq kg^{-1} has been shown to increase amino acid digestibility in pigs (Haydon and West, 1990). These authors suggested that the increased DEB may have modified the intestinal uptake of amino acids via Na^+-dependent transport systems.

It is noteworthy that phytase has been anecdotally linked to increased moisture in broiler excreta and poor litter quality in the field (Debicki-Garnier and Hruby, 2003; Pos et al., 2003), which could reflect changes in acid–base homeostasis. Therefore, mechanisms underlying the effects of phytate and phytase on movements of Na into the gut lumen demand further investigation. Finally, it seems possible that alterations to acid–base homeostasis and/or Na-dependent transport systems, induced by phytate and phytase, may influence intestinal uptakes of glucose and certain amino acids.

8. Manipulating phytase responses in poultry

It is probable that a number of factors could be manipulated to enhance phytase efficacy and responses in poultry. In broilers, intermittent lighting regimes may increase digesta retention in the crop (Hooppaw and Goodman, 1976); logically, given that the crop is probably the major site of activity, this simple procedure could facilitate phytate hydrolysis

by phytase. Recommended inclusion rates of contemporary phytase feed enzymes may be conservative but, as discussed, appropriate dietary formulations may be needed to realise benefits from higher additions. It remains possible that inherently more effective phytase feed enzymes, with the capacity to degrade the majority of phytate in broiler diets, will be developed.

Alternatively, the simultaneous inclusion of phytase with other exogenous enzymes may be beneficial, particularly if substrate access is enhanced. The combination of phytase and xylanase in wheat-based diets (Ravindran et al., 1999b; Zyla et al., 1999a,b) appears to be advantageous and it is possible that xylanase facilitates substrate access and the absorption of nutrients liberated by phytase. Combinations of different phytases and acid phosphatases have been evaluated (Zyla et al., 2004) and it may be that acid phosphatase accelerates phytase-induced dephosphorylation of phytate. The simultaneous inclusion of phytase with α-galactosidae, β-glucanase and xylanase has been investigated in maize, barley and wheat-based diets, respectively, by Juanpere et al. (2005) and Cowieson and Adeola (2005). The data suggest that phytase in combination with carbohydrase and protease has additive effects in nutritionally marginal broiler diets. Thus, further research into enzyme 'cocktails' is justified.

8.1. *The influence of calcium on phytase efficacy*

Importantly, Ballam et al. (1985) considered that Ca is the most underestimated factor contributing to phytate-P availability and, as reviewed by Angel et al. (2002), dietary levels of Ca (and Ca:P ratios) are crucial to phytase efficacy. Nevertheless, appropriate dietary calcium levels, and Ca:P ratios, in phytase-supplemented broiler diets still require proper definition; although, there is consensus that 'narrow' Ca:P ratios should be adopted. Ca:P ratios in the range of 1.1 to 1.4:1 have been recommended for turkeys (Qian et al., 1996b) and broilers (Qian et al., 1997). However, because increasing dietary Ca levels *per se* can generate negative effects, defining the impact of Ca on phytase is not straightforward. Qian et al. (1997) found that increasing Ca (5.61–10.20 g kg^{-1}) and Ca:P ratios (1.1–2.0:1) depressed weight gain (420 g/bird *versus* 553 g/bird) of broilers to 21 days of age. However, 900 FTU kg^{-1} phytase enhanced weight gain in diets with both narrow (615 g/bird *versus* 553 g/bird) and wide (541 g/bird *versus* 420 g/bird) Ca:P ratios.

Limestone is known to have a high acid-binding capacity (Lawlor et al., 2005) and, as a result, binds more acid and increases digesta pH in the proximal gut. The addition of Ca, as limestone, to broiler diets has been shown to increase crop pH (Shafey et al., 1991), which is the main site of phytate degradation by phytase feed enzymes. Depending on their spectrum of activity, variations in crop pH could directly influence phytase efficacy. It is noteworthy that phytase generated enhanced bone mineralisation in broilers following a reduction in crop pH (5.4 *versus* 6.4), induced by glutamic acid (Murai et al., 2001). Perhaps more importantly, elevations in crop pH may increase mineral–phytate complex formation, including Ca–phytate that reduces the susceptibility of phytate to hydrolysis (Wise, 1983; Maenz et al., 1999). Another possibility is that Ca is an inhibitor of phytase activity (Qian et al., 1996a), but the data on this aspect are conflicting (Mahajan and Dua, 1997). However, it is noteworthy that dietary Ca (9.0 g kg^{-1}) has been shown to reduce both mucosal phytase activity and ileal phytate degradation (Applegate et al., 2003a).

8.2. Differences in phytate hydrolysis between feed ingredients

It is likely that the susceptibility of phytate to phytase hydrolysis may vary between feed ingredients, but this possibility has received very little attention. Leske and Coon (1999) investigated the effects of 600 FTU kg^{-1} phytase activity on phytate (IP_6) degradation in a total tract assessment in broilers. Increased phytate hydrolysis (values in parentheses) varied amongst seven feed ingredients: canola meal (6.3%), rice bran (11.0%), wheat middlings (11.5%), barley (15.2%), wheat (17.8%), maize (28.2%) and soyabean meal (37.5%). Intrinsic phytase activity in wheat, wheat middlings and barley may have been a confounding factor. However, it would appear that phytase more readily hydrolyses phytate in soyabean meal and maize than in canola meal and rice bran, although the two latter feed ingredients contained higher levels of phytate. Further studies similar to this, preferably based on ileal degradation rates and taking phytate concentrations into consideration, should prove instructive.

8.3. The influence of feed additives on phytase efficacy

Various feed additives may complement the efficacy of phytase in broilers where Vitamin D_3 (cholecalciferol), hydroxylated D_3 compounds and citric acid have probably received the most attention. Edwards (1993) demonstrated that the inclusion of 1,25-dihyroxycholecalciferol in broiler diets enhanced phytate-P utilisation, which has been confirmed in subsequent studies (Biehl and Baker, 1997; Mitchell and Edwards, 1996; Driver et al., 2005b). Citric acid was shown to prevent rickets in rats (Shohl, 1937) and, more recently, to enhance phytate-P utilisation in broiler chicks (Rafacz-Livingston et al., 2005) but not layer hens (Boling et al., 2000b). Boling-Frankenbach et al. (2001b) concluded that 40–60 g citric acid kg^{-1} in maize–soy broiler diets decreased P requirements by 1.0 g kg^{-1}.

Instructively, Snow et al. (2004) evaluated combinations of 1α-hydroxycholecalciferol (up to 15 μg kg^{-1}), citric acid (up to 40 g kg^{-1}) and *A. niger* phytase (300 FTU kg^{-1}) in low P diets (1.3 g non-phytate-P kg^{-1}, 2.6 g phytate-P kg^{-1}) offered to broiler chicks from 8 to 21 days. Based on responses in growth performance and tibia ash, all three feed additives increased phytate-P utilisation and their positive effects were generally additive. In contrast, it has been shown that other feed additives may have deleterious effects on phytase efficacy. For example, high levels of zinc (Augspurger et al., 2004) and copper (Banks et al., 2004) in broiler diets have been shown to have negative influences in this context.

The incorporation of mineral chelating agents into poultry diets has the potential to enhance phytate degradation by microbial phytase. From *in vitro* investigations by Maenz et al. (1999), numerous cations (Zn^{2+}, Fe^{2+}, Mn^{2+}, Fe^{3+}, Ca^{2+}, Mg^{2+}) have the potential to form mineral–phytate complexes, which are resistant to phytate hydrolysis at neutral pH. However, acidification of the media to pH 4 decreased the inhibitory potency of divalent cations. Moreover, chelating agents, including EDTA, citric acid and phthalic acid, have the capacity to render phytate more susceptible to phytase hydrolysis. Further investigations into the combined use of phytase and mineral chelating agents in poultry diets are merited, although the inclusion cost of chelating agents is clearly a critical consideration.

8.4. Feed ingredients with reduced phytate-P contents

Supplementation of poultry diets with phytase is one approach to reduce the negative impact of phytate; another, is the formulation of diets containing reduced levels of phytate. Particular interest, as initially reported by Huff et al. (1998), has been expressed in high available phosphorus maize, which has been the subject of several, subsequent evaluations (Douglas et al., 2000; Li et al., 2000; Waldroup et al., 2000; Yan et al., 2000). An alternative approach is to pre-treat feed ingredients with phytase to reduce phytate concentrations (dephytinisation) and several aquacultural experiments with dephytinised feed ingredients have been reported. Newkirk and Classen (1998, 2001) have evaluated dephytinisation procedures and the inclusion of dephytinised canola meal in broiler diets. Both approaches hold promise but their practical adoption will depend on the global acceptance of genetically modified crops in the first instance and, in the second, the costs and benefits of dephytinised feedstuffs for pigs and poultry.

From a scientific standpoint, dephytinised feedstuffs could prove to be a valuable means to define the anti-nutritive properties of phytate. It is noteworthy that Newkirk and Classen (2001) found higher CAID for amino acids in maize–soy broiler diets containing 300 g dephytinised canola meal kg^{-1} in comparison to placebo-treated canola meal. This substitution with dephytinised canola meal increased the mean CAID coefficient of 17 amino acids by 17.4% (0.752 *versus* 0.648).

9. Phytase supplementation of layer diets

Compared to broiler chicks, phytase inclusion in diets for laying hens has been the subject of less research. The underlying reason may be that the P requirement for layers has not been established and it is likely that the NRC (1994) recommendation of 250 mg non-phytate-P/hen/day is excessive (Keshavarz, 2000). This may be complicated by the failure to recognise the contribution of digestible phytate-P in layers (Boorman and Gunaratne, 2001). Fundamentally, it is probable that layers are better equipped to accommodate the nutritional insults of phytate than broilers.

Liebert et al. (2005) contend that the benefits of phytase supplementation of layer diets are 'still under discussion'. However, van der Klis et al. (1997) demonstrated the efficacy of phytase in layers in a study in which an HPLC method was used to determine phytate. These researchers reported that in maize–soy diets, containing 2.4 g phytate-P kg^{-1}, 500 FTU phytase activity kg^{-1} substantially increased ileal degradation of phytate (0.661 *versus* 0.081). Thus, phytase degraded 0.58 of dietary phytate (IP_6) and increased ileal P absorption (0.545 *versus* 0.262); in contrast, Ca absorption (0.706 *versus* 0.720) was not influenced. It is likely, therefore, that, with judicious application, phytase inclusion in layer diets will be of benefit.

Anecdotally, the substitution of tricalcium phosphate (TCP) with microbial phytase in layer diets in Asia was the first time acceptance of this feed enzyme extended beyond the Netherlands to any real extent. Of relevance is that TCP is an inorganic P source, thought to be contaminated with fluoride, which has adverse effects on egg production. For example, Um and Paik (1999) reduced TCP in maize–soy layer diets from 14.0 to 7.0 and 0 g kg^{-1},

which were supplemented with 500 FTU kg^{-1} phytase. Phytase significantly increased egg production (86.3% *versus* 84.5%) in the high P diet and egg production in the lower P, supplemented diets was not compromised. There were no differences in eggshell strength and Haugh units, only subtle differences in specific gravity and eggshell thickness, and phytase reduced P excretion by up to 41% in this study. In another study involving TCP, Lim et al. (2003) concluded that phytase supplementation improved egg production and reduced percentages of broken and soft eggs and P excretion. However, it was concluded that dietary levels of Ca and non-phytate-P could significantly influence the effects of phytase supplementation.

A number of evaluations of phytase supplementation of layer diets have been completed with more conventional inorganic P supplements than TCP. These include studies by Gordon and Roland (1997, 1998), Carlos and Edwards (1998), Scott et al. (1999, 2000, 2001), Boling et al. (2000a), Keshavarz (2000, 2003), Sohail and Roland (2000), Jalal and Scheideler (2001), Ceylan et al. (2003), Francesch et al. (2005) and Panda et al. (2005). These 14 studies are summarised in Table 7 and the thrust of these studies, in which diets were based mainly on maize and soyabean meal, is that phytase supplementation of layer diets permits reductions in non-phytate-P for layers, and consequently P excretion, without compromising performance and egg quality. It is noteworthy that phytase inclusion levels (250–300 FTU kg^{-1}) in layer diets are generally lower than for broilers and this may be related to the longer retention of feed in the crop, which facilitates phytate degradation. It is possible that phytase enhances Ca availability (Sohail and Roland, 2000) and Ca influences phytase efficacy (Scott et al., 1999). It may be instructive to focus attention on the effects of dietary Ca levels when evaluating phytase supplementation of layer diets. Also, it may be possible to reduce additional nutrient specifications in association with phytase supplementation (Scott et al., 2001), which would be economically advantageous.

The effects of phytase on protein digestibility have received little attention in layers. However, van der Klis and Versteegh (1991) reported that the addition of 250–300 FTU phytase kg^{-1} to layer diets resulted in small, but significant, improvements in apparent ileal absorption of nitrogen. Snow et al. (2003) investigated the inclusion of 300 FTU phytase kg^{-1} on amino acid digestibility in moulted layers using acid insoluble ash (15 g kg^{-1}) as the marker. The results are curious, as phytase tended to depress the average digestibility of 17 amino acids by 2.3% (0.848 *versus* 0.868) in a maize–soy diet. However, with the addition of either meat-and-bone meal (75 g kg^{-1}) or wheat middlings (100 g kg^{-1}) to the basal diet, phytase numerically increased average amino acid digestibilities by 3.1% (0.852 *versus* 0.826) and 3.7% (0.898 *versus* 0.866), respectively. There is no obvious explanation for this apparent dichotomy and significant diet type × phytase interactions for alanine, glycine, leucine and methionine were observed. In an earlier study (Jalal et al., 1999), phytase enhanced the digestibility of alanine, cystine, glutamic acid and methionine, but not other amino acids, in maize–soy layer diets.

10. Future directions and implications

The present usage of phytase feed enzymes by poultry producers is substantially greater than anticipated when they were first introduced. Increasing ecological concerns in relation

Table 7
Summary of phytase supplementation of diets for laying hens

Reference	Phytase (FTU kg^{-1})	Non-phytate-P ($g\,kg^{-1}$)	P source	Ca ($g\,kg^{-1}$)	Comments
Gordon and Roland (1997)	300	1.0–5.0	DCP	40.0	Phytase supplementation of 1.0 $g\,kg^{-1}$ npP corrected adverse effects. Phytase did not improve performance in diets with higher npP levels
Gordon and Roland (1998)	300	1.0 and 3.0	DCP	25.0–31.0	Phytase × npP interactions observed for eggshell quality, feed consumption and egg production. Phytase compensated/reduced adverse effects of low dietary npP and Ca
Carlos and Edwards (1998)	600	3.3 tP	Nil	30.0	Phytase and 5 $\mu g\,kg^{-1}$ 1,25-$(OH)_2D_3$ and phytase assessed in two experiments. Phytase had positive effects on bodyweight, plasma P, tibia ash and phytate-P retention
Scott et al. (1999)	250, 500	2.0 and 4.0, 1.1 and 2.2	M/DCP	37 and 40	From 55 to 67 weeks, 500 FTU kg^{-1} phytase in 2.2 $g\,kg^{-1}$ npP diets depressed body wt., egg wt. and FCR. Ca:available P impacted on shell quality. Ca impacted on phytase
Scott et al. (2000)	250, 500, 250, 500	2.0 and 4.0, 1.1 and 2.2	M/DCP	37 and 40	Wheat-based diets, average 629 $g\,kg^{-1}$. Phytase had little effect, which was attributed to plant phytase activity in wheat (~821) or mean of 516 FTU kg^{-1} in complete diets
Scott et al. (2001)	300	2.0–4.2	M/DCP	~37.5	Standard and modified (matrix values) maize or wheat-based diets. From 50 to 62 weeks. Phytase accommodated reductions to energy, CP, P and Ca with maize (not wheat)
Boling et al. (2000a)	300	1.0–4.5	NA[a]	38.0	Diets containing 1.5 or 1.0 $g\,kg^{-1}$ available P plus 300 FTU kg^{-1} supported optimal egg production and the latter reduced P excretion by ~50% *vs.* 4.5 $g\,kg^{-1}$ avail P diets
Keshavarz (2000)	300	1.0–4.0	M/DCP	38.0	From 30 to 66 weeks layers on the lowest P regimen + phytase performed as well as controls. Phytase increased P retention by 15% and reduced P excretion by 34–47%
Keshavarz (2003)	300	1.0–4.0	M/DCP	38.0	Phytase supplementation completely addressed the adverse effects of 2.0 $g\,kg^{-1}$ npP; at 1.0 and 1.5 $g\,kg^{-1}$ effects were partially addressed. P excretion was 21–43% less
Sohail and Roland (2000)	300	3.0	DCP	31.0–37.0	Study designed to determine phytase effect on Ca. Phytase improved Ca availability, and eggshell quality at 34 $g\,kg^{-1}$ Ca; as indicated by improved egg specific gravity
Jalal and Scheideler (2001)	A 250, B 300	1.0–3.5	DCP	38.5	Phytase improved feed intake, conversion, egg mass in normal diets and shell quality and egg components at 1.0 $g\,kg^{-1}$ npP. A *vs.* B: differences in Ca and P digestibility
Ceylan et al. (2003)	300	2.0–4.0	DCP	38.0	HAP and standard maize. Phytase supplementation of 2.0 $g\,kg^{-1}$ npP diet did not improve egg production parameters. HAP maize permits reductions in DCP levels
Francesch et al. (2005)	150–450	1.1–3.2	DCP	36.0	Experimental phytase, barley and maize-based diets. Phytase compensated for lower npP levels and reduced P excretion by 49%. Phytase linearly increased P absorption
Panda et al. (2005)	500	1.2–3.0	DCP	34.8	There was no advantage in increasing npP above 1.8 $g\,kg^{-1}$ or adding phytase. Phytase permits 1.2 $g\,kg^{-1}$ npP diets, eliminates added iP and reduces P excretion

[a] Not available.

to P pollution, a better appreciation of the application of microbial phytases, and their decreasing inclusion costs, has contributed to this increasing acceptance. During the past 15 years, research on the evaluation of microbial phytases in diets for simple-stomached species has rapidly expanded, but much of the focus of this research has been on the evaluation of various phytases from different sources rather than the investigation of the underlying factors causing variability in phytase responses. Fundamental information in respect of phytate and phytase is lacking in many aspects, which needs to be generated and integrated for a more complete understanding of this subject.

Clearly there is an urgent need to clarify and define the P requirements of poultry accurately and to develop appropriate terminology to express these requirements uniformly (Angel et al., 2002). Ironically, this objective is being complicated by the introduction of 'low-phytate' feed ingredients and the acceptance of phytase feed enzymes. Existing recommended requirements for both P and Ca may have to be re-defined in relation to these developments. As noted previously, there is a need to develop more relevant and standard definitions of phytase activity and, also, for rapid and sensitive assays to determine the substrate, phytate. Accurate determinations of phytate concentrations in complete diets, ileal digesta and excreta are not straightforward and, arguably, this has limited progress in research into, and practical application of, microbial phytases. Also, phytate itself requires further study, particularly from a structural point of view in different feed ingredients and its susceptibility to hydrolysis by phytase activity.

Dietary manipulations to facilitate the activity of exogenous phytases should be considered and applied appropriately. Low dietary levels of Ca and P and narrow Ca:P appear advantageous. The simultaneous inclusion of phytase and xylanase in wheat-based diets has been shown to generate synergistic increases in digestibility of some amino acids (Ravindran et al., 1999b; Selle et al., 2003c). Phytate is concentrated in the aleurone layer of wheat (Ravindran et al., 1995a) and it may be that xylanase facilitates access of phytase to its substrate in the aleurone, which is the case for proteolytic enzymes (Parkkonen et al., 1997). The simultaneous use of 3-phytase, 6-phytase and acid phosphatase in broilers, as investigated by Zyla et al. (2004), may also increase phytate degradation rates in poultry. It is possible that combinations of phytase and various enzymes may be similarly advantageous for other feed ingredients (Cowieson and Adeola, 2005). It is also possible that chelating agents may facilitate phytate degradation with economic viability and this option needs to be further explored. It is generally assumed that the 'nutrient release or equivalency' values for phytase is valid independent of the raw material used. Clearly this is not the case and it is known that responses to phytase addition vary in different raw materials. Most nutrient release values have been generated from studies with broiler starters. But the efficacy of microbial phytase may vary with the age of birds, though this has not been considered in practical context, perhaps for reasons of simplicity. The possible influence of gender on phytase responses has also not been studied.

The original phytase feed enzymes were produced mainly from fungi. But recent developments in the production and/or expression of enzymes in other forms of microorganisms, such as bacteria and yeast, have resulted in new exogenous phytases. There is suggestive evidence that such bacterial phytases may be more efficacious in broiler chickens. For example, Augspurger et al. (2003) reported that a bacterial phytase derived from *E. coli* liberated more P in broilers than two recombinant fungal phytases, based on increases in

tibia ash relative to inorganic P supplementation. It has been reported that the *E. coli* phytase is more resistant to pepsin activity than fungal phytases (Rodriguez et al., 1999; Igbasan et al., 2000), which may explain the increased liberation of phytate-bound P. Nevertheless, it remains possible that 'second-generation' phytase feed enzymes with an inherently greater capacity to hydrolyse dietary phytate, which would further reduce P excretion and generate greater amino acid and energy responses, will be developed in the foreseeable future. The ideal enzyme would have high specific catalytic activity (per unit of protein), good thermostability during feed processing, high activity under wide ranges of gut pH, resistance to proteolysis and good stability under ambient temperatures.

Finally, it is important to recognise that the transit time and pH limitations within the digestive tract of poultry do not permit complete dephosphorylation of phytic acid to *myo*-inosotol and inorganic P moieties. Exogenous phytase probably hydrolyses less than 0.35 of dietary phytate in broilers and clearly there is considerable scope to develop strategies that will increase phytate-P degradation in poultry. Thus, it appears that considerable advantage remains to be realised from further reductions of phytate concentrations either inherently in feed ingredients and/or in the gastrointestinal tract by enzymatic hydrolysis or other means. Dephytinisation, or pre-treatment of feed ingredients with phytase, could become a practical alternative, particularly with oilseed meals. The use of low-phytate feed ingredients would also reduce dietary phytate contents. Presently, there is considerable interest in low-phytate maize, which has been developed following the identification of mutant genes that suppress phytate synthesis in the kernel without affecting total P concentrations (Raboy et al., 1990). Future developments in molecular biology may increase phytase efficacy, reduce phytate accumulation in plants or increase endogenous phytase synthesis in both plants and animals. Further research into dephytinisation of ingredients is justified, as it is likely that the negative influence of phytate, particularly on protein and energy utilisation, is of a greater magnitude than presently appreciated.

References

Abelson, P.H., 1999. A potential phosphate crisis. Science 283, 2015.

Adedokun, S.A., Sands, J.S., Adeola, A., 2004. Determining the equivalent phosphorus released by an *Escherichia coli*-derived phytase in broiler chicks. Can. J. Anim. Sci. 84, 437–444.

Aggrey, S.E., Zhang, W., Bakalli, R.I., Pesti, G.M., Edwards, H.M., 2002. Genetics of phytate phosphorus bio-availability in poultry. In: 7th World Congress on Genetics Applied to Livestock Production, INRA, Montpellier, France, pp. 277–279.

Angel, R., Tamim, N.M., Applegate, T.J., Dhandu, A.S., Ellestad, L.E., 2002. Phytic acid chemistry: influence on phytin-phosphorus availability and phytase efficacy. J. Appl. Poult. Res. 11, 471–480.

Angel, R., Saylor, W.W., Dhandu, A.S., Powers, W., Applegate, T.J., 2005. Effects of dietary phosphorus, phytase and 25-hydroxycholecalciferol on performance on broiler chickens grown in floor pens. Poult. Sci. 84, 1031–1044.

Applegate, T.J., Angel, R., Classen, H.L., 2003a. Effect of dietary calcium, 25-hydroxycholecalciferol, or bird strain on small intestinal phytase activity in broiler chickens. Poult. Sci. 82, 1140–1148.

Applegate, T.J., Joern, B.C., Nussbaum-Wagler, D.L., Angel, R., 2003b. Water-soluble phosphorus in fresh broiler litter is dependent upon phosphorus concentration fed but not on fungal phytase supplementation. Poult. Sci. 82, 1024–1029.

Applegate, T.J., Webel, D.M., Lei, X.G., 2003c. Efficacy of a phytase derived from *Escherichia coli* and expressed in yeast on phosphorus utilization and bone mineralization in turkey poults. Poult. Sci. 82, 1726–1732.

Atteh, J.O., Leeson, S., 1984. Effects of dietary saturated or unsaturated fatty acids and calcium levels on performance and mineral metabolism in broilers. Poult. Sci. 63, 2252–2260.

Augspurger, N.R., Webel, D.M., Lei, X.G., Baker, D.H., 2003. Efficacy of an *E. coli* phytase expressed in yeast for releasing phytate-bound phosphorus in young chicks and pigs. J. Anim. Sci. 81, 474–483.

Augspurger, N.R., Baker, D.H., 2004. High dietary phytase levels maximize phytate-phosphorus utilization but do not affect protein utilization in chicks fed phosphorus- or amino acid-deficient diets. J. Anim. Sci. 82, 1100–1107.

Augspurger, N.R., Spencer, J.D., Webel, D.M., Baker, D.H., 2004. Pharmacological zinc levels reduce the phosphorus-releasing efficacy of phytase in young pigs and chickens. J. Anim. Sci. 82, 1732–1739.

Austic, R.E., Calvert, C.C., 1981. Nutritional interrelationships between electrolytes and amino acids. Fed. Proc. 40, 63–67.

Averill, H.P., King, C.G., 1926. The phytin content of foodstuffs. JAOCS 48, 724–728.

Baker, D.H., 1998. Beyond phosphorus: phytase effects on protein, energy and trace-mineral utilization of swine and poultry. In: BASF Technical Symposium. BASF Corporation, Mount Olive, NJ, pp. 48–62.

Ballam, G.C., Nelson, T.S., Kirby, L.K., 1985. Effect of different levels of calcium and phosphorus on phytate hydrolysis by chicks. Nutr. Reports Int. 32, 909–913.

Banks, K.M., Thompson, K.L., Jaynes, P., Applegate, T.J., 2004. The effects of copper on the efficacy of phytase, growth and phosphorus retention in broiler chicks. Poult. Sci. 83, 1335–1341.

Bedford, M.R., Schulze, H., 1998. Exogenous enzymes of pigs and poultry. Nutr. Res. Rev. 11, 91–114.

Bedford, M.R., 2003. New enzyme technologies for poultry feeds. Br. Poult. Sci. 44 (Suppl. 1), S14–S16.

Biehl, R.R., Baker, D.H., 1997. Utilization of phytate and nonphytate phosphorus in chicks as affected by source and amount of Vitamin D_3. Poult. Sci. 75, 2986–2993.

Boling, S.D., Douglas, M.W., Johnson, M.L., Wang, X., Parsons, C.M., Koelkebeck, K.W., Zimmerman, R.A., 2000a. The effects of dietary available phosphorus levels and phytase on performance of young and older laying hens. Poult. Sci. 79, 224–230.

Boling, S.D., Webel, D.M., Mavromichalis, I., Parsons, C.M., Baker, D.H., 2000b. The effects of citric acid on phytate-phosphorus utilization in young chicks and pigs. Poult. Sci. 78, 682–689.

Boling-Frankenbach, S.D., Peter, C.M., Douglas, M.W., Snow, J.L., Parsons, C.M., Baker, D.H., 2001a. Efficacy of phytase for increasing protein efficiency ratio values of feed ingredients. Poult. Sci. 80, 1578–1584.

Boling-Frankenbach, S.D., Snow, J.L., Parsons, C.M., Baker, D.H., 2001b. The effect of citric acid on the calcium and phosphorus requirements of chicks fed corn–soybean meal diets. Poult. Sci. 80, 783–788.

Boorman, K.N., Gunaratne, S.P., 2001. Dietary phosphorus supply, egg-shell deposition and plasma inorganic phosphorus in laying hens. Br. Poult. Sci. 42, 81–91.

Cabahug, S., Ravindran, V., Selle, P.H., Bryden, W.L., 1999. Response of broiler chickens to microbial phytase as influenced by dietary phytic acid and non-phytate phosphorus levels. I. Effects on bird performance and toe ash content. Br. Poult. Sci. 40, 660–666.

Camden, B.J., Morel, P.C.H., Thomas, D.V., Ravindran, V., Bedford, M.R., 2001. Effectiveness of exogenous microbial phytase in improving the bioavailabilities of phosphorus and other nutrients in maize–soya-bean meal diets for broilers. Anim. Sci. 73, 289–297.

Campbell, G.L., Bedford, M.R., 1992. Enzyme applications for monogastric feeds: a review. Can. J. Anim. Sci. 72, 449–466.

Carlos, A.B., Edwards, H.M., 1998. The effects of 1,25-dihydroxycholecalciferol and phytase on the natural phytate phosphorus utilization by laying hens. Poult. Sci. 77, 850–858.

Cawley, R.W., Mitchell, T.A., 1968. Inhibition of wheat α-amylase by bran phytic acid. J. Sci. Food Agric. 19, 106–108.

Ceylan, N., Scheideler, S.E., Stilborn, H.L., 2003. High available phosphorus corn and phytase in layer diets. Poult. Sci. 82, 789–795.

Champagne, E.T., 1988. Effects of pH on mineral–phytate, protein–mineral–phytate and mineral–fiber interactions. Possible consequences of atrophic gastritis on mineral bioavailability from high fiber foods. J. Am. Coll. Nutr. 7, 499–508.

Chesson, A., 1993. Feed enzymes. Anim. Feed Sci. Technol. 45, 65–79.
Correll, D.L., 1999. Phosphorus: a rate limiting nutrient in surface waters. Poult. Sci. 78, 674–682.
Cosgrove, D.J., 1966. The chemistry and biochemistry of inositol polyphosphates. Rev. Pure Appl. Chem. 16, 209–224.
Cowieson, A.J., Acamovic, T., Bedford, M.R., 2004. The effects of phytase and phytic acid on the loss of endogenous amino acids and minerals from broiler chickens. Br. Poult. Sci. 45, 101–108.
Cowieson, A.J., Adeola, O., 2005. Carbohydrases, protease, and phytase have an additive beneficial effect in nutritionally marginal diets for broiler chicks. Poult. Sci. 85, 1860–1867.
Debicki-Garnier, A.M., Hruby, M., 2003. The effect of phytase and betaine on broiler performance and excreta characteristics. In: 14th European Symposium on Poultry Nutrition, World's Poultry Science Association, Lillehammer, Norway, pp. 14–15.
de Boever, J.L., Eeckhout, W., Boucque, Ch.V., 1994. The possibilities of near infrared spectroscopy to predict total-phosphorus, phytate-phosphorus and phytase activity in vegetable feedstuffs. Neth. J. Agric. Sci. 42, 357–369.
Demjen, A.P., Thompson, L.U., 1991. Calcium and phytic acid independently lower the glycemic response to a glucose load. In: Proc. 34th Canad. Fed. Biol. Sci., p. 53 (Abstract).
Denbow, D.M., Ravindran, V., Kornegay, E.T., Yi, Z., Hulet, R.M., 1995. Improving phosphorus availability in soybean meal for broilers by supplemental phytase. Poult. Sci. 74, 1831–1842.
Desphande, S.S., Cheryan, M., 1984. Effects of phytic acid, divalent cations and their interactions on α-amylase activity. J. Food Sci. 49, 516–519.
Dilger, R.N., Onyango, E.M., Sands, J.S., Adeola, O., 2004. Evaluation of microbial phytase in broiler diets. Poult. Sci. 83, 962–970.
Douglas, M.W., Peter, C.M., Boling, S.D., Parsons, C.M., Baker, D.H., 2000. Nutritional evaluation of low phytate and high protein corns. Poult. Sci. 79, 1586–1591.
Driver, J.P., Pesti, G.M., Bakalli, R.I., Edwards, H.M., 2005a. Effects of calcium and nonphytate phosphorus concentrations on phytase efficacy in broiler chicks. Poult. Sci. 85, 1406–1417.
Driver, J.P., Pesti, G.M., Bakalli, R.I., Edwards, H.M., 2005b. Phytase and 1α-hydroxycholecalciferol supplementation of broiler chickens during the starting and growing/finishing phases. Poult. Sci. 85, 1616–1628.
Driver, J.P., Antencio, A., Edwards, H.M., Pesti, G.M., 2006. Improvements in nitrogen-corrected apparent metabolisable energy of peanut meal in response to phytase supplementation. Poult. Sci. 85, 96–99.
Edwards, H.M., 1993. Dietary 1,25-dihydroxycholecalciferol supplementation increases natural phytate phosphorus utilization in chickens. J. Nutr. 123, 567–577.
Edwards, H.M., Carlos, A.B., Kasim, A.B., Toledo, R.T., 1999. Effects of steam pelleting and extrusion on phytate phosphorus utilization in broiler chickens. Poult. Sci. 78, 96–101.
Eeckhout, W., de Paepe, M., 1991. The quantitative effects of an industrial microbial phytase and wheat phytase on the apparent phosphorus absorbability of a mixed feed by piglets. Med. Fac. Landb. Rijk. Gent. 56, 1643–1647.
Eeckhout, W., de Paepe, M., 1994. Total phosphorus, phytate-phosphorus and phytase activity in plant feedstuffs. Anim. Feed Sci. Technol. 47, 19–29.
Ellis, R., Morris, E.R., Philpot, C., 1977. Quantitative determination of phytate in the presence of high inorganic phosphate. Anal. Biochem. 77, 536–539.
Engelen, A.J., van der Heeft, F.C., Randsdorp, P.H.G., Smit, E.L.C., 1994. Simple and rapid determination of phytase activity. J. AOAC Int. 77, 760–764.
Esteve-Garcia, E., Perez-Vendrell, A.M., Broz, J., 2005. Phosphorus equivalence of a *Consensus* phytase produced by *Hansenula polymorpha* in diets for young turkeys. Arch. Anim. Nutr. 59, 53–59.
Farrell, D.J., Martin, E., du Preez, J.J., Bongarts, M., Betts, M., Sudaman, A., Thomson, E., 1993. The beneficial effects of a microbial phytase in diets of broiler chickens and ducklings. J. Anim. Physiol. Anim. Nutr. 69, 278–283.
Farrell, D.J., 1997. Where in the world will we find the ingredients to feed our livestock by the year 2007? In: Recent Advances in Animal Nutrition in Australia. University of New England, Armidale, NSW, pp. 136–145.
Feil, B., 2001. Phytic acid. J. New Seeds 3, 1–35.
Francesch, M., Broz, J., Brufau, J., 2005. Effects of an experimental phytase on performance, egg quality, tibia ash content and phosphorus bioavailability in laying hens fed on maize- or barley-based diets. Br. Poult. Sci. 46, 340–348.

Frolich, W., Drakenberg, T., Asp, N.-G., 1986. Enzymic degradation of phytate (*myo*-inositol hexaphosphate) in whole grain flour suspension and dough. A comparison between ^{31}P NMR spectroscopy and a ferric ion method. J. Cereal Sci. 4, 325–334.

Gal-Garber, O., Mabjeesh, S.J., Sklan, D., Uni, Z., 2003. Nutrient transport in the small intestine: Na^+, K^+-ATPase expression and activity in the small intestine of the chicken as influenced by dietary sodium. Poult. Sci. 82, 1127–1133.

Garrett, J.B., Kretz, K.A., O'Donoghue, E., Kerovuo, J., Kim, W., Barton, N.R., Hazlewood, G.P., Short, J.M., Robertson, D.E., Gray, K.A., 2004. Enhancing the thermal tolerance and gastric performance of a microbial phytase for use as a phosphate-mobilizing monogastric-feed supplement. Appl. Environ. Microbiol. 70, 3041–3046.

Garriga, C., Moreto, M., Planas, J.M., 2000. Effects of resalination on intestinal glucose transport in chickens adapted to low Na^+ intakes. Expt. Physiol. 85 (4), 371–378.

Godoy, S., Chicco, C., Meschy, F., Requena, F., 2005. Phytic phosphorus and phytase activity of animal feed ingredients. Interciencia 30, 24–28.

Gordon, R.W., Roland, D.A., 1997. Performance of commercial laying hens fed various phosphorus levels, with and without supplemental phytase. Poult. Sci. 76, 1172–1177.

Gordon, R.W., Roland, D.A., 1998. Influence of supplemental phytase on calcium and phosphorus utilization in laying hens. Poult. Sci. 77, 290–294.

Gracia, M.I., Aranibar, M.J., Lazaro, R.N., Medel, P., Mateos, G.G., 2003. α-Amylase supplementation of broiler diets based on corn. Poult. Sci. 82, 436–442.

Greiner, R., Konietzny, U., Jany, K.-D., 1993. Purification and characterization of two phytases from *Escherichia coli*. Arch. Biochem. Biophys. 303, 107–113.

Haefner, S., Knietsch, A., Scholten, E., Braun, J., Lohscheidt, M., Zelder, O., 2005. Biotechnological production and application of phytases. Appl. Microbial. Biotechnol. 68, 588–597.

Harland, B.F., Oberleas, D., 1977. A modified method for phytate analysis using an ion-exchange procedure: application to textured proteins. Cereal Chem. 54, 827–832.

Harland, B.F., Morris, E.R., 1995. Phytate: a good of bad food component? Nutr. Res. 19, 947–961.

Hartig, T., 1855. Uber das Klebermehl. Botanische Z. 13, 881–885.

Haug, W., Lantzsch, H.-J., 1983. Sensitive method for the rapid determination of phytate in cereal and cereal products. J. Sci. Food Agric. 34, 1423–1426.

Haydon, K.D., West, J.W., 1990. Effect of dietary electrolyte balance on nutrient digestibility at the end of the small intestine and over the total digestive tract in growing pigs. J. Anim. Sci. 68, 3687–3693.

Heubner, W., Stadler, H., 1914. Uber eine Titration-methode zur Bestimmung des Phytins. Biochem. Z. 64, 432–437.

Hooppaw, P.W., Goodman, B.L., 1976. The influence of intermittent light on growth performance and other traits in young chicks. Poult. Sci. 55, 2285–2289.

Huff, W.E., Moore, P.A., Waldroup, P.W., Waldroup, A.L., Balog, J.M., Huff, G.R., Rath, N.C., Daniel, T.C., Raboys, V., 1998. Effect of dietary phytase and high available phosphorus corn on broiler chick performance. Poult. Sci. 77, 1899–1904.

Igbasan, F.A., Simon, O., Milksch, G., Manner, K., 2000. Comparative studies of the *in vitro* properties of phytases from various microbial origins. Arch. Anim. Nutr. 53, 353–373.

Jagger, S., Wiseman, J., Cole, D.J.A., Craigon, J., 1992. Evaluation of inert markers of ileal and faecal apparent digestibility values in the pig. Br. J. Nutr. 68, 729–739.

Jalal, M.A., Scheideler, S.E., Wyatt, C., 1999. Effects of phytase supplementation on eeg production parameters and amino acid digestibilities. Poult. Sci. 78 (Suppl. 1), 74 (Abstract).

Jalal, M.A., Scheideler, S.E., 2001. Effect of supplementation of two different sources of phytase on egg production parameters in laying hens and nutrient digestibility. Poult. Sci. 80, 1463–1471.

Johnson, R.J., Karunajeewa, H., 1985. The effects of dietary minerals and electrolytes on the growth and physiology of the young chick. J. Nutr. 115, 1680–1690.

Johnston, S.L., Williams, S.B., Southern, L.L., Bidner, T.D., Bunting, L.D., Matthews, J.O., Olcott, B.M., 2004. Effect of phytase addition and dietary calcium and phosphorus levels on plasma metabolites and ileal and total tract nutrient digestibility in pigs. J. Anim. Sci. 82, 705–714.

Jongbloed, A.W., Kemme, P.A., 1990. Effect of pelleting mixed feeds on phytase activity and the apparent absorbability of phosphorus and calcium in pigs. Anim. Feed Sci. Technol. 28, 233–242.

Juanpere, J., Perez-Vendrell, A.M., Angulo, E., Brufau, J., 2005. Assessment of potential interactions between phytase and glycosidase enzyme supplementation on nutrient digestibility in broilers. Poult. Sci. 84, 571–580.

Kasim, A.B., Edwards, H.M., 1998. The analysis for inositol phosphate forms in feed ingredients. J. Sci. Food Agric. 76, 1–9.

Kerr, M.J., Classen, H.L., Newkirk, R.W., 2000. The effects of gastrointestinal tract micro-flora and dietary phytase on inositol hexaphosphate hydrolysis in the chicken. Poult. Sci. 79 (Suppl. 1), 11 (Abstract).

Keshavarz, K., 2000. Nonphytate phosphorus requirement of laying hens with and without phytase on a phase feeding program. Poult. Sci. 79, 748–763.

Keshavarz, K., 2003. Effects of continuous feeding of low-phosphorus diets with and without phytase during the growing and laying periods of two strains of leghorns. Poult. Sci. 82, 1444–1456.

Khrehbiel, C.R., Matthews, J.C., 2003. Absorption of amino acids and peptides. In: D'Mello, J.P.F. (Ed.), Amino Acids in Animal Nutrition, second ed. CABI, pp. 41–70.

Kies, A.K., Gerrits, W.J.J., Schrama, J.W., Heetkamp, M.J.W., van der Linden, K.L., Zandstra, T., Verstegen, M.W.A., 2005. Mineral absorption and excretion as affected by microbial phytase, and their effect on energy metabolism in young piglets. J. Nutr. 135, 1131–1138.

Kirby, L.K., Nelson, T.S., 1988. Total and phytate phosphorus content of some feed ingredients derived from grains. Nutr. Reports Int. 37, 277–280.

Knuckles, B.E., Kuzmicky, D.D., Gumbmann, M.R., Betschart, A.A., 1989. Effect of myo-inositol phosphate esters on *in vitro* and *in vivo* digestion of protein. J. Food Sci. 54, 1348–1350.

Kocher, A., Choct, M., Ross, G., Chung, T.K., 2003. Effects of phytase on apparent metabolisable energy in broiler diets based on wheat or sorghum. Proc. Aust. Poult. Sci. Symp. 15, 112.

Konietzny, U., Greiner, R., 2002. Molecular and catalytic properties of phytate-degrading enzymes (phytases). Int. J. Food Sci. Technol. 37, 791–812.

Kornegay, E.T., 1996. Effect of Natuphos® phytase on protein and amino acid digestibility and nitrogen retention in poultry. In: Phytase in Animal Nutrition and Waste Management. BASF Corporation, Mount Olive, NJ, pp. 493–514.

Kornegay, E.T., Denbow, D.M., Yi, Z., Ravindran, V., 1996. Response of broilers to graded levels of microbial phytase added to maize–soyabean meal-based-diets containing three levels of non-phytate phosphorus. Br. J. Nutr. 75, 839–852.

Kornegay, E.T., Zhang, Z., Denbow, D.M., 1999. Influence of microbial phytase supplementation of a low protein/amino acid diet on performance, ileal digestibility of protein and amino acids, and carcass measurements of finishing broilers. In: Phytase in Animal Nutrition and Waste Management, second revised ed. BASF Corporation, Mount Olive, NJ, pp. 557–572.

Kozloski, G.V., de Moraes Flores, E.M., Martins, A.F., 1998. Use of chromium oxide in digestibility studies: variations of the results as a function of the measurement method. J. Sci. Food Agric. 76, 373–376.

Kwanyuen, P., Burton, J.W., 2005. A simple and rapid procedure for phytate determination in soybeans and soy products. JAOCS 82, 81–85.

Lasztity, R., Lasztity, L., 1990. Phytic acid in cereal technology. Adv. Cereal Sci. Technol. 10, 309–371.

Latta, M., Eskin, M., 1980. A simple and rapid colorimetry method for phytate determination. J. Agric. Food Chem. 26, 1313–1315.

Lawlor, P.G., Lynch, P.B., Caffrey, P.J., O'Reilly, J.J., O'Connel, M.K., 2005. Measurements of the acid binding capacity of ingredients used in pig diets. Irish Vet. J. 58, 447–452.

Leeson, S., 1993. Recent advances in fat utilisation by poultry. In: Recent Advances in Animal Nutrition in Australia. The University of New England, Armidale, NSW, pp. 170–1981.

Lei, X.G., Stahl, C.H., 2000. Nutritional benefits of phytase and dietary determinants of its efficacy. J. Appl. Anim. Res. 17, 97–112.

Leske, K.L., Coon, C.N., 1999. A bioassay to determine the effect of phytase on phytate phosphorus hydrolysis and total phosphorus retention of feed ingredients as determined with broilers and laying hens. Poult. Sci. 78, 1151–1157.

Li, Y.C., Ledoux, D.R., Veum, T.L., Raboy, V., Ertl, D.S., 2000. Effects of low phytic acid corn on phosphorus utilization performance and bone mineralization in broiler chicks. Poult. Sci. 79, 1444–1450.

Liebert, F., Wecke, C., Schoner, F.J., 1993. Phytase activities in different gut contents of chickens as dependent on level of phosphorus and phytase supplementations. In: Proceedings of 1st European Symposium Enzymes in Animal Nutrition, pp. 202–205.

Liebert, F., Htoo, J.K., Sunder, A., 2005. Performance and nutrient utilization of laying hens fed low-phosphorus corn–soybean and wheat–soybean diets supplemented with microbial phytase. Poult. Sci. 84, 1576–1583.

Lim, H.S., Namkung, H., Paik, I.K., 2003. Effects of phytase supplementation on the performance, egg quality, and phosphorus excretion of laying hens fed different levels of dietary calcium and nonphytate phosphorus. Poult. Sci. 82, 92–99.

Lonnerdal, B., Sandberg, A.-S., Sandstrom, B., Kunz, C., 1989. Inhibitory effects of phytic acid and other inositol phosphates on zinc and calcium absorption in suckling rats. J. Nutr. 119, 211–214.

Lott, J.N.A., Ockenden, I., Raboy, V., Batten, G.D., 2000. Phytic acid and phosphorus in crop seeds and fruit: a global estimate. Seed Sci. Res. 10, 11–33.

Lowe, J.T., Steenbock, H., Keiger, C.H., 1939. Cereals and rickets. IX. The availability pf phytin-P to the chick. Poult. Sci. 18, 40–44.

Maddaiah, V.T., Hulett, B.J., Kurnick, A.A., Reid, B.L., 1963. Availability of calcium phytate, sodium phytate and dicalcium phosphate in chicks, hens, and rats. Poult. Sci. 42, 1286 (Abstract).

Maenz, D.D., Classen, H.L., 1998. Phytase activity in the small intestinal brush border membrane of the chicken. Poult. Sci. 77, 557–563.

Maenz, D.D., Engele-Schann, C.M., Newkirk, R.W., Classen, H.L., 1999. The effect of minerals and mineral chelators on the formation of phytase-resistant and phytase-susceptible forms of phytic acid in solution and in a slurry of canola meal. Anim. Feed Sci. Technol. 77, 177–192.

Mahajan, A., Dua, S., 1997. Nonchemical approach for reducing antinutritional factors in rapseed (*Brassica campestris* var. Toria) and characterization of enzyme phytase. J. Agric. Food Chem. 45, 2507–2508.

Martin, E.A., Nolan, J.V., Nitsan, Z., Farrell, D.J., 1998. Strategies to improve the nutritive value of rice bran in poultry diets. IV. Effects of addition of fish meal and a microbial phytase to duckling diets on bird performance and amino acid digestibility. Br. Poult. Sci. 39, 612–621.

Matyka, S., Korol, W., Bogusz, G., 1990. The retention of phytin phosphorus from diets with fat supplements in broiler chickens. Anim. Feed Sci. Technol. 31, 223–230.

McCuaig, L.W., Davies, M.I., Motzok, I., 1972. Regulation of intestinal alkaline phosphatase and phytase of chicks: effects of dietary magnesium, calcium, phosphorus and throactive casein. Poult. Sci. 51, 526–530.

McCuaig, L.W., Motzok, I., 1972. Regulation of intestinal alkaline phosphatase by dietary phosphate. Can. J. Physiol. Pharmacol. 50, 1152–1156.

McGrath, J.M., Sims, J.T., Maguire, R.O., Saylor, W.W., Angel, C.R., Turner, B.L., 2005. Broiler diet modification and litter storage: impacts on phosphorus in litters, soils and runoff. J. Environ. Qual. 34, 1896–1909.

Miles, R.D., Nelson, T.S., 1974. The effect of enzymatic hydrolysis of phytate on the available energy content of feed ingredients for chickens and rats. Poult. Sci. 53, 1714–1717.

Miles, D.M., Moore, P.A., Smith, D.R., Rice, D.W., Stilborn, H.L., Rowe, D.R., Lott, B.D., Branton, S.L., Simmons, J.D., 2003. Total and water-soluble phosphorus in broiler litter over three flocks with alum litter treatment and dietary inclusion of high available phosphorus corn and phytase supplementation. Poult. Sci. 82, 1544–1549.

Miller, G.A., Youngs, V.L., Oplinger, E.S., 1980. Environmental and cultivar effects on oat phytic acid concentration. Cereal Chem. 57, 189–191.

Mitchell, R.D., Edwards, H.M., 1996. Additive effects of 1,25-dihyroxycholecalciferol and phytase on phytate phosphorus utilization and related parameters in broiler chickens. Poult. Sci. 75, 111–119.

Mullaney, E.J., Daly, C.B., Ullah, A.H.J., 2000. Advances in phytase research. Adv. Appl. Microbiol. 47, 157–199.

Munster, D.J., Bagshaw, P.F., Wilson, J.G., 1987. Peptic erosion of gastric mucus in the rat. Comp. Biochem. Physiol. 87A, 509–513.

Murai, A., Kita, K., Tsuruta, S., Okumara, J., 2001. Interaction of phytase and glutamic acid on bone ash response in chicken thigh. J. Poult. Sci. 38, 146–151.

Namkung, H., Leeson, S., 1999. Effect of phytase enzyme on dietary nitrogen-corrected apparent metabolisable energy and the ileal digestibility of nitrogen and amino acids. Poult. Sci. 78, 1317–1319.

National Research Council, 1994. Nutrient Requirements of Poultry, nineth revised ed. National Academy Press, Washington, DC.

Nelson, T.S., 1967. The utilization of phytate phosphorus by the chick—a review. Poult. Sci. 46, 862–871.

Nelson, T.S., Ferrara, L.W., Storer, N.L., 1968a. Phytate phosphorus content of feed ingredients derived from plants. Poult. Sci. 47, 11372–11374.

Nelson, T.S., McGillivray, J.J., Shieh, T.R., Wodzinski, R.J., Ware, J.H., 1968b. Effect of phytate on the calcium requirement of chicks. Poult. Sci. 47, 1985–1989.

Nelson, T.S., Shieh, T.R., Wodzinski, R.J., Ware, J.H., 1968c. The availability of phytate phosphorus in soybean meal before and after treatment with a mold phytase. Poult. Sci. 47, 1842–1848.

Nelson, T.S., Shieh, T.R., Wodzinski, R.J., Ware, J.H., 1971. Effect of supplemental phytase on utilization of phytate phosphorus by chicks. J. Nutr. 101, 1289–1294.

Newkirk, R.W., Classen, H.L., 1998. *In vitro* hydrolysis of phytate in canola meal with purified ande crude sources of phytase. Anim. Feed Sci. Technol. 72, 315–327.

Newkirk, R.W., Classen, H.L., 2001. The non-mineral nutritional impact of phytate in canola meal fed to broiler chicks. Anim. Feed Sci. Technol. 91, 115–128.

Nyman, M.E., Bjork, I.M., 1989. *In vivo* effects of phytic acid and polyphenols on the bioavailability of polysaccharides and other nutrients. J. Food Sci. 54, 1332–1335.

Oberleas, D., Li, Y.-C., Stoecker, B.J., Henley, S.A., Keim, K.S., Smith, J.C., 1990. The rate of chromium transit through the gastrointestinal tract. Nutr. Res. 10, 1189–1194.

Olaffs, K., Cossa, J., Jeroch, H., 2000. The importance of native phytase activity in wheat on the phosphorus utilization in broilers and laying hens. Arch. Geflugelk. 64, 157–191.

Onyango, E.M., Bedford, M.R., Adeola, O., 2005a. Efficacy of an evolved *Escherichia coli* phytase in diets for broiler chicks. Poult. Sci. 84, 248–255.

Onyango, E.M., Bedford, M.R., Adeola, O., 2005b. Phtase activity along the digestive tract of the broiler chick: a comparative study of an *Escherichia coli*-derived and *Peniophora lycii* phytase. Can. J. Anim. Sci. 85, 61–68.

Orban, J.I., Adeola, O., Stroshine, R., 1999. Microbial phytase in finisher diets of white Pekin ducks: effect on growth performance, plasma phosphorus concentration, and leg bone characteristics. Poult. Sci. 78, 366–377.

Paik, I., 2003. Application of phytase, microbial or plant origin, to reduce phosphorus excretion in poultry production. Asian–Aust. J. Anim. Sci. 16, 124–135.

Panda, A.K., Rama Rao, S.V., Raju, M.V.L.N., Bhanja, S.K., 2005. Effect of microbial phytase on production performance of white leghorn layers fed on a diet low in non-phytate phosphorus. Br. Poult. Sci. 46, 464–469.

Park, W.-Y., Matsui, T., Yano, F., Yano, H., 2000. Heat treatment of rapeseed meal increases phytate flow into the duodenum of sheep. Anim. Feed Sci. Technol. 88, 31–37.

Parkkonen, T., Tervila-Wilo, A., Hopeakoski-Nurminen, M., Morgan, A., Poutanen, K., Autio, K., 1997. Changes in wheat microstructure following *in vitro* digestion. Acta Agric. Scand. Section B Soil Plant Sci. 47, 43–47.

Persson, H., Turk, M., Nyman, M., Sandberg, A.-S., 1998. Binding of Cu^{2+}, Zn^{2+}, and Cd^{2+} to inositol tri-, tetra-, penta-, and hexaphosphates. J. Agric. Food Chem. 46, 3194–3200.

Peter, C.M., Yan, Y., Boling-Frankenbach, S.D., Parsons, C.M., Baker, D.H., 2000. Limiting order of amino acids and the effects of phytase on protein quality in corn gluten meal fed to young chicks. Poult. Sci. 78, 2150–2156.

Peter, C.M., Baker, D.H., 2001. Microbial phytase does not improve protein-amino acid utilization in soybean meal fed to young chickens. J. Nutr. 131, 1792–1797.

Phillippy, B.Q., Johnston, M.R., 1985. Determination of pbytic acid in foods by ion chromatography with post-column derivatization. J. Food Sci. 50, 541–542.

Phillippy, B.Q., 1999. Susceptibility of wheat and *Aspergillus niger* phytases to inactivation by gastrointestinal enzymes. J. Agric. Food Chem. 47, 1385–1388.

Pintar, J., Homen, B., Gazic, K., Janjecic, Z., Sikiric, M., Cerny, T., 2005. Effects of supplemental phytase on nutrient excretion and retention in broilers fed different cereal based diets. Czech. J. Anim. Sci. 50, 40–46.

Pos, J., Enting, H., Veldman, A., 2003. Effect of phytase and dietary calcium level on litter quality and broiler performance. In: 14th European Symposium on Poultry Nutrition, World's Poultry Science Association, Lillehammer, Norway, pp. 17–18.

Potter, L.M., 1988. Bioavailability of phosphorus from various phosphates based on body weight and toe ash measurements. Poult. Sci. 67, 96–102.

Qian, H., Gregory, E.M., Kornegay, E.T., 1996a. Characterization if *Aspergillus niger* phytase and investigation of the inhibitory effect of cations on the phytase activity. J. Anim. Sci. 74 (Suppl. 1), 8 (Abstract).

Qian, H., Kornegay, E.T., Denbow, D.M., 1996b. Phosphorus equivalence of microbial phytase in turkey diets as influenced by calcium to phosphorus ratios and phosphorus levels. Poult. Sci. 75, 69–81.

Qian, H., Kornegay, E.T., Denbow, D.M., 1997. Utilization of phytate phosphorus and calcium as influenced by microbial phytase, cholecalciferol, and the calcium:total phosphorus ratio in broiler diets. Poult. Sci. 76, 37–46.

Raboy, V., Dickinson, D.B., Neuffer, M.G., 1990. A survey of kernel mutants for varioation in phytic acid. Maydica 35, 385–390.

Rafacz-Livingston, K.A., Martinez-Amezcua, C., Parsons, C.M., Baker, D.H., Snow, J., 2005. Citric acid improves phytate phosphorus utilization in crossbred and commercial broiler chickens. Poult. Sci. 84, 1370–1375.

Rajendran, S., Prakash, V., 1993. Kinetics and thermodynamics of the mechanism of interaction of sodium phytate with α-globulin. Biochemistry 32, 3474–3478.

Rapp, C., Lantzsch, H.-J., Drochner, W., 2001. Hydrolysis of phytic acid by intrinsic plant or supplemented microbial phytase (*Aspergillus niger*) in the stomach and small intestine of minipigs fitted with re-entrant cannulas. 3. Hydrolysis of phytic acid (IP_6) and occurrence of hydrolysis products (IP_5, IP_4, IP_3 and IP_2). J. Anim. Physiol. Anim. Nutr. 85, 420–430.

Ravindran, V., Ravindran, G., Sivalogan, S., 1994. Total and phytate phosphorus contents of various foods and feedstuffs of plant origin. Food Chem. 50, 133–136.

Ravindran, V., 1995. Phytases in poultry nutrition. An overview. Proc. Aust. Poult. Sci. Symp. 7, 135–139.

Ravindran, V., Bryden, W.L., Kornegay, E.T., 1995a. Phytases: occurrence, bioavailability and implications in poultry nutrition. Poult. Avian Biol. Rev. 6, 125–143.

Ravindran, V., Kornegay, E.T., Denbow, D.M., Yi, Z., Hulet, R.M., 1995b. Response of turkey poults to tiered levels of Natuphos® phytase added to soyabean meal-based semi-purified diets containing three levels of nonphytate phosphorus. Poult. Sci. 74, 1843–1854.

Ravindran, V., Cabahug, S., Ravindran, G., Bryden, W.L., 1999a. Influence of microbial phytase on apparent ileal amino acid digestibility in feedstuffs for broilers. Poult. Sci. 78, 699–706.

Ravindran, V., Selle, P.H., Bryden, W.L., 1999b. Effects of phytase supplementation, individually and in combination, on the nutritive value of wheat and barley. Poult. Sci. 78, 1588–1595.

Ravindran, V., Cabahug, S., Ravindran, G., Selle, P.H., Bryden, W.L., 2000. Response of broiler chickens to microbial phytase supplementation as influenced by dietary phytic acid and non-phytate phosphorus levels. II. Effects on apparent metabolisable energy, nutrient digestibility and nutrient retention. Br. Poult. Sci. 41, 193–200.

Ravindran, V., Selle, P.H., Ravindran, G., Morel, P.C.H., Kies, A.K., Bryden, W.L., 2001. Microbial phytase improves performance, apparent metabolisable energy and ileal amino acid digestibility of broilers fed a lysine-deficient diet. Poult. Sci. 80, 338–344.

Ravindran, V., Morel, P.C.H., Partridge, G.G., Hruby, M., Sands, J.S., 2006. Influence of an *E. coli*-derived phytase on nutrient utilization in broiler starter fed diets containing varying concentrations of phytic acid. Poult. Sci. 85, 82–89.

Reddy, N.R., Sathe, S.K., Salunkhe, D.K., 1982. Phytates in legumes and cereals. Adv. Food Res. 28, 1–91.

Rickard, S.E., Thompson, L.U., 1997. Interactions and biological effects of phytic acid. In: Shaidi, F. (Ed.), Antinutrients and Phytochemicals in Food. American Chemical Society, Washington, DC, pp. 294–312.

Riley, W.W., Austic, R.E., 1989. Influence of dietary electrolytes on lysine and arginine absorption in chick intestine. Poult. Sci. 68, 1255–1262.

Ritz, C., Hulet, R.M., Self, B.B., Denbow, D.M., 1995. Growth and intestinal morphology of male turkeys as influenced by dietary supplementation of amylase and xylanase. Poult. Sci. 74, 1329–1334.

Rodriguez, E., Porres, J.M., Han, Y., Lei, X.G., 1999. Different sensitivity of recombinant *Aspergillus niger* phytase (r-PhyA) and *Escherichia coli* pH 2.5 acid phosphatase (r-AppA) to trypsin and pepsin *in vitro*. Arch. Biochem. Biophys. 365, 262–267.

Rojas, S.W., Scott, M.L., 1969. Factors affecting the nutritive value of cottonseed meal as a protein source in chick diets. Poult. Sci. 48, 819–835.

Rosen, G., 2003. Microbial phytase in broiler nutrition. In: Garnsworthy, P.C., Wiseman, J. (Eds.), Recent Advances in Animal Nutrition. Nottingham University Press, Nottingham, UK, pp. 105–117.

Rounds, M.A., Nielsen, S.S., 1993. Anion-exchange high-performance liquid chromatography with post-column detection for the analysis of phytic acid and other inositol phosphates. J. Chromatogr. A 653, 148–152.

Rutherfurd, S.M., Chung, T.K., Moughan, P.J., 2002. The effect of microbial phytase on ileal phosphorus and amino acid digestibility in broiler chickens. Br. Poult. Sci. 44, 598–606.

Rutherfurd, S.M., Chung, T.K., Morel, P.C.H., Moughan, P.J., 2004. Effect of microbial phytase on ileal digestibility of phytate phosphorus, total phosphorus, and amino acids in a low-phosphorus diet fro broilers. Poult. Sci. 83, 61–68.

Saha, D.C., Gilbreath, R.L., 1991. Analytical recovery from diet and faeces determined by colorimetry and atomic absorption spectrophotometry. J. Sci. Food Agric. 55, 433–446.

Sales, J., Janssens, G.P.J., 2003. The use of markers to determine energy metabolizability and nutrient digestibility in avian species. World's Poult. Sci. J. 59, 314–327.

Schoner, F.-J., Hoppe, P.P., Schwartz, G., 1991. Comparative effects of microbial phytase and inorganic phosphorus on performance and retentions of phosphorus, calcium and crude ash in broilers. J. Anim. Physiol. Anim. Nutr. 66, 248–255.

Schoner, F.-J., Hoppe, P.P., Schwartz, G., Weische, H., 1993. Comparison of microbial phytase and inorganic phosphate in male chickens: the influence on performance data, mineral retention and dietary calcium. J. Anim. Physiol. Anim. Nutr. 69, 235–244.

Scott, T.A., Kampen, R., Silversides, F.G., 1999. The effect of phosphorus, phytase enzyme, and calcium on the performance of layers fed corn-based diets. Poult. Sci. 78, 1742–1749.

Scott, T.A., Kampen, R., Silversides, F.G., 2000. The effect of phosphorus, phytase enzyme, and calcium on the performance of layers fed wheat-based diets. Poult. Sci. 80, 183–190.

Scott, T.A., Kampen, R., Silversides, F.G., 2001. The effect of adding exogenous phytase to nutrient-reduced corn- and wheat-based diets on performance and egg quality of two strains of laying hens. Can. J. Anim. Sci. 81, 393–401.

Sebastian, S., Touchburn, S.P., Chavez, E.R., Lague, P.C., 1997. Apparent digestibility of protein and amino acids in broiler chickens fed a corn–soybean diet supplemented with microbial phytase. Poult. Sci. 76, 1760–1769.

Selle, P.H., Ravindran, V., Pittolo, P.H., Bryden, W.L., 1999. An evaluation of microbial phytase in sorghum-based broiler diets. Proc. Aust. Poult. Sci. Symp. 11, 97–100.

Selle, P.H., Ravindran, V., Caldwell, R.A., Bryden, W.L., 2000. Phytate and phytase: consequences for protein utilisation. Nutr. Res. Rev. 13, 255–278.

Selle, P.H., Pittolo, P.H., Bryden, W.L., 2001. Individual and combined supplementation of wheat-based broiler diets with phytase and xylanase. Proc. Aust. Poult. Sci. Symp. 13, 246.

Selle, P.H., Cadogan, D.J., Bryden, W.L., 2003a. Effects of phytase supplementation of phosphorus-adequate, lysine-deficient, wheat-based diets on growth performance of weaner pigs. Aust. J. Agric. Res. 54, 323–330.

Selle, P.H., Ravindran, V., Pittolo, P.H., Bryden, W.L., 2003b. Effects of phytase supplementation of diets with two tiers of nutrient specifications on growth performance and protein efficiency ratios of broiler chickens. Asian–Aust. J. Anim. Sci. 16, 1158–1164.

Selle, P.H., Ravindran, V., Ravindran, G., Pittolo, P.H., Bryden, W.L., 2003c. Influence of phytase and xylanase supplementation on growth performance and nutrient utilisation of broilers offered wheat-based diets. Asian–Aust. J. Anim. Sci. 16, 394–402.

Selle, P.H., Walker, A.R., Bryden, W.L., 2003d. Total and phytate-phosphorus contents and phytase activity of Australian sourced feed ingredients for pigs and poultry. Aust. J. Expt. Agric. 45, 475–479.

Selle, P.H., Ravindran, V., Ravindran, G., Bryden, W.L., 2005. Amino acid digestibility and growth performance interactions to phytase and lysine supplementation of lysine-deficient broiler diets. Proc. Aust. Poult. Sci. Symp. 17, 234–237.

Shafey, T.M., McDonald, M.W., Dingle, J.G., 1991. Effects of dietary calcium and available phosphorus concentration on digesta pH and on the availability of calcium, iron, magnesium and zinc from the intestinal contents of meat chickens. Br. Poult. Sci. 32, 185–194.

Sharpley, A., 1999. Agricultural phosphorus, water quality and poultry production: are they compatible? Poult. Sci. 78, 660–673.

Shelton, J.L., Southern, L.L., Gaston, L.A., Foster, A., 2004. Evaluation of nutrient matrix values for phytase in broilers. J. Appl. Poult. Res. 13, 213–221.

Shen, Y., Yin, Y., Chavez, E.R., Fan, M.Z., 2005. Methodological aspects of measuring phytase activity and phytate phosphorus content in selected cereal grains and feces of pigs. J. Agric. Food Chem. 53, 853–859.

Shirley, R.B., Edwards, H.M., 2003. Graded levels of phytase past industry standards improves broiler performance. Poult. Sci. 82, 671–680.

Shohl, A.T., 1937. The effect of acid–base content of the diet upon the production and cure of rickets with special reference to citrates. J. Nutr. 14, 69–83.

Simon, O., Igbasan, F., 2002. *In vitro* properties of phytases from various microbial origins. Int. J. Food Sci. Technol. 37, 813–822.

Simons, P.C.M., Versteegh, H.A.J., Jongbloed, A.W., Kemme, P.A., Slump, P., Bos, K.D., Wolters, M.G.E., Beudeker, R.F., Verschoor, G.J., 1990. Improvement of phosphorus availability by microbial phytase in broilers and pigs. Br. J. Nutr. 64, 525–540.

Sklan, D., Noy, Y., 2000. Hydrolysis and absorption in the small intestines of posthatch chicks. Poult. Sci. 79, 1306–1310.

Skoglund, E., Carlsson, N.-G., Sandberg, A.-S., 1997a. Determination of isomers of inositol mono- to hexaphopshates in selected foods and intestinal contents using high performance ion chromatography. J. Agric. Food Chem. 45, 431–436.

Skoglund, E., Carlsson, N.-G., Sandberg, A.-S., 1997b. Analysis of inositol mono- and diphopshate isomers using high-performance ion chromatography and pulsed amperometric detection. J. Agric. Food Chem. 45, 4668–4673.

Skoglund, E., Carlsson, N.-G., Sandberg, A.-S., 1998. High-performance chromatographic separation of inositol phosphate isomers on strong anion exchange columns. J. Agric. Food Chem. 46, 1877–1882.

Smith, T.N., Pesti, G.M., Bakalli, R.I., Kilburn, J., Edwards, H.M., 2001. The use of near-infrared reflectance spectroscopy to predict the moisture, nitrogen, calcium, total phosphorus, gross energy, and phytate phosphorus contents of broiler excreta. Poult. Sci. 80, 314–319.

Snow, J.L., Douglas, M.W., Parsons, C.M., 2003. Phytase effects on amino acid digestibility in molted laying hens. Poult. Sci. 82, 474–477.

Snow, J.L., Baker, D.H., Parsons, C.M., 2004. Phytase, citric acid, and 1α-hydroxycholecalciferol improve phytate phosphorus utilization in chicks fed a corn–soybean meal diet. Poult. Sci. 83, 1187–1192.

Sohail, S.S., Roland, D.A., 2000. Influence of phytase on calcium utilization in commercial layers. J. Appl. Poult. Res. 9, 81–87.

Sooncharernying, S., Edwards, H.M., 1993. Phytate content of excreta and phytate retention in the gastrointestinal tract of young chickens. Poult. Sci. 72, 1906–1916.

Suzuki, U., Yoshimura, K., Takaishi, M., 1907. Uber ein Enzym "Phytase" das Anhydro-oxy-methylen-diphosphosaure spaltet. Coll. Agric. Bull. Tokyo Imp. Univ. 7, 503–505.

Swick, R.A., Ivey, F.J., 1992. Phytase: the value of improving phosphorus retention. Feed Manage. 43, 8–17.

Takemasa, M., Murakami, H., Yamazaki, M., 1996. Reduction of phosphorus excretion in chicks by addition of yeast phytase. Jpn. Poult. Sci. 33, 104–111.

Tamim, N.M., Angel, R., 2003. Phytate phosphorus hydrolysis as influenced by dietary calcium and macro-mineral source in broiler diets. J. Agric. Food Chem. 51, 4687–4693.

Tamim, N.M., Angel, R., Christman, M., 2004. Influence of dietary calcium and phytase on phytate phosphorus hydrolysis in broiler chickens. Poult. Sci. 83, 1358–1367.

Thompson, L.U., Button, C.L., Jenkins, D.J.A., 1987. Phytic acid and calcium affect the *in vitro* rate of navy bean starch digestion and blood glucose response in humans. Am. J. Clin. Nutr. 46, 467–473.

Thompson, L.U., 1988. Antinutrients and blood glucose. Food Technol. 42, 123–131.

Toor, G.S., Peak, J.D., Sims, J.T., 2005. Phosphorus speciation in broiler litter and turkey manure produced from modified diets. J. Environ. Qual. 34, 687–697.

Um, J.S., Paik, I.K., 1999. Effects of microbial phytase supplementation on egg production, eggshell quality, and mineral retention of laying hens fed different levels of phosphorus. Poult. Sci. 78, 75–79.

Ummadi, P., Chenoweth, W., Bennink, M., Ng, P.K.W., 1995a. The effects of extrusion processing on solubility and distribution of protein, phytate and iron in relation to iron dialyzability of Durum wheat. FASEB J. 8, A714.

Ummadi, P., Chenoweth, W., Ng, P.K.W., 1995b. Changes in solubility and distribution of Semolina proteins due to extrusion processing. Cereal Chem. 72, 564–567.

Usmani, N., Jafri, A.K., 2003. Influence of dietary phytic acid on the growth, conversion efficiency, and carcass composition of mrigal *Cirrhinus mrigala* (Hamilton) fry. J. World Aqua. Soc. 33, 199–204.

Vaintraub, I.A., Bulmaga, V.P., 1991. Effect of phytate on the *in vitro* activity of digestive proteinases. J. Agric. Food Chem. 39, 859–861.

van der Klis, J.D., Versteegh, H.A.J., 1991. Ileal Absorption of Phosphorus in Lightweight Laying Hens Using Microbial Phytase and Various Calcium Contents in Laying Hen Feed. Spelderholt, Beekbergen, The Netherlands, Spelderholt Publication No. 562.

van der Klis, J.D., Versteegh, H.A.J., Simons, P.C.M., Kies, A.K., 1997. The efficacy of phytase in corn–soybean meal-based diets for laying hens. Poult. Sci. 76, 1535–1542.

Viveros, A., Centeno, C., Brenes, A., Canales, R., Lozano, A., 2000. Phytase and acid phosphatase activities in plant feedstuffs. J. Agric. Food Chem. 48, 4009–4013.

Vohra, A., Satanarayana, T., 2003. Phytases: microbial sources, production, purification, and potential biotechnological applications. Crit. Rev. Biotechnol. 23, 29–60.

Waldroup, P.W., 1999. Nutritional approaches to reducing phosphorus excretion in poultry. Poult. Sci. 78, 683–691.

Waldroup, P.W., Kersey, J.H., Saleh, E.A., Fritts, C.A., Yan, F., Stilborn, H.L., Crum, R.C., Raboy, V., 2000. Nonphytate phosphorus requirements and phosphate excretion of broiler chicks fed diets composed of normal or high available phosphate corn with and without microbial phytase. Poult. Sci. 79, 1451–1459.

Warden, W.K., Schaible, P.J., 1962. Preliminary investigations concerning utilization of phytin phosphorus by the chick. Poult. Sci. 41, 1692 (Abstract).

Weremko, D., Fandrejewski, H., Zebrowska, T., Han, K., Kim, J.H., Cho, W.T., 1997. Bioavailability of phosphorus in feeds of plant origin for pigs. Review. Asian–Aust. J. Anim. Sci. 10, 551–566.

Wheeler, E.L., Ferrel, R.E., 1971. A method for phytic acid determination in wheat and wheat fractions. Cereal Chem. 48, 312–320.

Wise, A., 1983. Dietary factors determining the biological activity of phytates. Nutr. Abstr. Rev. Clin. Nutr. 53, 791–806.

Wodzinski, R.J., Ullah, A.H.J., 1996. Phytase. Adv. Appl. Microbiol. 42, 263–303.

Wu, Y.B., Ravindran, V., Morel, P.C.H., Hendriks, W.H., Pierce, J., 2005. Evaluation of a microbial phytase, produced by solid-state fermentation, in broiler diets. 1. Influence on performance, toe ash contents, and phosphorus equivalency estimates. J. Appl. Poult. Res. 13, 373–383.

Wyss, M., Pasamontes, L., Remy, R., Kohler, J., Kusznir, E., Gradient, M., Muller, F., van Loon, A.P.G.M., 1998. Comparison of the thermostability properties of three acid phosphatases from molds: *Aspergillus fumigatus* phytase, *A. niger* phytase, and *A. niger* pH 2.5 acid phosphatase. Appl. Environ. Microbiol. 64, 4446–4451.

Yan, F., Kersey, J.H., Fritts, C.A., Waldroup, P.W., Stilborn, H.L., Crum, R.C., Rice, D.W., Raboy, V., 2000. Evaluation of normal yellow dent corn and high available phosphorus corn in combination with reduced dietary phosphorus and phytase supplementation for broilers grown to market weights in litter pens. Poult. Sci. 79, 1282–1289.

Yi, Z., Kornegay, E.T., Denbow, D.M., 1996a. Effect of microbial phytase on nitrogen and amino acid digestibility and nitrogen retention of turkey poults fed corn–soybean meal diets. Poult. Sci. 75, 979–990.

Yi, Z., Kornegay, E.T., Ravindran, V., Denbow, D.M., 1996b. Improving phytate phosphorus availability in corn and soybean meal for broilers using microbial phytase and calculation of phosphorus equivalency values for phytase. Poult. Sci. 75, 240–249.

Yin, Y.-L., McEvoy, J.D.G., Schulze, H., Hennig, U., Souffrant, W.-B., McCracken, K.J., 2000. Apparent digestibility (ileal and overall) of nutrients as evaluated with PTVC-cannulated or ileal-rectal anastomised pigs fed diets containing two indigestible markers. Livestock Prod. Sci. 62, 133–141.

Yonemochi, C., Takagi, H., Hanazumi, M., Hijikuro, S., Koide, K., Ina, T., Okada, T., 2000. Improvement of phosphorus availability by dietary supplement of phytase in broiler chicks. Jpn. Poult. Sci. 37, 154–161.

Zhang, X., Roland, D.A., McDaniel, G.R., Rao, S.K., 1999. Effect of Natuphos® phytase supplementation to feed on performance and ileal digestibility of protein and amino acids in broilers. Poult. Sci. 78, 1567–1572.

Zhang, W., Aggrey, S.E., Pesti, G.M., Edwards, H.M., Bakalli, R.I., 2003. Genetics of phytate phosphorus bioavailability: heritability and genetic correlations with growth and feed utilization traits in a randombred chicken population. Poult. Sci. 82, 1075–1079.

Zhang, W., Aggrey, S.E., Pesti, G.M., Bakalli, R.I., Edwards, H.M., 2005. Correlated responses to divergent selection for phytate phosphorus bioavailability in a randombred chicken population. Poult. Sci. 84, 536–542.

Zyla, K., Gogol, D., Koreleski, J., Swiatkiewicz, S., Ledoux, D.R., 1999a. Simultaneous application of phytase and xylanase to broiler feeds based on wheat: *in vitro* measurements of phosphorus and pentose release from wheats and wheat-based feeds. J. Sci. Food Agric. 79, 1832–1840.

Zyla, K., Gogol, D., Koreleski, J., Swiatkiewicz, S., Ledoux, D.R., 1999b. Simultaneous application of phytase and xylanase to broiler feeds based on wheat: feeding experiment with growing broilers. J. Sci. Food Agric. 79, 1841–1848.

Zyla, K., Koreleski, J., Swiatkiewicz, S., Piironen, J., Ledoux, D.R., 2001. Influence of supplemental enzymes on the performance and phosphorus excretion of broilers fed wheat-based diets to 6 weeks of age. Anim. Feed Sci. Technol. 89, 13–118.

Zyla, K., Mika, M., Stodolak, B., Wikiera, A., Koreleski, J., Swiatkiewicz, S., 2004. Towards complete dephosphorylation and total conversion of phytates in poultry feed. Poult. Sci. 83, 1175–1186.

Tovar, R. L.

Plant Foods Hum Nutr (2008) 63:189–194
DOI 10.1007/s11130-008-0089-5

ORIGINAL PAPER

Pulque, An Alcoholic Drink from Rural Mexico, Contains Phytase. Its *in vitro* Effects on Corn Tortilla

Luis Raul Tovar · Manuel Olivos · Ma. Eugenia Gutierrez

Published online: 29 August 2008

Abstract *Pulque* is made by fermenting the agave sap or *aguamiel* of *Agave atrovirens* with a whole array of microorganisms present in the environment including several lactic acid bacteria and yeasts such as *Saccharomyces cerevisiae*. Ascorbic acid was determined in *pulque* and *aguamiel*, respectively. Phytase activity in lees, liquid and freeze-dried *pulque* was assayed by measuring the appearance of phosphate from phytate by a colorimetric method likewise phosphate from phytate present in fresh corn tortilla was measured after *in vitro* incubation with *pulque*. Iron, zinc, calcium, magnesium and selenium contents were measured in *pulque* and corn tortilla as well as in nixtamalized corn flour (NCF), the latter is used to make instant tortilla, since corn provides most of the energy as well as most of the phytate in the Mexican rural diet. *Pulque* showed phytase activity but much less ascorbic acid and iron than previously reported; additionally, phytase in *pulque* hydrolyzed most of phytate's corn tortilla. Lees, which is mostly made of *pulque's* microbiota, significantly accumulated iron and zinc but no selenium. NCF was fortified with iron by the manufacturers but poorly blended. There were significant differences on selenium content between tortillas samples, apparently some soils in central Mexico are selenium deficient. Moderate *pulque* intake appears to increase the bioavailability of iron and zinc bound by phytate in corn.

Keywords *Aguamiel* · Corn tortilla · *Pulque* · Phytase · Phytate · Iron · Zinc · Selenium

L. R. Tovar (✉) · M. Olivos · M. E. Gutierrez
Center for the Environment and Development,
National Polytechnic Institute,
Calle 30 de Junio de 1520 S/N,
Barrio La Laguna Ticoman 07340 D.F., Mexico
e-mail: lrtovar@hotmail.com

Abbreviations

NCF	Nixtamalized corn flour
Pi	inorganic phosphate

Introduction

Pulque is a beverage brewed mainly from the agave sap of *Agave atrovirens* and *Agave salmiana*. There are reports that it has been made since 990 C.E. [1], it is made and consumed mostly by the rural population of central Mexico. The agave juice called *aguamiel*, literally means "honey water", is removed by suction from the plants when they are 8 to 10 years old by previously cutting off the flower stalk; each plant yields about 1500 l of *aguamiel*. The main sugars identified in *aguamiel* are glucose, sucrose, fructose and several pentoses [2]. Fermentation of the sap takes place due to the presence of microorganisms in the environment or by pitching the agave juice with the lees which is the sediment of prior fermented *pulque*. During fermentation *aguamiel* turns sour as lactic acid and other organic acids are formed. The final product, *pulque*, is a white, viscous liquid with about 45 g l^{-1} of ethanol and a pH of 3.4.

One study carried out among pregnant women in rural central Mexico showed that the relationships of *pulque* intake to predict infant size and Bayley mental performance at 6 months of age, were highest when consumption of *pulque* by mothers was about 250 ml/day; these findings were attributed to ascorbic acid and iron supposedly present in *pulque* [3]. To confirm this finding, we determined ascorbic acid level and iron content in several samples of *aguamiel* and *pulque*.

On the other hand, median phytate (*myo*-inositol hexakisphosphate) intake by non-pregnant women in central

Mexico, predominantly from corn tortillas, was 4170 mg/day [4]. Phytate in cereals and legumes inhibits absorption of iron, zinc, calcium and magnesium [5–8], including that of phosphorous.

We tested the hypothesis that *pulque* contains phytase since this beverage includes several species of *Lactobacillus* as well as yeasts like *Saccharomyces cerevisiae* [9] which are known extracellular phytase producers [10]. Furthermore, corn tortilla was incubated in the presence of *pulque* and inorganic phosphate (Pi) was quantified.

In addition iron, zinc, calcium, magnesium and selenium contents were measured in *pulque, aguamiel* and the lees as well as in corn tortillas and nixtamalized corn flours (NCF) used to prepare instant tortillas. Nixtamalization is the lime-treatment of corn. Either corn tortilla or NCF contains about 870 mg of phytate per 100 g of either food on a dry basis [11]. Selenium was determined in *pulque* and corn tortilla because in rural areas consumption of animal foods, which are good sources of it [12, 13], is marginal.

Materials and Methods

Samples Sources

Pulque and *aguamiel* samples were obtained from several producers located in the states of Tlaxcala, Puebla and Hidalgo. Samples from each supplier were pooled and stored at 4 °C at the laboratory. Liquid *pulques* 1 and 2 were assayed at the third and fourth day after being produced. Samples of lees, *pulque* and *aguamiel* were freeze dried. *Pulque* samples were as follows: fresh, X_1; 3 days old, X_2 and 4 days old, X_3.

One source of corn was a gift from farmers at Tepoztlan, Morelos and tortillas were prepared by the traditional method (Cravioto et al. [14]; Corn $Tortilla_1$); another source of corn tortilla came from a tortilla shop in the north of Mexico City (Corn $Tortilla_2$). Both kinds of tortillas were dried overnight in an oven at 50 °C and ground before analysis. Six packages, 1 kg each, of NCF made either by Maseca (NCF_1) or Minsa (NCF_2), the main nixtamalized corn flour producers in the country, were purchased at different groceries in Mexico City. Samples from each brand were pooled for analysis. Moisture was determined on each of the samples according to the AOAC (method 945.380) [15].

Deionized water was used to prepare all solutions.

Ascorbic Acid in Pulque and Aguamiel

Ascorbic acid was determined by triplicate in three pooled samples of liquid *pulque* and two pooled samples of *aguamiel*, according to the AOAC (method 967.21) [15].

Phytase Assays

Phytase activity was determined in the samples using sodium phytate (Sigma Chemical Co., St. Louis, MO, USA) as a substrate and the Pi liberated in the reaction was measured by the method of Fiske and Subbarow [16]. Pi was read at 660 nm with a Perkin Elmer Lambda 20 spectrophotometer (Perkin Elmer, Inc., Shelton, CT, USA). A phosphate standard curve was made from a 1 mM stock solution of KH_2PO_4 which was diluted to prepare a range of phosphate standard solutions with concentrations from 200 to 1000 nmol ml^{-1} Pi. A blank was prepared by separate incubation of substrate and sample at 50 °C over the 30 min assay period. One unit (U) of phytase was defined as the amount of enzyme required to liberate 1 μmol Pi min^{-1} under standard assay conditions. Day-to-day variability in the assay was assessed by monitoring the activity of 0.625 mg ml^{-1} phytase preparation crude, from *Aspergillus ficuum* (3.5 U/mg; Sigma Chemical Co.) dissolved in 200 mM sodium acetate buffer pH 5.5.

Recently made corn tortilla was dried, ground and dissolved 0.05 g ml^{-1} in 0.1 M glycine HCl pH 2.5 then incubated with 100 mg ml^{-1} of a pooled sample of fresh liquid *pulque* dissolved in 200 mM sodium acetate buffer (pH 5.5) at 37 °C for 30 min therefore hydrolysis of phytate present in corn was determined. The assay was run by triplicate. Samples with no *pulque* added were also assayed.

Background Phosphate Assays

Often there was background phosphate in the samples before the enzymatic assay was carried out, deionized water instead of the 0.1% (*w/v*) sodium phytate solution was used and the above procedure followed.

Digestion of Samples for Atomic Absorption Spectroscopy

Digestion of the samples took place in an Anton Paar Physica microwave oven (Graz, Austria) model Multiwave. Each sample was run by triplicate.

Iron, zinc, calcium and magnesium in the samples were quantified by flame atomic absorption spectroscopy according to the AOAC (method 985.35) [15] using a Perkin Elmer atomic absorption spectrophotometer, model Aanalyst 100, with the appropriate hollow cathode lamp for each element. Calcium was determined adding to the samples up to 100 μl of a 5% lanthanum oxide solution (Aldrich Chemical Co., Milwaukee, WI, USA) dissolved in concentrated HCl at 60 °C. Selenium was determined by hydride generation with a Perkin Elmer hydride system model MHS-10. A 1% sodium borohydride (Sigma Chemical Co.) solution dissolved in 0.1 M NaOH was used for the formation of the volatile hydride [17]. Calibration curves

were prepared using certified standard solutions for the five elements (AccuStandard Inc., New Haven, CT, USA). Detection limits, expressed in mg l^{-1}, were as follows: Fe, 0.0003; Zn, 0.0008; Mg, 0.00001; Ca, 0.001; Se, 0.00002.

Statistical Analysis

Significance of the differences among means of phytase activity, inorganic phosphate liberated from phytate and background phosphate of the lees and the freeze-dried *pulque* as well as that of liquid samples, including *aguamiel*, was determined by one-way analysis of variance coupled with the Duncan's multiple range test. Differences with $p<0.05$ were considered significant. Differences among mean values of trace elements and mineral contents of the liquid *pulque* samples as well as those of corn tortillas and NCF's were determined likewise.

Results

Ascorbic Acid Content in Pulque and Aguamiel

Two of the liquid samples of *pulque* from different dates contained 2.66±0.12 mg of ascorbic acid per 100 ml but in the third one ascorbic acid was negligible. On the other hand, *aguamiel* contained in two different samples 2.01± 0.10 mg of ascorbic acid per 100 ml, this indicates that ascorbic acid is an endogenous metabolite of the agave. The recommended dietary allowance (RDA) for this vitamin during pregnancy and lactation is 85 and 120 mg/day, respectively [18].

Moisture of corn tortillas and NCF's was less than 10%, whereas *pulque* showed a moisture content of 93.3%.

Phytase Activity in Pulque and Other Samples

The results for phytase activity in the freeze-dried and liquid *pulque* samples are outlined in Table 1. Phytase activity is expressed as unit per gram of freeze dried sample or unit per 100 ml of *pulque* or *aguamiel*. Pi produced by hydrolysis of phytate with phytase present in each sample and the background phosphate present in the latter are also depicted in the third and fourth columns of Table 1, respectively. Phytase activity in sample X_1 was significantly the highest, probably because the enzyme in X_2 and X_3 had been either inactivated during the freeze-drying step or repressed by the background phosphate [19], or both since their concentrations of background phosphate per g of sample were significantly higher than those of X_1 and the lees. Another explanation of this difference is the samples' age; additionally, phytase activities in X_2 and X_3 were not statistically significant.

On regards to the liquid samples, phytase activity in *aguamiel* was significantly lower than that in fresh and liquid *pulque*. Phytase activity in *aguamiel*, 0.933±0.26 U/ 100 ml, suggests that fermentation was already taking place. Fresh *pulque* showed the highest phytase activity among the liquid samples, 3.0±0.16 U/100 ml, and the lowest content of background phosphate, 1.7±0.04 mg/ 100 ml, both being statistically significant ($p<0.05$). Phytases initiate the removal of phosphate groups from phytate at the carbon ring positions 1, 3 or 6, producing inorganic phosphate, the free cations previously chelated to phytate and *myo*-inositol or its intermediate metabolites [20]. Over a typical course of 12 assays, phytase activity from *A. ficuum* diluted solution yielded 1370±30 Ug^{-1} which was in closed agreement with values previously reported [19].

In the *in vitro* assay, *pulque* released 5.0±0.35 mg of Pi per g of fresh corn tortilla. This indicates that 78% of the Pi from phytate was hydrolyzed; probably iron, zinc and magnesium present in corn tortilla were released by phytase. Pi in the blank was less than 1 mg/g of fresh corn tortilla.

Trace Elements and Mineral Contents in Pulque, Aguamiel, Lees, Corn Tortilla and NCF's

Iron, zinc, magnesium, calcium and selenium contents of the *pulque* samples are depicted in Table 2. Apparently, the microorganisms that make the lees provided *pulque* with most of the iron and zinc but just a fraction of selenium. Zinc content was much less than iron in the freeze dried samples furthermore, no zinc whatsoever was detected in any of the liquid samples of *pulque*. It is surprising the highly significant contents of iron and zinc in the lees compared with the freeze-dried *pulque* samples. Further work is required to determine availability of these trace elements from the lees in humans.

Iron content in fresh *pulque* was one order of magnitude less than what the Mexican food composition database reported [21], whereas in the case of the two other liquid *pulque* samples it was about four times less. In contrast, *aguamiel* contained traces of iron but zinc was not detected. Calcium and magnesium were present in *aguamiel* since the very beginning of the fermentation but only slight differences in their contents were observed among the liquid *pulque* samples, the same trend was found with the freeze-dried samples though no differences were observed. However, the lees had significantly less magnesium than the freeze-dried *pulque* samples. Selenium content in all *pulque* samples followed a similar trend as calcium and magnesium.

Both commercial NCF's were iron fortified (Table 2) but the sample variance of iron obtained in the NCF's was rather large, apparently because iron stirring in the flours did not produce a smooth blend particularly for NCF_1. The

Table 1 Phytase activity of freeze-dried *pulque*, lees, liquid *pulque* and *aguamiel*, as well as inorganic phosphate hydrolyzed from phytate (PA) under the assay conditions and background phosphate in the samples[a]

Sample	U[b] g^{-1}	Pi from PA mg $h^{-1}g^{-1}$	PO_4^{-3c} mg g^{-1}	U 100 ml^{-1}	Pi from PA mg h^{-1} 100 ml^{-1}	PO_4^{-3c} mg 100 ml^{-1}
X_1	4.042±0.017a	32.98±0.14 a	6.49±0.18 c			
X_2	0.329±0.050c	2.68±0.40 c	11.90±0.35 b			
X_3	0.550±0.054 c	4.48±0.43 c	16.18±0.15 a			
Lees	1.057±0.066b	8.62±0.54 b	4.65±0.38d			
Liquid *pulque* 1				1.57±0.067x	12.70±0.55 x	17.0±0.10 v
Liquid *pulque* 2				1.65±0.03 x	13.49±0.25 x	9.6±0.20 y
Fresh *pulque*				3.01±0.16v	24.55±1.31v	1.7±0.04z
Aguamiel				0.933±0.26y	7.61±2.12 y	15.5±0.025 x

[a] Mean values ± SD of replicate samples were analyzed in triplicate. Values within the same column with unlike letters either a to d or v to z are significantly different ($p<0.05$)
[b] U=μmol Pi min^{-1}
[c] Background PO_4^{-3} as KH_2PO_4

same trend was observed in calcium and selenium but not in magnesium and zinc since the amounts of lime used in the nixtamalization process varied from batch to batch. Moreover, lime, depending on its source, contains selenium traces as an impurity (data not shown), fact that explains the variability observed for selenium contents in both NCF's. As it was expected, iron and zinc contents between corn tortillas were not significantly different. Likewise zinc content of corn tortillas and the NCF's also was not significantly different.

Table 2 shows that Corn $Tortilla_1$ contained significantly more selenium than the other samples. Selenium content in Corn $Tortilla_2$ was not detected; this tortilla was made with corn grown in the state of Morelos located in central Mexico. Selenium content in the NCF's ranged from 1 to 10 μg/100 g; however, most of the corn used to make the latter comes from abroad. These results suggest that it is very likely that selenium intake among the rural population of central Mexico is below the RDA which is 60 and 70 μg/day in pregnancy and lactation, respectively [18].

Table 2 Iron, zinc, magnesium, calcium and selenium content of *pulque*, lees, *aguamiel*, corn tortilla and nixtamalized corn flours (NCF's)[a]

Sample	Trace elements and minerals				
	Fe	Zn	Mg	Ca	Se
	mg/100 g				μg/100 g
X_1	2.06±0.4c	0.78±0.06 b	298±70 a	491±119 a	10.8±0.77 a
X_2	2.28±0.2 c	0.56±0.04 c	282±51 a	356±52 a	12.2±1.4 a
X_3	5.32±1.2b	0.92±0.25 b	270±45 a	368±92 a	5.91±3.41b
Lees	16.0±0.9a	11.7±0.3 a	160±22 b	261±83 a	6.90±2.4 b
	mg/100 ml				μg/100 ml
Liquid *pulque* 1	0.20±0.01p	nd	21.4±0.1 p	26.0±7.3 p	2.58±0.2p
Liquid *pulque* 2	0.16±0.07 p	nd	14.4±2.2q	22.0±6.4pq	1.61±0.1 q
Fresh *pulque*	0.07±0.03q	nd	16.4±8.9 pq	20.4±1 q	1.82±0.2 q
Aguamiel	0.03±0.03q	nd	13.8±4.4 q	25.8±5 pq	1.31±0.3 q
	mg/100 g				μg/100 g
Corn $Tortilla_1$	2.64±0.14y	2.01±0.12 x	117.6±2.2 y	227.6±8.8 x	33±2.7v
Corn $Tortilla_2$	2.41±0.30 y	2.52±0.3 x	139.6±2.5 x	373±7.4v	nd
NCF_1	11.3±7.0 x	2.56±0.78 x	160±36 xy	462±324vx	5.31±3.8y
NCF_2	4.7±1.7 x	2.15±0.76 x	149.5±1.4 x	324±316vx	5.39±4.9 y

[a] Mean values ± SD of replicate samples analyzed in triplicate. Mean values within the same column followed by different letters a to d, p to s, and v to z are significantly different ($p<0.05$)
nd Not detected

Table 3 shows an approximation of the daily contributions of iron, zinc, magnesium, calcium, selenium, phosphorous and vitamin C from *pulque* and corn tortillas in the amounts they are consumed by lactating women in rural Mexico. It is interesting to observe that the RDA's for magnesium, phosphorous and iron are above 100% as well as the adequate intake for calcium; however, the bioavailability of these nutrients is considerably reduced by phytate and probably by calcium contained in corn tortilla [22] unless phytase, present in *pulque*, liberates the covalently bound phosphate groups of corn tortilla's phytate from the inositol ring as well as the trace elements chelated to them as it seems to happen *in vitro*. Whether this occurs in the gastrointestinal tract of humans remains to be determined.

Discussion

It is the first time, to the best of our knowledge, that phytase in *pulque* is reported. It is tempting to speculate that the contents of background phosphate and phytase activity are related to the age of the beverage, fact that may have practical implications in the quality of *aguamiel* and *pulque*. It is very likely that the background phosphate observed in *aguamiel* which seems to be produced within the agave was fully utilized by microorganisms during the fermentation to *pulque*.

Sandberg and Andersson [23] showed that wheat-bran containing phytase hydrolyzed phytate in the stomach and small intestine of two healthy men with established ileostomy. Backstrand et al. [4] found, from a logistic regression model, that each milligram intake of ascorbic acid from *pulque* by nonpregnant women was associated with a 5% decrease in the risk of low ferritin and low hemoglobin values. In contrast, our findings suggest that phytase from *pulque's* microbiota possibly dephosphorylates phytate in the gastrointestinal tract of humans improving the bioavailability of iron and zinc, the most deficient micronutrients in a corn tortilla-based diet.

Furthermore, the assumptions of Backstrand et al. [3, 4] that *pulque* improves iron status because it contains ascorbic acid, alcohol and nonheme iron are inaccurate instead, the consumption of *pulque* appears to enhance iron and probably zinc absorption by the presence of viable microorganisms with phytase activity.

So far we do not have an explanation on how lees accumulate iron and zinc (Table 2) and whether these trace elements are available after *pulque* is consumed.

Table 3 shows that the nutrients provided by *pulque* are much less than what corn tortilla provides, iron intake is within recommended levels although iron absorption from corn tortilla is only 1.93% [24], presumably the inhibitory effect of phytate on trace elements and mineral absorption is reduced when *pulque's* phytase cleaves the phosphate groups from phytate, even if the latter is partially hydrolyzed [25]. Vitamin C intake appears to be deficient in the Mexican rural diet which is somewhat puzzling because of the wide variety of fruits and vegetables available throughout the year in central Mexico.

In another study done in human subjects drinking a corn and sorghum beer, iron absorption in the latter was more than 12-fold greater compared to a gruel made from the same cereals used to prepare the beer, apparently because ethanol and lactic acid were present in the final brew as well as live brewer's yeast [26]. This beverage certainly resembles *pulque*.

On regards to the iron fortification of corn flours, it is a matter of concern the high amount of iron added by industry, particularly to NCF_1 (Table 2) since tortillas made from Maseca flours, will reach both individuals in need of additional iron and individuals without that need.

Average selenium concentration in soils of the state of Tlaxcala, in central Mexico, was about 0.048 ppm, goats grazing in these fields showed a marginal selenium status [27]. Coincidentally, the state of Morelos is fairly close to Tlaxcala, fact that partially explains the absence of selenium in corn tortillas made with corn grown in that state (Table 2). Since selenium content in plant foods varies greatly depending upon the amount available from the soil, continued effort to analyze periodically for selenium in foods grown throughout central Mexico should be helpful to avoid deficiency among the rural population.

It can be concluded that vitamin C, selenium, zinc and iron are deficient in the diet of lactating women in rural central Mexico, albeit moderate *pulque* drinking appears to ameliorate iron and zinc deficiencies by the presence of phytase from live bacteria in the latter.

Table 3 Estimated percentage of the recommended dietary allowances of several nutrients provided by *pulque* and corn tortilla for lactating women (19 to 30 years)[a]

Nutrient	Fresh pulque, 300 ml	Corn tortilla, 350
	%	
Iron	3.3	98.0
Zinc	nd	67.3
Magnesium	18.6	160
Calcium[b]	8.24	105
Selenium	9.1	17.1
Phosphorous	4.0	105.7
Vitamin C	7.8	nd

[a] National Academy of Sciences [18].
[b] Adequate intake instead of RDA.
nd Not detected.

Acknowledgments Two of us, LRT and MEG, are fellows from the Commission for the Promotion of Academic Activities, an organiza-

tion that belongs to the National Polytechnic Institute. We appreciate the reviewers' comments on this article.

References

1. Gonçalves de Lima O (1978) El maguey y el pulque en los códices mexicanos, 2ª edición. Fondo de Cultura Económica, México, pp 32–33, 80
2. Sanchez-Marroquin A, Hope P (1953) Fermentation and chemical composition studies of some species. J Agric Food Chem 1:246–249, doi:10.1021/jf60003a007
3. Backstrand JR, Allen LA, Martinez E, Pelto GH (2001) Maternal consumption of *pulque*, a traditional central Mexican alcoholic beverage: relationship to infant growth and development. Public Health Nutr 4:883–891, doi:10.1079/PHN2001130
4. Backstrand JR, Allen LA, Black AK, de Mata M, Pelto GH (2002) Diet and iron status of nonpregnant women in rural Central Mexico. Am J Clin Nutr 76:156–164
5. Hallberg L, Rossander L, Skanberg A-B (1987) Phytates and the inhibitory effect of bran on iron absorption in man. Am J Clin Nutr 45:988–996
6. Oberleas D, Muherer ME, O'Dell BL (1966) Dietary metal-complexing agents and zinc availability in the rat. J Nutr 90:56–62
7. Heaney RP, Weaver CM, Fitzsimmons ML (1991) Soybean phytate content: effect on calcium absorption. Am J Clin Nutr 53:745–747
8. Bohn T, Davidsson L, Walcyzyk T, Hurrell RF (2004) Phytic acid added to white-wheat bread inhibits fractional apparent magnesium absorption in humans. Am J Clin Nutr 79:418–423
9. Sanchez-Marroquin A, Larios C, Vierna L (1967) Estudios sobre la microbiología del pulque XIX. Elaboración de la bebida mediante cultivos puros en planta piloto. Rev Latinoam Microbiol Parasitol (Mex) 9:83–85
10. Vohra A, Satyanarayana T (2003) Phytases: microbial sources, production, purification, and potential biotechnological applications. Crit Rev Biotechnol 23:29–60, doi:10.1080/713609297
11. Tovar LR, Larios-Saldaña A (2005) Iron and zinc fortification of corn tortilla made either at the household or at industrial scale. Int J Vitam Nutr Res 75:142–148, doi:10.1024/0300-9831.75.2.142
12. Levander OA (1996) Selenium. In: World Health Organization (ed) Trace Elements in Human Nutrition and Health. World Health Organization, Geneva, pp 105–122
13. Keck A-S, Finley JW (2006) Databases values do not reflect selenium contents of grain, cereals, and other foods grown or purchased in the upper Midwest of the United States. Nutr Res 26:17–22, doi:10.1016/j.nutres.2005.11.009
14. Cravioto RO, Anderson RK, Lockhart EE, Miranda FP, Harris R (1945) Nutritive value of the Mexican tortilla. Science 102:91–93, doi:10.1126/science.102.2639.91
15. AOAC (2005) Official methods of analysis, 18th edn. Association of Official Analytical Chemists, Gaithersburg
16. Fiske CH, Subbarow Y (1925) The colorimetric determination of phosphorous. J Biol Chem 66:375–400
17. Campbell AD (1992) A critical survey of hydride generation techniques in atomic spectroscopy. Pure Appl Chem 64:227–244, doi:10.1351/pac199264020227
18. Institute of Medicine (2000) Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids. National Academy of Sciences, Washington, D.C., p 506
19. Shieh TR, Ware JH (1968) Survey of microorganisms for the production of extracellular phytase. Appl Environ Microbiol 16:1348–1351
20. Lei XG, Stahl CH (2001) Biotechnological development of effective phytases for mineral nutrition and environmental protection. Appl Microbiol Biotechnol 57:474–481, doi:10.1007/s002530100795
21. Hernández M, Chavez A, Bourges H (1977) Valor nutritivo de los alimentos mexicanos. Instituto Nacional de la Nutrición, México, pp 19–20
22. Instituto Nacional de Salud Pública (2001) Encuesta Nacional de Nutrición 1999. Estado nutricio de niños y mujeres en México, Cuernavaca, pp 185–186
23. Sandberg A-S, Andersson H (1988) Effect of dietary phytase on the digestion of phytate in the stomach and small intestine of humans. J Nutr 118:469–473
24. Mendoza C, Viteri FE, Lönnerdal B, Young KA, Raboy V, Brown KH (1998) Effect of genetically modified, low phytic acid maize on absorption of iron from tortillas. Am J Clin Nutr 68:1123–1127
25. Hallberg L, Brune M, Rossander L (1989) Iron absorption in man: ascorbic acid and dose-dependent inhibition by phytate. Am J Clin Nutr 49:140–144
26. Charlton RW, Jacobs P, Seftel HC, Bothwell TH (1964) Effect of alcohol on iron absorption. Br J Med 2:1427–1429
27. Ramirez-Bribiesca JE, Tortora JE, Huerta M, Aguirre A, Hernandez LM (2001) Diagnosis of selenium status in grazing dairy goats on the Mexican plateau. Small Rumin Res 41:81–85, doi:10.1016/S0921-4488(01)00188-2

van Dijck, P. W.M.

P63

ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Regulatory Toxicology and Pharmacology 38 (2003) 27–35

Regulatory Toxicology and Pharmacology

www.elsevier.com/locate/yrtph

On the safety of a new generation of DSM *Aspergillus niger* enzyme production strains

Piet W.M. van Dijck,[a,*] Gerard C.M. Selten,[b] and Rixta A. Hempenius[a]

[a] *DSM Food Specialties, Department of Regulatory Affairs, P.O. Box 1, 2600 MA Delft, Netherlands*
[b] *DSM Food Specialties, Department of Genetics, P.O. Box 1, 2600 MA Delft, Netherlands*

Received 7 October 2002

Abstract

Consumers safety of enzyme preparations is determined by three variables: the producing organism, the raw materials used in the production, and the production process itself. The latter one is embedded in current Good Manufacturing Practice (cGMP) and Hazard Analysis of Critical Control Points (HACCP); therefore the safety focus can be directed to raw materials and the producing organism. In this paper, we describe the use of novel genetically modified strains of *Aspergillus niger*—made by a design and build strategy—from a lineage of classically improved strains with a history of safe use in enzyme production. The specifics of the host strain allow for integration and over-expression of any gene of interest at a targeted integration site implying that the rest of the host genome is not affected by this integration. Furthermore due to the fact that the newly integrated gene copies are put under the genetic regulation of the host's own glucoamylase promoter, the recipe of the production process of any new production strain can be kept constant with respect to the raw materials composition. Consequently the safety of a new enzyme product from these novel genetically modified strains is determined by the background of the production organism. The use of a strain with a history of safe use and targeted integration according to the concept described above has consequences for the safety studies on the final product. If a known enzymatic activity is over-expressed the safety of a new enzyme preparation is covered by the results of the safety studies performed for other strains from this specific *Aspergillus niger* strain lineage. In this paper an overview is given on the available toxicity tests with these strains. We conclude that for new enzyme products produced with strains from this lineage using the design and build technology no new sub-acute/chronic oral toxicity studies are needed. This also has the benefit that no longer test animals are needed to demonstrate the safety of products produced by these strains.

Keywords: Enzyme safety; Micro-organism; *Aspergillus niger*; Genetic engineering; Secondary metabolites

1. Introduction

Microbial enzymes are since many decades used in many food processing and animal feed applications. Although they are sold for a specific enzymatic function they seldom are pure enzyme products. The same holds for enzyme preparations derived from plant or animal origin. Microbial enzymes may contain other enzymatic proteins, metabolites from the production organism, remnants of the fermentation raw materials as well as added materials such as preservatives and stabilizers.

Enzymes in general are consumed in large amounts in fresh as processed foods such as meat, eggs, nuts and grains, dairy products, fruits, and vegetables. Enzymes are proteins and upon ingestion are degraded to peptides and amino acids, which are completely metabolised by humans. Enzymes in the diet are as a rule completely non-toxic (Aunstrup et al., 1979; Reed, 1975) and can be considered intrinsically safe (Noordervliet and Toet, 1987). Therefore, the safety assessment of microbial enzymes for the food processing and animal feed industry focuses more on the "contaminants" in an enzyme preparation than on the enzyme itself (IFBC, 1990; Kessler et al., 1992; Pariza and Foster, 1983; Pariza and Johnson, 2001). Toxic contaminants may be introduced during the whole production process by the

* Corresponding author. Fax: +31-15-2793614.
E-mail address: piet.dijck-van@dsm.com (P.W.M. van Dijck).

0273-2300/03/$ - see front matter
doi:10.1016/S0273-2300(03)00049-7

production organism, the fermentation process (substrates, raw materials etc.), and the isolation and purification procedures. Note that the latter ones are embedded in current Good Manufacturing Practice (cGMP) and Hazard Analysis of Critical Control Points (HACCP).

For many decades, *Aspergillus niger* has been safely used in the commercial production of various food enzymes, such as glucose oxidase, pectinase, α-amylase, and glucoamylase. Industrial production of citric acid by *A. niger* has taken place since 1919 (Röhr et al., 1983).

This long experience of industrial use has resulted in a good knowledge of the characteristics of *A. niger* and understanding of the metabolic reactions. Recently its genome has been elucidated (Groot et al., 2002). Genetically modified industrial strains of *A. niger* have been used as a host to over-express food and feed enzymes such as phytase and xylanase. Traditionally, the genes encoding these enzymes are integrated in a random fashion in the genome of the host organism. These enzymes are on the market now for almost a decade.

New technological developments have enabled us to construct a new generation of strains according to a design and build concept, in which the genes are introduced targeted to a predetermined region in the genome of the strain. The safety of this novel generation of enzymes for the consumer and the consequences with regard to safety studies are discussed in this paper.

2. The safety of *A. niger*

A. niger is known to naturally occur in foods. The fungus is commonly present in products like rice, seeds, nuts, olives, and dried fruits.

The long industrial use and wide distribution of *A. niger* in nature has never led to any pathogenic symptoms. Its non-pathogenic nature has been confirmed by several experimental studies, as reviewed by Schuster et al. (2002). *A. niger* is therefore generally accepted as a non-pathogenic organism.

Even though products from *A. niger* have been used in food for many decades, there has never been any evidence that the industrial strains used are able to produce detectable levels of toxins. The non-toxicogenicity has been confirmed by a large amount of toxicological tests, as well as batch testing of the various end products for toxins. This non-toxicogenicity does, however, not hold for all *A. niger* strains. A small percentage of natural isolates have been shown to possess the potential to produce ochratoxin A, a nephrotoxic, and carcinogenic mycotoxin (Abarca et al., 2001; Varga et al., 2000). Other secondary metabolites isolated from *A. niger* strains comprise nigragillin, the malformins, and the naphtho-γ-pyrones. These compounds are not considered to be mycotoxins.

The whole body of knowledge from the literature is carefully taken into consideration when testing industrial strains for any possible risk during the development of a fermentation process. Whenever possible the production organism is chosen from strains which have been in use for many years and which are examined for their ability to produce known toxins under the fermentation conditions. In addition, the products are regularly checked that they meet the requirements of the health authorities as given in the Food Chemical Codex (1996) or in the FAO/WHO Joint Expert Committee on Food Additives (JECFA) specifications (1992).

When a production process at our company DSM is developed with a new fungal strain the strain is routinely tested for its potential to produce toxic secondary metabolites, including mycotoxins. To this end the strains are cultured on agar-media, which are known from literature to give optimal expression of these toxic secondary metabolites (Frisvad and Filtenborg, 1989; Frisvad and Thrane, 1993). All cultures are incubated for 14 days in darkness at 24 °C. For metabolite analysis, the samples are extracted by the method described by Frisvad and Thrane (1987) and analysed by high performance liquid chromatography (HPLC) equipped with a diode array detection (DAD) system (Frisvad and Thrane, 1993). The metabolites found are compared to a spectral UV library made from authentic standards run at the same conditions, and retention indices were compared with those of the standards. An extra control run is carried out with a fluorescence monitor that detects ochratoxin.

3. The DSM *A. niger* GAM strain lineage

The DSM industrial strain used as a host for over-expression of enzymes is *A. niger* DS03043. Strain DS03043 is an over-producer of the enzyme glucoamylase, which is shortened to GAM. Consequently this lineage of strains is called the GAM strain lineage and is derived by classical strain improvement techniques (mutagenic treatment followed by selection for improved isolates) from the original strain *A. niger* NRRL3122 from the Culture Collection Unit of the Northern Utilization Research and Development Division, US Department of Agriculture, Peoria, IL, USA. The strain NRRL3122 and its classical derivatives have been in use for the production of glucoamylase (and acid amylase) by Wallerstein Laboratories since the sixties. Wallerstein was acquired by Baxter-Travenol and later on divested to Gist-brocades, now part of DSM.

Strain DS03043 was used as a suitable host strain to over-express enzymes of interest for DSM. For over-expression of phytase, the phytase gene was cloned from the donor strain *A. niger* NRRL3135 (also known as *Aspergillus ficuum*), put in an expression cassette under

the control of the host-own *gla*A promoter. Multiple copies of the phytase *phy*A gene were randomly incorporated into the genome of the host. The research yielded two phytase production strains, designated *A. niger* DS25956 and DS27301. These strains are being used in large-scale production of phytase, and marketed under the brandname Natuphos since 1991.

Using the same procedures the gene encoding endo-1,4-β-xylanase gene from a xylanase overproducing strain of *A. niger* (strain DS16813) was over-expressed in DS03043 by random integration of multiple copies of the xylanase expression cassette. The resulting strain *A. niger* DS26538 is used since 1995 for larger scale production of xylanase, marketed under the brandname Natugrain Wheat.

In recent years the strain DS03043 was genetically modified to serve as a suitable host for targeted integration of genes of interest to DSM and over-expression of the genes. Genetic analysis of the classical improved GAM lineage of strains showed that the increase in glucoamylase production capacity is, at least partly, due to an increase in the number of gene copies in the strain. Whereas NRRL3122 only contained one gene copy of the glucoamylase *gla*A gene, the DS03043 strain contains seven *gla*A gene copies. Many of the production strains that have undergone strain improvement by classical mutation and selection techniques have appeared to contain multiple copies of the gene of interest.

A recombinant strain was derived from DS03043, in which the seven glaA loci (i.e., the promoter and coding sequences) were deleted, creating so-called Δ*gla*A loci. Each of these loci was designed in such a way that it can be individually detected on a gel electrophoresis system (Fig. 1). The strain, in which all seven loci had been deleted, was used as the primary DSM host strain for over-expression of other enzymes. From this strain other host strains were derived with additional modifications such as inactivation of the major protease *pep*A and with improved enzyme production capability. As specific demands require new host strains will be derived from this *A. niger* strain lineage. The present set of *A. niger* host strains have been approved as GMO self-clones[1] by the Dutch competent authorities.

In order to obtain accurate integration and expression of any desired gene in one of the above-mentioned host strains, standard expression units are used, where the gene of interest (call it gene X) is inserted between the host-own glucoamylase promoter and glucoamylase terminator elements in a proprietary *Escherichia coli* vector. Also the *amd*S gene, to be used as a dominant selection marker, was put in such a standard unit. For details, see Selten et al. (1995, 1998).

[1] Self-cloning as defined in the European Directive on the Contained Use of Genetically Modified Micro-organisms 98/81/EC (revision of 90/219/EC).

Prior to the transformation the two vectors (the amdS vector and the vector containing gene X) are linearized using the appropriate restriction enzymes and all sequences derived from *E. coli* are removed by purification on agarose gels. The linearized expression cassettes for gene X and the *amd*S marker are then introduced into the host strain. The gene X and *amd*S expression units—both completely free of any *E. coli* DNA—are integrated into the genome of the host by co-transformation. Due to the homology in the 3′- and 3″-*gla*A parts of the two expression units, these expression units are targeted to one of the seven Δ*gla*A loci. Transformants are selected on agar plates containing acetamide as the sole carbon source. Only those clones are able to grow that have acquired at least one copy of the *amd*S expression unit.

Transformants are selected that contain an *amd*S expression unit with several in tandem integrated gene X expression units in one of the seven Δ*gla*A loci. The selection of these transformants is done by PCR analyses, applying gene X and *gla*A specific primers. Subsequently, by counter-selection on fluoro-acetate containing plates, a natural variant is selected in which the *amd*S selection marker is deleted as a result of a natural internal recombination event. This is schematically depicted in Fig. 2. In the recombination event the strain also may lose a number of gene X expression units. The absence of the *amd*S marker is confirmed by Southern analysis. The resulting organism is thus not only totally free of *E. coli* DNA, but also of the *amd*S selection marker.

For further multiplication of the remaining gene X expression units in this marker-free organism, the naturally occurring gene conversion is used to fill up other Δ*gla*A loci. Gene conversion is a natural spontaneous process, not involving any mutagenic treatment of the strain (Selten et al., 1998).

Strains can be selected in which up to all seven Δ*gla*A loci are occupied with multiple copies of the expression unit in question, arranged in tandem (see Fig. 3).

Since the integration of new gene copies into the genome is targeted there is no disturbance of other parts in the genome, so there is no possibility of e.g. activation of mycotoxin gene(s).

Due to the fact that the newly introduced gene copies are under the genetic regulation of the glucoamylase promoter the production processes for new enzyme products can be kept the same with respect to the raw materials used. This eliminates the chances of new safety hazards for the enzyme product brought in to the system by the raw materials.

This innovative technology has been used by DSM to design and build a lineage of novel marker free production strain lineages, including the following:

- The ABF lineage, e.g., strain DS34552, over-expressing the *abf*A arabinofuranosidase gene from the DSM classical arabinofuranosidase production strain *A. niger* DS06846.

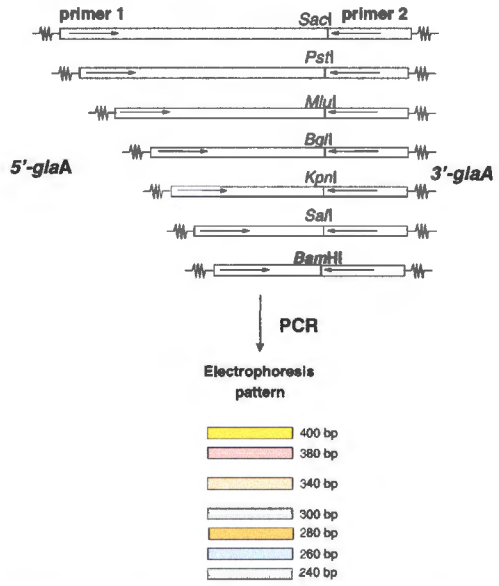


Fig. 1. Visualization of all seven marked *gla*A loci of *A. niger* strains, in which the *gla*A genes were removed. All gene sequences of the seven glucoamylase loci were removed by using proprietary technology. Subsequently they were partly filled using the same methodology with sequences from the 3′ region of the original locus. Due to the fact that these sequences varied in length and were decorated with a specific restriction site the seven marked loci can detected individually by PCR using a set of specific primers and separation on a gel electrophoretic system.

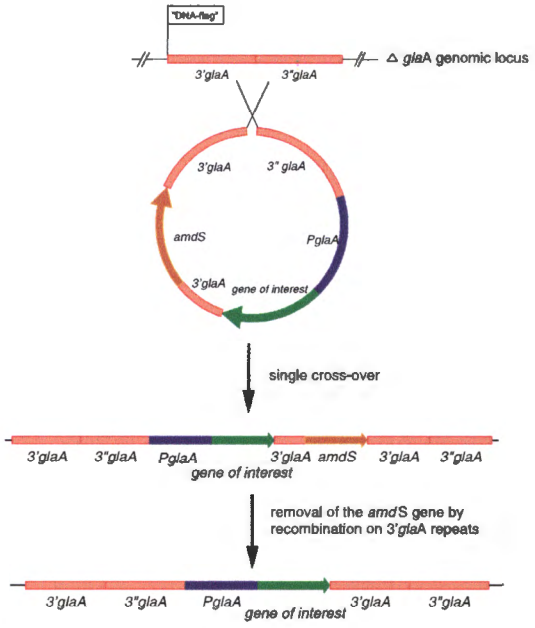


Fig. 2. Example of the marker-gene free insertion of an expression unit. The expression unit, a linear piece of DNA, is integrated into one of the seven marked loci by homology of the 3′ regions of the loci. By varying the conditions of transformation of the expression units multiple copies of the gene of interest arranged in tandem can be integrated in a marked locus. Current practise is that both the gene of interest and the *amd*S selection marker are on different expression units. So the transformation is carried out with a mixture of expression units carrying the gene of interest and expression units carrying the *amd*S marker. By selection on agarplates containing acetamide as sole carbon source the transformants are selected. By counter-selection on agarplates containing fluoro-acetate variants can be selected from these transformants which have lost the *amd*S marker but which have retained (multiple copies of) the gene of interest.

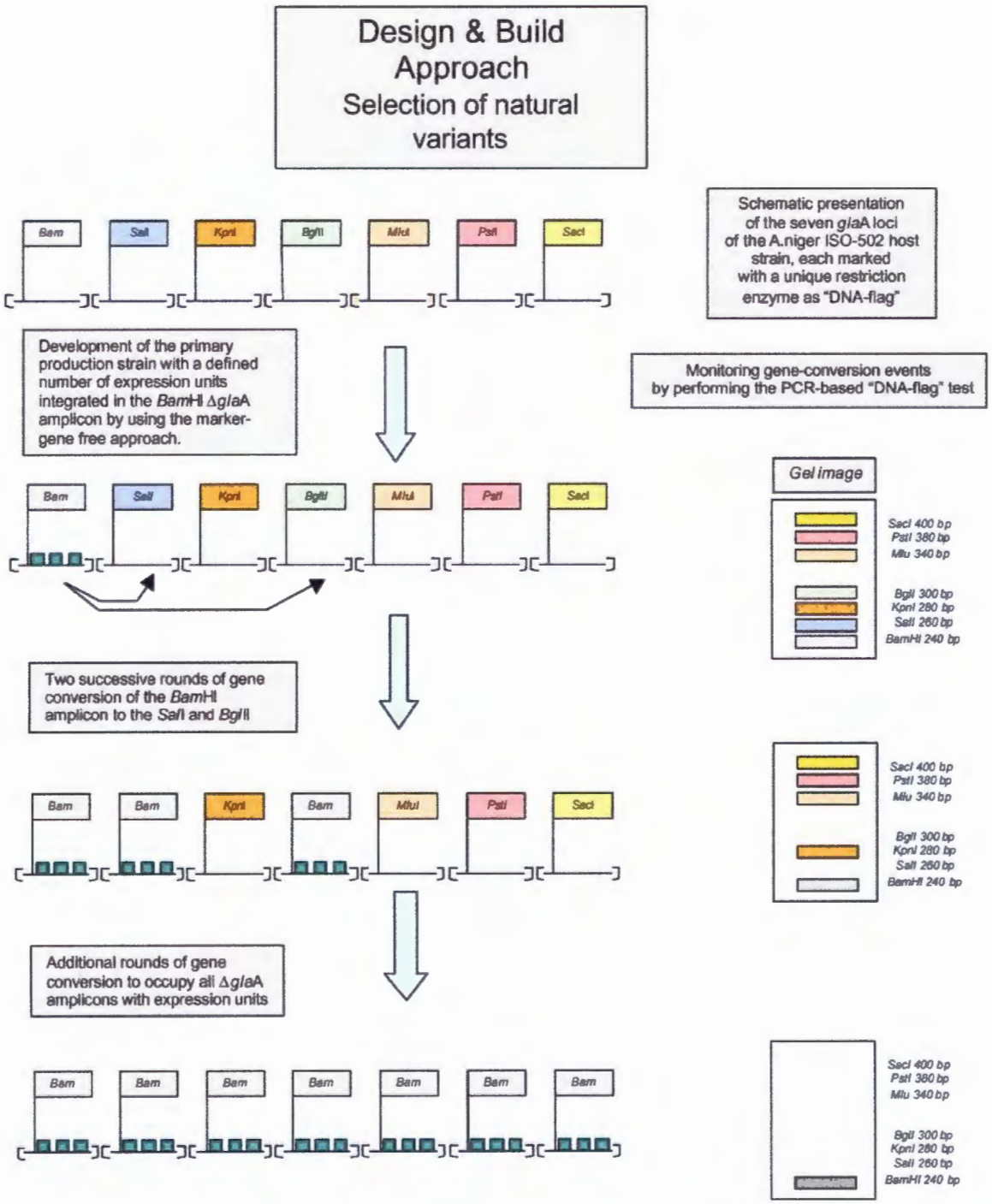


Fig. 3. Selection of natural variants. The natural process of gene conversion is used to select for variants that have acquired a set of multiple gene copies of the gene of interest in any the remaining empty marked loci. The filling-up process in the several selection rounds can be followed by analysis in a gel electrophoretic system.

- The NPH lineage, e.g., strain DS35387, over-expressing the *phy*A phytase gene from *A. niger* NRRL3135 (also known as *Aspergillus ficuum*).
- The PME lineage, e.g., strain DS34553, over-expressing the *pme*A pectin methyl esterase gene from the DSM classical *A. niger* pectinase production strain.
- The PLA lineage, e.g., strain DS35496, over-expressing the phospholipase A_2 gene obtained as cDNA from porcine pancreas.
- The GLA and AMY lineages, e.g., strains DS36728 and DS37099, respectively, over-expressing the *gla*A glucoamylase gene and the *amy*A acid amylase gene

from *A. niger* DS03043, the DSM glucoamylase production strain and parental strain of the above described novel host strains.

- The XEA lineage, e.g., strain DS38163, over-expressing a heterologous xylanase gene.

Several new production strain lineages are under development.

The GMO strains from the PME, ABF, NPH, GLA, and AMY lineages have been approved by the Dutch competent authorities as GMO self-cloned strains.

Based on the genetic modifications performed, there are no reasons to assume that the new recombinant production strains should be less safe than the original *A. niger* DS03043 strain.

4. Secondary metabolites produced by strains from the GAM lineage

It has been shown (Van Dijck et al., 2002) that the *A. niger* ancestor strain NRRL3122 has the potency to produce nigragillin and naphtho-γ-pyrones as secondary metabolites. This potency remains more or less the same with the classically improved strains from the GAM lineage, although the number and the amount of secondary metabolites decrease. Introduction of genes by random integration of phytase genes (DS25956 and DS27301) or xylanase genes (DS26538) into the genome of DS03043 does not change this pattern.

We expect that targeted introduction of genes into the genome of the novel *A. niger* host strains, by design should not have any influence on the pattern of secondary metabolites. That this indeed is the case was demonstrated for all production strains that were developed so far. All these GMO strains, if tested under conditions optimal for mycotoxin production, showed a pattern of secondary metabolites similar to that of the original host strain DS03043. In none of the strains could aflatoxin, trichothecenes, or ochratoxin A be detected. (Van Dijck et al., 2002).

The strains were also tested under conditions representative for large-scale production conditions. All secondary metabolites, normally found in the strains under stress conditions, such as nigragillin and naphtho-γ-pyrones, were not detectable either in broth samples, taken at the end of the fermentation, or in the final (unformulated) enzyme products (Van Dijck et al., 2002).

5. Safety studies on the classical strain

The toxicological studies performed on various enzyme preparations from *A. niger* provided the basis for a safety evaluation by the Joint Expert Committee on Food Additives (JECFA) of the FAO/WHO. Since the enzyme preparations tested were of different activities and forms, and of most of the organic materials in the preparations are not the enzyme per se JECFA estimated a numerical Acceptable Daily Intake (ADI) expressed in terms of total organic solids (TOS). Although all toxicological studies for enzyme preparations of *A. niger* showed no-observed-effect levels greater than 100 mg TOS/kg bw/day in 90-day studies in rats, JECFA nevertheless allocated an ADI of 0–1 mg TOS/kg bw for each enzyme preparation. This was merely based on the fear that some strains may produce unknown toxins (JECFA, 1988). However, in view of the long history of use as an enzyme source, the numerous toxicological studies and two expert reports that showed that toxin production is very unlikely (Bennett, 1988; Moss, 1988), JECFA reconsidered its decision and changed the ADI for enzyme preparations derived from *A. niger* into "not specified" (JECFA, 1991).

Among the safety studies evaluated by JECFA (1975) were Baxter-Travenol's unpublished safety studies carried out on a carbohydrase product (Garvin and Merubia, 1959; unpublished report submitted to WHO by Baxter Laboratories; Garvin et al., 1972; unpublished report from Travenol Laboratories submitted to WHO by Gist-brocades). This study was a 90-day oral toxicity study with rats and showed a NO(A)EL (No Observed Adverse Effect Level) of 10% in the diet. In the early seventies strain numbers were not mentioned in the reports of the safety studies. We can only assume but not prove that the safety studies were done on a carbohydrase product obtained from a fermentation with *A. niger* NRRL3122. Besides amyloglucosidase the product also exhibited α-amylase activity. Since the DSM GAM-lineage of strains originates from the Wallerstein (= Baxter-Travenol) amyloglucosidase production strain, the result of the study bears relevance for the DSM GAM lineage of strains. Table 1 gives an overview of all the safety studies performed on the DSM GAM lineage of strains.

6. Safety studies on strains obtained by random integration of genes

Representative batches of phytase produced either with strain DS25956 or strain DS27301 or xylanase produced with strain DS26538 have been subjected to safety studies as required by the guidelines of the EU Scientific Committee for Food (1992). Two mutagenicity tests (Ames test and an in vitro chromosome aberration study) and a 90-day subchronic oral toxicity test in rats have been done. In all three cases the NOAEL was the highest enzyme dose tested and was above the 100 mg TOS/kg bw/day, which was the threshold for safe enzyme products according to JECFA. For an overview of the safety data, see Table 1. From these data we conclude that strains derived from strains from the GAM

Table 1
Overview of oral toxicity studies on enzyme preparations produced with strains from the *A. niger* GAM strain lineage

Enzyme	Production organism	Type of integration	Oral toxicity study		
			Year of study	Duration	NO(A)EL[a] (mg TOS/kg bw/day)
General (JECFA)	*A. niger*		—	90	>100
Glucoamylase	*A. niger* NRRL-3122	—	1972	90	
Phytase	*A. niger* DS25956	Random	1991	90	>1260
Phytase	*A. niger* DS27301	Random	2000	90	>1206
Xylanase	*A. niger* DS26538	Random	1995	90	>4095
Phytase	*A. niger* DS35387	Targeted	2000	90	>833
Phosholipase A2	*A. niger* DS35496	Targeted	2000	90	>1350
Xylanase	*A. niger* DS38163	Targeted	2001	90	>1850
Pectin methylesterase	*A. niger* DS34553	Targeted	2000	14	>133
Arabinofuranosidase	*A. niger* DS34552	Targeted	2000	14	>103
Amyloglucosidase	*A. niger* DS36728	Targeted	2001	14	>1640

[a] In all cases the NOAEL (No-observed-adverse-effect level) was the highest dose level tested.

lineage do not produce any harmful compounds that may end up in the enzyme product.

7. Safety studies on the novel DSM *A. niger* strains

With enzyme products produced with the novel DSM *A. niger* strains in three cases, besides the two mutagenicity studies, a 90-day subchronic oral toxicity study in rats was carried out. This was done for phytase, produced with *A. niger* DS35387, phospholipase A2 produced with a strain from the *A. niger* PLA lineage and xylanase, produced with *A. niger* DS38163. In all three cases the NOAEL was the highest enzyme dose tested and was above the 100 mg TOS/kg bw/day.

For other enzyme products made with these novel strains only a 14-day oral toxicity study was carried out. This holds for arabinofuranosidase, produced with DS34552, pectin-methyl esterase, produced with DS35387, and gluco-amylase, produced with a strain from the GLA lineage. Also with these three products the results showed that the NOAEL was the highest enzyme dose tested (i.e., >100 mg TOS/kg/day). In all cases the mutagenicity studies were negative.

8. Discussion

In this paper we have shown that the *A. niger* GAM lineage and the derived GMO strains can be considered as a safe strain lineage. There are safety data available from the classical ancestor strain, from the DS03043 derived GMO strains, where the expression cassettes were randomly integrated into the genome of DS03043 and from the novel GMO strains, where the expression cassettes were integrated into the genome of the host at a targeted site.

The finding that in the strains obtained by random integration of expression cassettes no change in the pattern of secondary metabolites is found makes it highly unlikely that random integration of genetic material in the genome, due to pleiotropic effects, induces the formation of novel toxic metabolites. This often is raised as a hypothetical possibility by regulatory authorities. In strains obtained by targeted integration of expression cassettes, both *cis* and *trans* activations of toxin producing genes are hard to imagine and can be ruled out for all practical purposes.

The fact that the pattern of secondary metabolites does not change can also be taken as an indication that the over-expression of enzymes does not lead to induction of metabolic stress to such an extent that secondary metabolites are formed. Metabolic stress due to over-expression of proteins has been found in bacterial and yeast systems (Gill et al., 2000; Patil and Walter, 2001). With the DSM *A. niger* GMO strains the over-expression occurs in a strain that is derived from an industrial strain, which has already the capacity to over-produce and secrete copious amounts of enzyme protein. Any stress that is inflicted upon these GMO strains apparently does not lead to any detectable toxin production. This can be concluded from the analyses of the secondary metabolites under actual large-scale fermentation conditions.

The fact that the genes of interest are over-expressed under the same genetic regulatory system enables the fermentation process of all these products to be constant with respect to the raw materials composition of the fermentation medium. Consequently no new safety hazards are introduced into the product background. This is supported by the safety studies performed on these novel enzymes. In all cases the highest dose level tested was the NOAEL. Using standard methods for the calculation of the expected human consumption (Douglas et al., 1997;

Löwik et al., 1998) the Margin of Safeties were 250 up to 400,000 in case of a 90-day toxicity study and 500 up to 2750 for the 14-day toxicity studies.

We conclude that with all safety data available we cover the safety of new enzymes to be produced using a GMO strain derived from the novel DSM *A. niger* strain lineage. Provided of course that with the gene of interest we do not introduce additional non-characterized gene sequences into the strain. PCR techniques allow us to restrict the DNA fragment to be transformed only to the coding sequence of the gene of interest. When we apply the case of an enzyme product belonging to a class of enzymes which have a history of safe use in food processing (see Table 1 in the Pariza and Johnson paper) made according to the principles described in the previous pages to the Decision Tree put forward by Pariza and Johnson (2001) it is clear that the production strain is genetically modified and that recombinant techniques have been used in the introduction (Q1 and 2). The question, whether the expressed enzyme product, which is encoded for by the introduced DNA, has a history of safe use (Q3a), can be answered with yes. Since the novel DSM strains do not contain any heterologous markers such as antibiotic resistance markers (Q3c) and all introduced DNA is well characterized and free of attributes that would render it unsafe of constructing micro-organisms to be used in food-grade products (Q3e) the Decision Tree takes us to Q4. The introduced DNA is integrated in the genome of the host in a targeted manner (in one or more of the seven glucoamylase loci), which brings us to question 6: is the production strain derived from a safe strain lineage? For the GAM lineage of strains we have shown in the preceding paragraphs that sufficient data are available from safety studies on enzyme products made with strains from this lineage that the *A. niger* GAM lineage of strains truly can be called a safe strain lineage.

This affirmative answer to Q6 concludes the passage through the Decision Tree with the statement: the test article is ACCEPTED.

Thus, when assessing the potential safety hazards of a new enzyme product it would make no difference as to from what background the enzyme encoding gene of interest is isolated with PCR, provided that the enzyme itself has a history of safe use in food. The donor organism can be a safe micro-organism but it would not matter if the donor strain is pathogenic, toxicogenic or when the background of the gene is not known. So even in case of direct isolation of DNA by PCR techniques from a biological source, without the intermediary step of isolating and cultivating the donor micro-organism, the existing safety studies would cover the derived enzyme product and no new safety studies would be required. Consequently unnecessary use of testing animals would be prevented.

Acknowledgments

We acknowledge the critical comments of our (former) colleagues within DSM Regulatory Affairs, Henk van Dam, Henk Aalten, Danielle Praaning, and Albert de Leeuw. The paper is dedicated to the memory of Henk van Dam, who stimulated the discussions leading to writing this paper. Henk died of cancer at the age of 53 on May 9, 2002.

References

Abarca, M.L., Accensi, F., Bragulat, M.R., Cabanes, F.J., 2001. Current importance of ochratoxin A-producing *Aspergillus* spp. J. Food Prot. 64, 903–906.

Aunstrup, K., Andresen, O., Falch, E.A., Nielsen, T.K., 1979. Production of microbial enzymes. In: Peppler, H.J., Perlman, D. (Eds.), Microbial Technology, second ed. Microbial processes, vol. I. Academic Press, New York, pp. 281–309.

Bennett, J.W., 1988. Proposed acceptable daily intake (ADI) levels for enzymes from organisms not commonly considered to be constituents of food. Report for JECFA dated September 14, 1988.

Committee on Food Chemicals Codex, 1996. Food Chemicals Codex. National Academy Press. Washington, DC.

Douglas, J.S., Barraj, L.M., Tennant, D.R., Long, W.R., Chaisson, C.F., 1997. Evaluation of the budget method for screening food additive intakes. Food Add. Contamin. 14, 791–802.

Frisvad, J.C., Filtenborg, O., 1989. Terverticillate penicillia: chemotaxonomy and mycotoxin production. Mycologia 81, 837–861.

Frisvad, J.C., Thrane, U., 1987. Standardized high-performance liquid chromatography of 182 mycotoxins and other fungal metabolites based on alkylphenone retention indices and UV–Vis spectra (diode array detection). J. Chromatogr. 404, 195–214.

Frisvad, J.C., Thrane, U., 1993. Liquid column chromatography of mycotoxins. In: Betina, V. (Ed.), Chromatography of Mycotoxins. Techniques and Applications. Journal of Chromatography Library, vol. 54. Elsevier, Amsterdam, pp. 253–372.

Gill, R.T, Valdes, J.J., Bently, W.E., 2000. A comparative study of global stress gene regulation in response to overexpression of recombinant proteins in *Escherichia coli*. Metab. Eng. 2, 178–189.

Groot, G., Pel, H., van Ooyen, A.J.J., 2002. Exploring and exploiting the *Aspergillus niger* genome. SIM Annual Meeting abstracts S68, p. 77.

International Food Biotechnology Council (IFBC), 1990. Safety evaluation of foods and food ingredients derived from microorganisms in biotechnologies and food: assuring the safety of foods produced by genetic modification. Reg. Toxicol. Pharmacol. 12, S1–S196.

Joint FAO/WHO Expert Committee on Food Additives, 1975. Toxicological evaluation of some food colours, enzymes, flavour enhancers, thickening agents, and certain other food additives. WHO Food Additives Series 6, 124–128.

Joint FAO/WHO Expert Committee on Food Additives, 1988. Enzymes derived from *Aspergillus niger*. In Toxicological evaluation of certain additives. WHO Food Additives Series 22, 11–23.

Joint FAO/WHO Expert Committee on Food Additives, 1991. 3.1.2 Enzyme preparations. Enzyme preparations from *Aspergillus niger* + Annex 2. Toxicological evaluation of certain additives. Report 35th meeting JECFA. WHO Technical Report Series 789, pp. 15+44–46.

Joint FAO/WHO Expert Committee on Food Additives, 1992. General specifications for enzyme preparations used in food processing. Food and Nutrition Paper No 52/2, Annex 1.

Kessler, D.A., Taylor, M.R., Maryanski, J.H., Flamm, E.L., Kahl, L.S., 1992. The safety of foods developed by biotechnology. Science 256, 1747–1749, 1832.

Löwik, M.R.H., Hulshof, K.F.A.M., van der Heiijden, J.M., Brussaard, J.H., Burema, J., Kistemaker, C., de Vries, P.J.F., 1998. Changes in the diet in the Netherlands: 1987–1988 to 1992 (Dutch Nutrition Surveillance System). Int. J. Food Sci. Nutr. 49 (Suppl. 1), S1–S64.

Moss M.O., 1988. The occurrence and significance of mycotoxins. Report for JECFA dated September 1988.

Noordervliet, P.F., Toet, D.A., 1987. Safety in enzyme technology. In: Rehm, H.J., Reed, G. (Eds.), Biotechnology, vol. 7A. VCH Verlagsgesellschaft, Weinheim, Germany, pp. 711–741.

Pariza, M.W., Foster, E.M., 1983. Determining the safety of enzymes used in food processing. J. Food Prot. 46, 453–463.

Pariza, M.W., Johnson, E.A., 2001. Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. Reg. Toxicol. Pharmacol. 33, 1–14.

Patil, C., Walter, P., 2001. Intracellular signalling from the endoplasmic reticulum to the nucleus: the unfolded protein response in yeast and mammals. Curr. Opin. Cell. Biol. 13, 349–355.

Reed, G., 1975. Health and legal aspects of the use of enzymes. In: Reed, G. (Ed.), Enzymes in Food Processing, second ed. Academic Press, New York, pp. 549–554.

Röhr, M., Cubicek, C.P., Kominek, J., 1983. Citric acid. In: Rehm, H.J., Reed, G. (Eds.), Biotechnology, vol. 3. VCH Verlagsgesellschaft, Weinheim, Germany, pp. 419–454.

Schuster, E., Dunn-Coleman, N., Frisvad, J.C., van Dijck, P.W.M., 2002. On the safety of *Aspergillus niger*—a review. Appl. Microbiol. Biotechnol. 59, 426–435.

Scientific Committee for Food, 1992. Guidelines for the presentation of data on food enzymes. Report of the Scientific Committee for Food, 27th Report Series (EUR 14181 EN). Office for Official Publications of the EEC, pp. 13–22.

Selten, G.C.M, van Gorcom, R.F.M., Swinkels, B.W., 1995. Selection marker gene free recombinant strains: a method for obtaining them and the use of these strains. European Patent Application EPO0635574 A1.

Selten, G.C.M., Swinkels, B.W., Bovenberg, R.A.L., 1998. Gene conversion as a tool for the construction of recombinant industrial filamentous fungi. International Patent Application WO 98/46772.

Van Dijck, P.W.M., Spierenburg, H., Frisvad, J.C., Samson, R.A., 2002. The influence of strain improvement and genetic modification on the pattern of secondary metabolites in industrial strains of *Penicillium chrysogenum* and *Aspergillus niger*. Abstract 10th IUMS, Paris, June 27–July 1, M-60, p. 23.

Varga, J., Kevei, F., Hamari, Z., Tóth, B., Téren, J., Croft, J.H., Kozakiewiz, Z., 2000. Genotypic and phenotypic variability among black aspergilli. In: Samson, R.A., Pitt, J.I. (Eds.), Integration of Modern Taxonomic methods for Penicillium and Aspergillus Classification. Harwood Academic Publishers, Amsterdam, pp. 397–411.