Molecular and catalytic properties of phytate-degrading enzymes (phytases)

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Summary Phytate-degrading enzymes catalyse the step-wise release of phosphate from phytate, the principle storage form of phosphorus in plant seeds and pollen. They are widespread in nature, occurring in plants and micro-organisms, as well as in some animal tissues. Phytate-degrading enzymes have been studied intensively in recent years because of the great interest in such enzymes for reducing phytate content in animal feed and food for human consumption. Phytate-degrading enzymes are also of interest for producing defined breakdown products of phytate for kinetic and physiological studies. Certain *myo*-inositol phosphates have been proposed to have novel metabolic effects and therefore, the physiological role of different *myo*-inositol phosphates is presently undergoing extensive research. Generally, phytase behaves like a monomeric enzyme with molecular masses between 40 and 70 kDa. Up to now, two main types of phytate-degrading enzymes have been identified; acid phytate-degrading enzymes with an pH optimum around pH 5 and alkaline phytate-degrading enzymes with an pH optimum around pH 8. Most of the so far described phytate-degrading enzymes belong to the acidic type, and their optimal pH ranges from 4.5 to 6.0. This review summarises the molecular features as well as catalytic properties of phytate-degrading enzymes and also discusses enzymatic phytate degradation.

Keywords Animal tissue, micro-organism, phytate-degrading enzymes, plant, properties, purification, occurrence.

Introduction

Phytate [*myo*-inositol(1,2,3,4,5,6)hexakisphosphate] is degraded to *myo*-inositol pentakisphosphate and orthophosphate by enzymes that have been collectively called phytases [*myo*-inositol(1,2,3,4,5,6)hexakisphosphate phosphohydrolase (EC 3.1.3.8, EC 3.1.3.26)], but in no case, *myo*-inositol pentakisphosphate was identified as the final product of enzymatic phytate degradation. The classification is solely because of the *in vitro* capability of these enzymes to accept phytate as a substrate; the knowledge is rather limited about their *in vivo* function. This fact will be taken into consideration by replacing 'phytase' for 'phytate-degrading enzyme' throughout this review.

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Phytate-degrading enzymes have been studied intensively in recent years because of the great interest in such enzymes for reducing phytate content in animal feed and food for human consumption. Phytate-degrading enzymes were originally proposed as an animal feed additive to enhance the value of plant material in animal feed by liberating orthophosphate (Mitchell *et al.*, 1997). More recently, addition of phytate-degrading enzymes has also been seen as a way to reduce the level of phosphate pollution in areas of intensive lifestock management. A number of studies have already shown that addition of phytate-degrading enzymes enhances phosphate utilization from phytate and drastically reduces orthophosphate excretion (Cromwell *et al.*, 1995; Simons *et al.*, 1990). As phytate can act as an anti-nutrient by binding to proteins and by chelating minerals, such as zinc, iron, calcium

and magnesium (Cheryan, 1980), addition of phytate-degrading enzymes can improve the nutritional value of plant-based foods by enhancing protein digestibility and mineral availability through phytate hydrolysis during digestion in the stomach (Sandberg *et al*., 1996; Yi & Kornegay, 1996) or during food and feed processing (Reddy *et al*., 1989).

Phytate-degrading enzymes are also of interest for producing defined breakdown products of phytate for kinetic and physiological studies. The physiological role of different *myo*-inositol phosphates is presently undergoing extensive research. The effects observed with the different *myo*-inositol phosphate isomers seem to be very specific for a certain isomer. Certain *myo*-inositol phosphates have been proposed to have novel metabolic effects, such as amelioration of heart disease by controlling hypercholesterolaemia and atherosclerosis (Jariwalla *et al*., 1990; Potter, 1995), prevention of renal stone formation (Modlin, 1980; Ohkawa *et al*., 1984), and reduced risk of colon cancer (Baten *et al*., 1989; Ullah & Shamsuddin, 1990; Graf & Eaton, 1993; Vucenik *et al*., 1993; Yang & Shamsuddin, 1995; Shamsuddin *et al*., 1997). Furthermore, D-*myo*-inositol(1,2,6)trisphosphate has been studied with respect to the prevention of diabetes complications and treatment of chronic inflammations as well as cardiovascular diseases (Claxon *et al*., 1990; Ruf *et al*., 1991; Siren *et al*., 1991; Carrington *et al*., 1993). In addition, much attention has been focused on lower *myo*-inositol phosphates, in particular the intracellular second messengers D-*myo*-inositol(1,4,5)trisphosphate and D-*myo*-inositol(1,3,4,5)tetrakisphosphate, which affect cellular metabolism and secretion by stimulating intracellular release of calcium (Potter, 1990). Different phytate-degrading enzymes may result in different positional isomers of the lower *myo*-inositol phosphates and therefore in different physiological effects. After identifying individual phytate degradation products inducing physiological effects beneficial for health, the generating phytate-degrading enzymes may also find application in food processing to produce foods with improved nutritional value, health benefits and maintained sensory properties (functional foods).

Occurrence

Phytate-degrading enzymes are widespread in nature, occurring in plants, micro-organisms, as well as in some animal tissues. In barley (Greiner *et al*., 2000b), maize (Hübel & Beck, 1996; Maugenest *et al*., 1999), rice (Hayakawa *et al*., 1989), wheat (Nagai & Funahashi, 1962; Lim & Tate, 1971; Nakano *et al*., 1999), spelt (Konietzny *et al*., 1995), soybean (Hamada, 1996), rape seed (Houde *et al*., 1990), pumpkin (Goel & Sharma, 1979), lily (Baldi *et al*., 1988), as well as in *Aspergillus niger* (Hamada, 1994), *A. oryzae* (Fujita *et al*., 2000), *Escherichia coli* (Greiner *et al*., 1993), and *Saccharomyces cerevisiae* (Moore *et al*., 1995) multiple forms of phytate-degrading enzymes have been reported.

Micro-organisms

Microbial phytate-degrading activity is detected in numerous micro-organisms. Most of these micro-organisms produce only intracellular phytate-degrading enzymes. Extracellular phytate-degrading activity was observed nearly exclusively in filamentous fungi, whereby *A. niger* strains were identified as the best producers (Shieh & Ware, 1968; Gargova *et al*., 1997). It was also noted that many of these fungi exhibit intracellular phytate-degrading activity.

Phytate-degrading enzymes have also been detected in various bacteria, such as *Pseudomonas* sp. (Richardson & Hadobas, 1997), *Bacillus subtilis* (Powar & Jagannathan, 1982; Shimizu, 1992; Kerovuo *et al*., 1998), *B. amyloliquefaciens* (Kim *et al*., 1998a), *Klebsiella* sp. (Tambe *et al*., 1994; Greiner *et al*., 1997), *E. coli* (Greiner *et al*., 1993) and *Enterobacter* (Yoon *et al*., 1996). The only bacteria showing extracellular phytate-degrading activity are those of the genera *Bacillus* and *Enterobacter*. The phytate-degrading enzymes of *E. coli* have been reported to be periplasmatic enzymes. Furthermore, pure culture studies of anaerobic ruminal bacteria have clearly demonstrated phytate-degrading activity in numerous strains, particularly in *Selenomonas ruminantium*, *Megasphaera elsdenii*, *Prevotella* sp., and *Mitsuokella multiacidus* (Yanke *et al*., 1998). The phytate-degrading activity in *S. ruminantium* and

M. multiacidus was found to be associated with the outer membrane (D'Silva *et al.*, 2000).

In addition, microorganisms used in food fermentation have been investigated for their capability to degrade phytate during the fermentation process. Some yeast, such as *S. cerevisiae* (Nakamura *et al.*, 2000) and *Schwanniomyces castellii* (Lambrechts *et al.*, 1992), have been shown to produce intracellular as well as extracellular phytate-degrading enzymes. With lactic acid bacteria the results were inconsistent; a few strains seem to have a quite low phytate-degrading activity (Sreeramulu *et al.*, 1996; Zamudio *et al.*, 2001), but with the majority of strains the detection of phytate-degrading activity failed. Therefore, reduction in phytate content in plant-based foods during lactic acid fermentation might be because of co-precipitation of phytate and protein or activation of endogenous plant phytate-degrading enzymes as a result of lowering pH caused by lactic acid production (Shirai *et al.*, 1994).

Production of extracellular fungal phytate-degrading enzymes has been shown to be under tight regulatory inhibition by orthophosphate levels in the growth medium. This phenomenon was generally observed in all microbial producers of phytate-degrading enzymes with the exception of ruminal bacteria (Yanke *et al.*, 1998). With the majority of microorganisms it was demonstrated that the theoretically available level of phosphate may not be the only factor affecting the production of microbial phytate-degrading enzymes. For example, with submerged batch culture, the production of phytate-degrading enzymes in *A. niger* NRRL 3135 was inhibited by a high concentration of glucose and a low level of aeration (Shieh & Ware, 1968). In contrast to fungal phytate-degrading enzyme production, the synthesis of phytate-degrading enzymes in *B. subtilis* (Powar & Jagannathan, 1982) and *K. terrigena* (Greiner *et al.*, 1997) was induced by phytate in the cultivation medium and the synthesis of the periplasmatic phytate-degrading enzymes in *E. coli* was remarkably increased in the stationary phase and under anaerobic conditions (Greiner *et al.*, 1993).

To find optimal conditions for the production of phytate-degrading enzymes in a certain micro-organisms is therefore not an easy task; the regulation of production may differ from micro-organism to micro-organism. Thus, a failure to detect phytate-degrading activity does not necessarily imply that the microorganism under investigation is not a phytate-degrading enzyme producer at all, but that the culture conditions are disadvantageous for such a production. Furthermore, phytate was used only rarely as a test substrate during characterization of nonspecific phosphatases. Thus, some of these nonspecific phosphatases may exhibit phytate-degrading activity. Besides enzyme activity, isolation of putative genes encoding phytate-degrading enzymes by, for example, polymerase chain reaction (PCR) cloning using degenerate primers based on the protein sequences of several phytate-degrading enzymes has been shown suitable to identify a microorganism as a phytate-degrading enzyme producer (Mitchell *et al.*, 1997; Pasamontes *et al.*, 1997a, 1997b).

Plants

Phytate-degrading enzymes occur mostly in grains, seeds and pollen of higher plants, such as cereals, legumes, oilseeds and nuts, but a low phytate-degrading activity is also found in the roots of the plants. The phytate-degrading activity of grains, seeds and pollen has been shown to be responsible for phytate degradation during germination to make phosphate, minerals and *myo*-inositol available for the purpose of plant growth and development (Reddy *et al.*, 1989). Root phytate-degrading enzymes have been suggested as one of the mechanisms of plants to increase the availability of soil phosphorus, but in several studies it was shown that phytate appears to be only poorly utilized by plants (Hayes *et al.*, 2000). However, soil microorganisms producing extracellular phytate-degrading activity such as *Bacillus* sp. and *Enterobacter* could be significant factors in making phytate phosphorus available to the plant.

The highest phytate-degrading activities have been reported in cereals (rye, triticale, wheat, barley) and in pseudo-cereals (buckwheat, amaranth). In general, legumes and oilseeds exhibit a 10-fold lower phytate-degrading activity. Optimal pH value for phytate degradation was estimated to be about 5.0. In addition, a second optimum at about pH 8.0 was reported in legumes (Scott, 1991). In general, legumes exhibit a lower activity at pH 8.0 compared with pH 5.0.

Little information is available on the localization of phytate-degrading enzymes in plant grains, seeds and pollen. In cereals phytate-degrading activity was found to be mainly associated with the aleurone layer (Gabard & Jones, 1986), in legumes phytate-degrading activity was reported to be in the cotyledons (Gibson & Ullah, 1988; Hegeman & Grabau, 2001). Furthermore, the extraction of phytate-degrading activity is strongly enhanced by the presence of Triton X-100, suggesting at least an association with membrane structures partly (Scott & Loewus, 1986; Scott, 1991; Greiner & Egli, 2001).

Several molecular forms of phytate-degrading enzymes, which may be regulated in different ways, have been identified in a certain plant. Grains, seeds and pollen contain both constitutive and germination-inducible phytate-degrading enzymes (Lin *et al*., 1987; Greiner *et al*., 2000b). Although large increase in phytate-degrading activities have been extensively reported in germinating seeds as well as in germinating pollen, the biochemical mechanism leading to this rise in phytate-degrading activity is not well understood. In pollen, it was suggested that phytate-degrading enzymes induced during germination may be synthesized from long-lived, pre-existing messenger RNA (mRNA) (Jackson & Linskens, 1982; Lin *et al*., 1987). In cereals and legumes, the cause of the rise in phytate-degrading activity during germination is also in dispute. Some studies suggest *de novo* synthesis (Bianchetti & Sartirana, 1967), others simply suggest activation of pre-existing enzymes as the cause of the rise in phytate-degrading activity (Eastwood & Laidman, 1971).

Various levels of controlling phytate degradation in the germinating seed have been discussed. During germination phytate-degrading activity may be controlled by the action of gibberellic acid and phosphate, respectively. The action of gibberellic acid on the phytate-degrading enzymes of the germinating seed is not well understood. It is claimed that gibberellic acid merely increase the secretion of phytate-degrading enzymes but does not stimulate their synthesis (Gabard & Jones, 1986), thus giving phytate-degrading enzymes access to phytate, and that gibberellic acid stimulates phytate-degrading activity (Srivastava, 1964). Two main mechanisms appear to be involved in the regulation of phytate-degrading activity by phosphate. Acid phytate-degrading enzymes are strongly inhibited by phosphate, thus the enzyme activity itself may be controlled by phosphate. It was concluded that phosphate also acts at the transcription level (Sartirana & Bianchetti, 1967), as phosphate added early enough in the germination sequence can repress the increase in phytate-degrading activity.

Animal tissue

In comparison with the phytate-degrading enzymes of microorganisms and plants, investigation of animal phytate-degrading enzymes is very limited. The first report on animal phytate-degrading enzymes, found in liver and blood of calves, was given in 1908 (McCollum & Hart, 1908). However, further search for phytate-degrading activity in blood of mammals was unsuccessful; it was detected only in the blood of lower vertebrates, such as birds, reptiles and fishes (Rapoport *et al*., 1941).

Because dietary phytate has adverse nutritional consequences for animals including man, the presence of phytate-degrading activity in the gastrointestinal tract of various animals was investigated. Phytate-degrading activity has been detected in the mucosa extracts of the small intestine of rats, rabbits, guinea-pigs, chicken, calves and humans (Bitar & Reinhold, 1971; Cooper & Gowing, 1983; Iqbal *et al*., 1994). On the one hand, phytate-degrading activity of the mammalian intestine has been attributed to the action of nonspecific intestinal alkaline phosphatase (Davies & Motzok, 1972; Davies & Flett, 1978); on the other hand, it was suggested that intestinal alkaline phosphatase and intestinal phytate-degrading enzyme are different proteins (Bitar & Reinhold, 1971). However, intestinal phytate-degrading activity does not play a significant role in digestion of dietary phytate in monogastrics (Iqbal *et al*., 1994), but dietary phytate-degrading enzymes have been shown to be an important factor (Lantsch *et al*., 1992). Degradation of phytate in monogastrics is also caused by the microbial flora of the large intestine. An increased degradation of dietary phytate during digestion in monogastrics could be obtained by supplementing food and feed with phytate-degrading enzymes

(Simons *et al*., 1990; Cromwell *et al*., 1995). Phytate-degrading activity produced by anaerobic ruminal bacteria is probably responsible for the increased rate of phytate degradation in ruminants (Yanke *et al*., 1998).

Properties

Phytate-degrading enzyme could be found in various sources and is considered to be a special kind of phosphatase capable of releasing orthophosphate from phytate as well as other diversified phosphorylated compounds. It has distinct aspects in molecular features as well as catalytic properties depending on the source of origin. Only a few phytate-degrading enzymes have been purified to homogeneity or near homogeneity (Table 1).

Purification of phytate-degrading enzymes includes common biochemical techniques such as ammonium sulphate fractionation, acetone precipitation, gel filtration, ion-exchange chromatography, affinity chromatography and hydrophobic interaction. One major problem in the purification of phytate-degrading enzymes especially from plants is the separation of phytate-degrading enzymes from contaminating nonspecific acid phosphatases (Konietzny *et al*., 1995). In addition, purification of the enzymes from plant sources involves extended germination times. The limited stability in comparison with the microbial enzymes further renders purification of phytate-degrading enzymes from plant sources more difficult.

Microbial extracellular phytate-degrading enzymes have been easily obtained from the culture filtrate in rather high yields. To purify the phytate-degrading enzyme from *A. niger* NRRL 3135 a three-step protocol has been proposed, including ion-exchange chromatography and chromatofocusing. The enzyme was purified 22-fold with a recovery of 58% (Ullah & Gibson, 1987).

The two periplasmatic phytate-degrading enzymes from *E. coli* have been purified over 16 000-fold with a recovery of 7 and 18%, respectively, using a five-step purification protocol, including ammonium sulphate fractionation, ion-exchange chromatography and hydrophobic interaction (Greiner *et al*., 1993).

The purification of a phytate-degrading enzyme from oat (Greiner & Larsson-Alminger, 1999) and faba beans (Greiner *et al*., 2001b), respectively, was achieved using a seven-step purification protocol, including ammonium sulphate fractionation, acetone precipitation, ion-exchange chromatography and gel filtration. The enzyme from oat was

Table 1 Phytate-degrading enzymes purified to homogeneity or near homogeneity

Phytase source	Localization	Reference
Aspergillus niger	Extracellular	Ullah (1988a); Ullah & Gibson (1987)
Aspergillus terreus	Extracellular	Yamada *et al*. 1968
Aspergillus oryzae	Extracellular	Shimizu (1993)
Bacillus subtilis	Extracellular	Kerovou *et al*. (1998); Powar & Jagannathan (1982); Shimizu (1992)
Bacillus amyloliquefaciens	Extracellular	Kim *et al*. (1998a)
Escherichia coli	Periplasmatic	Greiner *et al*. (1993)
Klebsiella sp.	Cytoplasmatic	Greiner *et al*. (1997); Tambe *et al*. (1994)
Schwanniomyces castellii	Extracellular	Segueilha *et al*. (1992)
Penicillium simplicissimum	Extracellular	Tseng *et al*. 2000
Phaseolus aureus (mung bean)	Cotyledon	Maiti *et al*. (1974); Mandel *et al*. (1972)
Glycine max. (soybean)	Cotyledon	Gibson & Ullah (1988); Hegeman & Grabau (2001)
Zea mays (maize)	Seed, root	Hübel & Beck (1996); Laboure *et al*. (1993)
Lycopersicon esculentum (tomato)	Root	Li *et al*. 1997
Triticum spelta (spelt)	Seed	Konietzny *et al*. (1995)
Secale cereale (rye)	Seed	Greiner *et al*. (1998)
Hordeum vulgare (barley)	Seed	Greiner *et al*. (2000b)
Triticum aestivum (wheat)	Bran	Nakano *et al*. (1999)
Avena sativa (oat)	Seed	Greiner & Larsson Alminger (1999)
Vicia faba (faba bean)	Seed	Greiner *et al*. (2001b)
Lupinus albus (lupine)	Seed	Greiner (2001)
Allium fistulosum (scallion)	Leaves	Phillippy (1998)
Rat	Intestine	Yang *et al*. (1991b)

purified 5380-fold with a recovery of 23%, and that of faba beans 2190-fold with a recovery of 6%.

A six-step protocol, including butanol extraction, ethanol precipitation, ion-exchange chromatography and gel filtration, was used for the purification of the intestinal phytate-degrading enzyme of the rat. A 1136-fold purification with a recovery of 19% was achieved (Yang *et al*., 1991b).

Recently some microbial phytate-degrading enzymes have been accessible not by extraction and purification of the enzymes from the wild-type organisms, but by cloning and heterologously expressing the corresponding genes. The recombinant phytate-degrading enzymes from *A. terreus* (Wyss *et al*., 1999a, 1999b), *A. fumigatus* (Pasamontes *et al*., 1997b; Wyss *et al*., 1998; Wyss *et al*., 1999a, 1999b), *Emericella nidulans* (Wyss *et al*., 1999a, 1999b) as well as from the thermophilic fungi *Thermomyces lanuginosus* (Berka *et al*., 1998), *Myceliophthora thermophila*, and *Talaromyces thermophilus* (Wyss *et al*., 1999a, 1999b) have been biochemically characterized.

Phytate-degrading activity can be determined by measuring the orthophosphate liberated by the action of the enzyme using a method based upon colorimetric measurements of phosphomolybdate in acetone (Heinonen & Lahti, 1981). To demonstrate phytate-degrading activity the phytate preparation used for such an assay has to be free of lower *myo*-inositol phosphates or other phosphorylated compounds. As the enzymatic hydrolysis of phytate is a stepwise process in which each lower *myo*-inositol phosphate may become a substrate for further hydrolysis, the release of orthophosphate has to be limited. In the assay commonly used, the release of orthophosphate is limited to at maximum 4% of the totally available phytate phosphate (Konietzny *et al*., 1995); thus, dephosphorylation of lower *myo*-inositol phosphates in the assay mixtures by the phytate-degrading enzyme under investigation is to be excluded.

Recently, reverse phase C18 high-performance liquid chromatography (HPLC) has been developed for separation and quantitative determination of phytate and lower *myo*-inositol phosphates (Skoglund *et al*., 1997). This method is capable of detecting directly the reduction in phytate during enzymatic hydrolysis, but with respect to the determination of phytate-degrading activity the method is too time-consuming and significant amounts of lower *myo*-inositol phosphates in the assay mixtures have been generated until the reduction in phytate could be quantified with adequate accuracy.

Molecular properties

Molecular mass

Table 2 shows the molecular masses and the number of subunits of phytate-degrading enzymes from various sources. The majority of the so far characterized phytate-degrading enzymes behave like monomeric proteins with molecular masses between 40 and 70 kDa. However, some phytate-degrading enzymes appear to be built up of multiple subunits. The phytate-degrading enzyme from *Schwanniomyces castellii* was identified as a tetrameric protein with one large subunit (M_r 125 kDa) and three identical small subunits (M_r 70 kDa) (Segueilha *et al*., 1992) and that of the rat intestine was suggested to be an heterodimer consisting of 70- and 90-kDa subunit (Yang *et al*., 1991b). As both subunits were expressed differentially during the development of the rat (Yang *et al*., 1991a), these subunits may represent two different enzymes. The phytate-degrading enzymes isolated from maize roots (Hübel & Beck, 1996), germinating maize seeds (Laboure *et al*., 1993), tomato roots (Li *et al*., 1997b), soybean seeds (Hegeman & Grabau, 2001) and *A. oryzae* (Shimizu, 1993) were reported to be homodimeric proteins, whereas a homohexameric structure was proposed for the *A. terreus* enzyme (Yamamoto *et al*., 1972). In other studies there was no indication of an oligomeric structure of the phytate-degrading enzymes from *A. terreus* (Wyss *et al*., 1999b) and soybeans (Gibson & Ullah, 1988). This conflicting observation was suggested to be the result of the use of gel filtration or native PAGE (polyacrylamide gel electrophoresis) for molecular mass determination. As recently shown these methods overestimate the molecular mass and the error increases with the extent of glycosylation (Table 1) (Wyss *et al*., 1999b). Thus, the conclusion of the existence of oligomeric phytate-degrading enzymes should be regarded with caution at least until there is more convincing evidence that oligomeric forms occur.

Table 2 Molecular properties of phytate-degrading enzymes

Phytase source	M_r (Da) from sequence data	M_r (Da) from SDS–PAGE	M_r (Da) from gel filtration	Number of subunits	References
A. niger	48 423–48 973	66 360–100 000	82 360–103,030	1	Ullah & Gibson (1987); Wyss *et al.* (1999b)
A. terreus	48 189–49 166	60 550–82 100	79 900–115 100; 214 000	1, 6	Wyss *et al.* (1999b); Yamamoto *et al.* (1972)
A. fumigatus	48 270	60 000–76 000	90 500	1	Pasamontes *et al.* (1997b); Rodriguez *et al.* (2000); Wyss *et al.* (1999b)
A. oryzae	–	60 000	–	2	Shimizu (1993)
E.nidulans	49 034–49 360	66 430	77 850–86 930	1	Wyss *et al.* (1999b)
M. thermophila	50 524	62 890	73 800	1	Wyss *et al.* (1999b)
T. thermophilus	49 775	128 400	–	1	Wyss *et al.* (1999b)
T. lanuginosus	51 000	60 000	–	1	Berka *et al.* (1998)
P. simplicissimum	–	65 000–67 000	65 000	1	Tseng *et al.* (2000)
S. castellii	–	70 000; 125 000	490 000	3 + 1	Segueilha *et al.* (1992)
E. coli	45 846	42 000–47 270	39 190–43 000	1	Greiner *et al.* (1993)
K. terrigena	–	40 000	40 000	1	Greiner *et al.* (1997)
K. aerogenes	–	–	700 000; (10 000–13 000)	1	Tambe et al. (1994)
B. subtilis	–	38 000–42 000	36 000	1	Kerovuo *et al.* (1998); Shimizu (1992)
B. amyloliquefaciens	–	44 000	40 000	1	Kim *et al.* (1998a) phytase source
Wheat PHY1	–	68 000	71 000	1	Nakano *et al.* (1999)
Wheat PHY2	–	66 000	66 000	1	Nakano *et al.* (1999)
Spelt D21	–	68 000	65 000	1	Konietzny *et al.* (1995)
Rye	–	68 000	67 000	1	Greiner *et al.* (1998)
Oat	–	68 000	67 000	1	Greiner *et al.* 1999
Barley P1	–	66 000	67 000	1	Greiner *et al.* (2000b)
Barley P2	–	66 000	67 000	1	Greiner *et al.* (2000b)
Maize root	–	38 000	71 000	2	Hübel & Beck (1996)
Maize seedling	–	38 000	76 000	2	Laboure *et al.* (1993)
Soybean	–	59 000–60 000	50 000	1	Gibson & Ullah (1988)
	–	70 000–72 000	130 000	2	Hegeman & Grabau (2001)
Mung bean	–	–	158 000	1	Maiti *et al.* (1974)
Scallion leaves	–	72 000	–	1	Phillippy (1998)
Faba bean	–	67 000	65 000	1	Greiner *et al.* (2001b)
Lupine L11	–	57 000	58 000	1	Greiner (2001)
Lupine L12	–	57 000	58 000	1	Greiner (2001)
Lupine L2	–	64 000	65 000	1	Greiner (2001)
Tomato root	–	82 000	164 000	2	Li *et al.* (1997b)
Rat intestine	–	70 000; 90 000	–	1 + 1	Yang *et al.* (1991b)

Two different forms of phytate-degrading enzymes have been reported in *K. aerogenes* (Tambe *et al.*, 1994). One, possibly the native enzyme, has an exceptionally large size (700 kDa), whereas the other, probably a fraction of the native enzyme, exhibits an exceedingly small molecular mass (10–13 kDa) but a full complement of activity.

Fungal and several plant phytate-degrading enzymes have been found to be glycosylated. The N-linked mannose and galactose of native enzyme from *A. niger* NRRL 3135 account for 27.3% of its molecular mass (Ullah, 1988a). Glycosylation may have an effect on the catalytic properties, the stability or the isoelectric point of an enzyme. Surprisingly, different extents of glycosylation did not have any effect on the thermostability and refolding properties of the phytate-degrading enzymes from *A. fumigatus* and *A. niger* (Wyss *et al.*, 1999b). However, a complete

deglycosylation of the phytate-degrading enzyme from *A. niger* resulted in a 34% reduction in thermostability (Han *et al.*, 1999).

Isoelectric point

All of the fungal, bacterial and plant phytate-degrading enzymes investigated so far have acidic pI values with the exception of the *A. fumigatus* enzyme, which has a pI of about 8.6 (Table 3) (Wyss *et al.*, 1999b).

pH, temperature and protease stability

In general, phytate-degrading enzymes of microbial origin are more pH and thermostable than their plant counterparts. The stability of most of the plant enzymes decreased dramatically at pH values below 4 and above 7.5, whereas the majority of the corresponding microbial enzymes are rather stable even at pH values above 8.0 and below 3.0. For example, the phytate-degrading enzyme from *E. coli* did not lose any activity at

Table 3 Catalytic properties of phytate-degrading enzymes: part 1

phytase source	pI	pH-optimum	Temperature optimum (°C)	specific activity at 37 °C (U mg^{-1})	References
A. niger	4.5–5.2	2.2, 5.0–5.5	55–58	50–103	Ullah & Gibson (1987); Wyss *et al.* (1999a, b)
A. terreus	5.0	5.0–5.5	70	142–196	Wyss *et al.* (1999a, b); Yamada *et al.* (1968);
A. fumigatus	8.6	5.0–6.0	60	23–28	Pasamontes *et al.* (1997b); Rodriguez *et al.* (2000); Wyss *et al.* (1999a, b)
A. oryzae	4.2	5.5	50	11	Shimizu (1993)
E. nidulans	5.3	6.5	–	29–33	Wyss *et al.* (1999a, b)
M. thermophila	4.8	5.5	–	42	Wyss *et al.* (1999a, b)
T. lanuginosus	5.4	6.0	65	110	Berka *et al.* (1998)
P. simplicissimum	5.8	4.0	55	3	Tseng *et al.* 2000
S. castellii	–	4.4	77	418 (70 °C)	Segueilha *et al.* (1992)
E. coli	6.0–7.4	4.5	55–60	811–1800	Golovan *et al.* (2000); Greiner *et al.* (1993); Wyss *et al.* (1999a, b)
K. terrigena	5.5	5.0	58	205	Greiner *et al.* (1997)
K aerogenes	3.7, 10.5	4.5, 5.2	68	–	Tambe *et al.* (1994)
B. subtilis	6.3–6.5	6.0–7.5	55–60	9–15	Kerovuo *et al.* (1998); Shimizu (1992)
B. amyloliquefaciens	–	7.0–8.0	70	20	Kim *et al.* (1998a)
Wheat PHY1	–	6.0	45	127	Nakano *et al.* (1999)
Wheat PHY2	–	5.0	50	242	Nakano *et al.* (1999)
Spelt D21	–	6.0	45	262	Konietzny *et al.* (1995)
Rye	–	6.0	45	517	Greiner *et al.* (1998)
Oat	–	5.0	38	307	Greiner *et al.* 1999
Barley P1	–	5.0	45	117	Greiner *et al.* (2000b)
Barley P2	–	6.0	55	43	Greiner *et al.* (2000b)
Maize root	4.8–5.0	5.0–5.1	35–40	5.7	Hübel & Beck (1996)
Maize seedling	–	4.8	55	2.3	Laboure *et al.* (1993)
Soybean	5.5	4.5–4.8	55	2.4	Gibson & Ullah (1988),
	–	4.5–5.0	57	–	Hegeman & Grabau (2001)
Mung bean	–	7.5	58	0.5	Mandal *et al.* (1972)
Scallion leaves	–	5.5	57	500	Phillippy (1998)
Faba bean	–	5.0	51	636	Greiner *et al.* (2001b)
Lupine L11	–	5.0	50	539	Greiner (2001)
Lupine L12	–	5.0	50	607	Greiner (2001)
Lupine L2	–	5.0	50	498	Greiner (2001)
Tomato root	5.5	4.3	50	205	Li *et al.* (1997b)
Rat intestine	–	7.0, 7.5–8.0	45	–	Yang *et al.* (1991b)

pH 2.0 and 10.0 when exposed at 4 °C for 2 h (Greiner *et al*., 1993). In purified form, most of the phytate-degrading enzymes from plants have been irreversibly inactivated at temperatures above 70 °C within minutes, whereas most of the corresponding microbial enzymes retain significant activity even after prolonged incubation times. The phytate-degrading enzymes most resistant to high temperatures reported so far have been isolated from *A. fumigatus* (Pasamontes *et al*., 1997b) and *S. castellii* (Segueilha *et al*., 1992). Incubation of the *A. fumigatus* enzyme at 90 °C for 20 min resulted in only 10% loss of the initial activity. It was shown that the *A. fumigatus* enzyme is not thermostable, but had the remarkable property of being able to refold completely into native-like, fully active conformation after heat denaturation (Wyss *et al*., 1998).

For a technical application of phytate-degrading enzymes in feed and food processing it is of practical interest that a crude enzyme preparation as well as an enzyme present in the feed or food matrix is more heat resistant than the corresponding highly purified enzyme.

The effectiveness and limitations of food and feed supplementation with phytate-degrading enzymes may also depend on their sensitivity to protease digestion. It was shown that the phytate-degrading enzyme from *A. niger* was more stable in the presence of pepsin or pancreatin than the corresponding enzyme from wheat (Phillippy, 1999). Furthermore, the *Aspergillus* enzyme is more resistant to trypsin and less resistant to pepsin and pancreatin than the phytate-degrading enzyme from *E. coli* (Rodriguez *et al*., 1999b). Compared with the phytate-degrading enzyme from *E. coli*, the corresponding enzymes from *Bacillus* exhibit a similar sensitivity to pancreatin, but a much higher susceptibility to pepsin (Igbasan *et al*., 2000).

Protein sequence/primary structure

The primary sequences of several fungal phytate-degrading enzymes have been determined in recent years. The amino acid sequences of the phytate-degrading enzymes from *A. niger* var. awamori (Piddington *et al*., 1993), *A. fumigatus* (Pasamontes *et al*., 1997b), *A. terreus* (Mitchell *et al*., 1997), *Emericella nidulans*, *Talaromyces thermophilus* (Pasamontes *et al*., 1997a), *Thermomyces lanuginosus* (Berka *et al*., 1998), and *Myceliophthora thermophila* (Mitchell *et al*., 1997) exhibited 97, 66, 60, 61, 48 and 47% homology to the corresponding enzyme from *A. niger* NRRL 3135, whereas the bacterial phytate-degrading enzyme from *E. coli* did not show apparent sequence similarity (Rodriguez *et al*., 1999a). However, all this phytate-degrading enzymes share the highly conserved sequence motif RHGxRxP considered to be the phosphate acceptor site near the N-terminus. In addition, they contain near the C-terminus a conserved HD-motif where the aspartate is proposed to be the proton donor for the substrate leaving group. The amino acid sequence of the phytate-degrading enzymes from maize seedling appears to be very different from one of the corresponding enzyme from *A. niger*, however, a homologous region of 33 amino acids including the RHGxRxP-motif was identified (Maugenest *et al*., 1997). All these phytate-degrading enzymes belong to the subfamily of histidine acid phosphatases (Mitchell *et al*., 1997).

The amino acid sequence of the phytate-degrading enzymes from *B. amyloliquefaciens* (Kim *et al*., 1998b) and *B. subtilis* (Kerovuo *et al*., 1998) do not have homology to the sequences of any other phosphatase listed in the databases. Even the putative active site motifs RHGxRxP and HD found in histidine acid phosphatases are absent. Thus, the *Bacillus* enzymes represent a novel class of phosphatases.

Recently, it was reported that a phytate-degrading enzyme from soybeans also lacks the RHGxRxP-motif (Hegeman & Grabau, 2001). Instead, this enzyme contains motif characteristics of a large group of phosphoesterases, including purple acid phosphatases. Purple acid phosphatases contain binuclear Fe(III)-Me(II) centres where Me is Fe, Mn or Zn.

Crystal structure

Recently, the crystal structure of the phytate-degrading enzymes from *A. niger* (Kostrewa *et al*., 1997), *E. coli* (Lim *et al*., 2000), and *B. amyloliquefaciens* (Ha *et al*., 2000) have been determined. The structure of the *A. niger* and *E. coli* enzyme closely resembles the overall fold of other histidine acid phosphatases. These structures contain a conserved α/β-domain and a variable α-domain. The active site is located at the interface between

the two domains. Differences in substrate binding have been attributed to differences in the α-domain. The proposed structures also provides information about substrate binding and the catalytic mechanism on the molecular level. For the *E. coli* enzyme it was shown that the scissile phosphate is co-ordinated by the two arginine residues of the RHGxRxP-motif, as well as by conserved residues downstream, a further arginine residue and the histidine and aspartate residue of the HD-motif. Furthermore, the histidine residue of the RHGxRxP-motif was shown to be oriented for nucleophilic attack.

The structure of the phytate-degrading enzyme from *B. amyloliquefaciens*, a six-bladed propeller, represents a novel scaffold for phosphatases (Shin *et al.*, 2001). Binding of two calcium ions to high-affinity calcium binding sites results in a dramatic increase in thermostability by joining loop segments remote in the amino acid sequence. Binding of three additional calcium ions to low-affinity calcium binding sites at the top of the molecule turns on the catalytic activity of the enzyme by converting the highly negatively charged cleft into a favourable environment for the binding of phytate. The structure also explains the catalytic mechanism and the degradation pathway of phytate.

Catalytic properties

pH and temperature optimum

Until now, two main types of phytate-degrading enzymes have been identified, acid phytate-degrading enzymes with an pH optimum around pH 5 and alkaline phytate-degrading enzymes with an pH optimum around pH 8. Most of the so far described phytate-degrading enzymes belong to the acidic ones and their pH optima range from 4.5 to 6.0 (Table 3). In contrast to most other phytate-degrading enzymes, the one from *A. fumigatus* has a broad pH optimum; at least 80% of the maximal activity is observed at pH values between 4.0 and 7.3 (Wyss *et al.*, 1999a). Some bacterial phytases, especially those from *Bacillus*, have a pH optimum at 6.0–8.0 (Powar & Jagannathan, 1982; Shimizu, 1992; Kerovuo *et al.*, 1998; Kim *et al.*, 1998a). Alkaline phytate-degrading enzymes having a pH optimum at *c.* 8.0 have also been described in legume seeds (Scott, 1991), pollen of lily (Baldi *et al.*, 1988) and cattail (Hara *et al.*, 1985) as well as the rat intestine (Yang *et al.*, 1991b).

The temperature optima of phytate-degrading enzymes vary from 35 to 77 °C (Table 3). In general, phytate-degrading enzymes from plants exhibit maximum activity at lower temperatures compared with their microbial counterparts. The optimum temperature of the phytate-degrading enzymes from cereals have been reported as the lowest optimal temperature recorded among phytate-degrading enzymes from different sources.

The Arrhenius activation energy for the hydrolysis of sodium phytate by the phytate-degrading enzymes from different sources ranges from 25 to 53 kJ mol^{-1} (Table 4).

Effectors of enzyme activity

Metal ions have been shown to modulate phytate-degrading activity, but it is difficult to determine whether the inhibitory effect of various metal ions is the result of binding to the enzyme or the formation of poorly soluble metal ion-phytate complexes. However, the appearance of a precipitate while adding Fe^{2+} or Fe^{3+} to the assay mixtures suggests a decrease of the active substrate concentration by the formation of poorly soluble iron-phytate (Konietzny *et al.*, 1995). Most phytate-degrading enzymes characterized so far are greatly inhibited by Cu^{2+} and Zn^{2+}.

With the exception of the phytate-degrading enzyme from *A. fumigatus*, ethylene diamine tetraacetic acid (EDTA) had no major effects on the enzymatic activity of the other acid phytate-degrading enzymes. The enzyme of *A. fumigatus* was stimulated up to 50% in the presence of EDTA (Wyss *et al.*, 1999a), whereas alkaline phytate-degrading enzymes were greatly inhibited by EDTA (Hara *et al.*, 1985; Scott & Loewus, 1986; Shimizu, 1992; Kim *et al.*, 1998a; Kerovuo *et al.*, 2000a), indicating that a metal ion is needed for optimal activity. The enzymatic activity of the phytate-degrading enzymes of *B. subtilis* (Shimizu, 1992; Kerovuo *et al.*, 2000a), *B. amyloliquefaciens* (Kim *et al.*, 1998a), lily pollen (Scott & Loewus, 1986) and cattail pollen (Hara *et al.*, 1985) was reported to be Ca^{2+}-dependent.

Reducing reagents, such as 2-mercaptoethanol, dithiotreitol and reduced glutathione have no major effect on the enzymatic activity of phytate-degrading enzymes. This suggests that these

Table 4 Catalytic properties of phytate-degrading enzymes: part 2

Phytase source	K_M (μmol)	k_{cat} [s^{-1}]	*A* (kJ/mol)	Substrate specificity	References
A. niger	10–40	216	–	Rather specific	Ullah (1988b); Wyss *et al.* (1999a)
A. terreus	11–23	–	–	Rather specific	Wyss *et al.* (1999a)
A. fumigatus	<10	114	–	Broad	Pasamontes *et al.* (1997b); Rodriguez *et al.* (2000); Wyss *et al.* (1999a)
A. oryzae	330	–	43.2	Broad	Shimizu (1993)
E. nidulans	–	–	–	Broad	Wyss *et al.* (1999a)
M. thermophila	–	–	–	Broad	Wyss *et al.* (1999a)
T. lanuginosus	110	–	–	–	Berka *et al.* (1998)
P. simplicissimum	–	–	–	Broad	Tseng *et al.* 2000
S. castellii	38	–	39.3	Broad	Segueilha *et al.* (1992)
E. coli	130–630	1744	58.5	Rather specific	Golovan *et al.* (2000); Greiner *et al.* (1993); Wyss *et al.* (1999a)
K. terrigena	300	180	35.0	Middle	Greiner *et al.* (1997)
K aerogenes	62, 114	–	37.8	–	Tambe *et al.* (1994)
B. subtilis	50–500	–	48.6	Rather specific	Kerovuo *et al.* (1998); Shimizu (1992)
B. amyloliquefaciens	550	–	–	Rather specific	Kim *et al.* (1998a) phytase source
Wheat PHY1	0.48	–	–	Middle	Nakano *et al.* (1999)
Wheat PHY2	0.77	–	–	Middle	Nakano *et al.* (1999)
Spelt D21	400	368	52.9	Broad	Konietzny *et al.* (1995)
Rye	300	358	26.6	Broad	Greiner *et al.* (1998)
Oat	30	356	25.1	Broad	Greiner *et al.* 1999
Barley P1	72	136	27.5	Broad	Greiner *et al.* (2000b)
Barley P2	190	<10	42.0	Broad	Greiner *et al.* (2000b)
Maize root	24–43	<10	–	–	Hübel & Beck (1996)
Maize seedling	117	<10	–	–	Laboure *et al.* (1993)
Soybean	48	–	35.3	–	Gibson & Ullah (1988)
	61	–	–	Broad	Hegeman & Grabau (2001)
Mung bean	650	–	–	Broad	Mandal *et al.* (1972)
Scallion leaves	200	–	–	Broad	Phillippy (1998)
Faba bean	148	704	–	Broad	Greiner *et al.* (2001b)
Lupine L11l	80	523	–	Broad	Greiner (2001)
Lupine L12	300	589	–	Broad	Greiner (2001)
Lupine L2	130	533	–	Broad	Greiner (2001)
Tomato root	38	–	–	Broad	Li *et al.* (1997b)
Rat intestine	210	–	–	Broad	Yang *et al.* (1991b)

enzymes either do not have any free and accessible sulphydryl group or that free sulphydryl groups play a negligible role in enzyme activity and structure. This interpretation is supported by the fact that most mature microbial phytate-degrading enzymes have an even number of cysteine residues which might be implicated in disulphide bridges as shown for the phytate-degrading enzymes from *A. niger* (Kostrewa *et al.*, 1997) and *E. coli* (Lim *et al.*, 2000). Furthermore, from unfolding studies it was concluded that the function of disulphide bonds in the phytate-degrading enzyme from *A. niger* are necessary for the structure and activity of the enzyme and play an important role in the folding of the protein (Ullah & Mullaney, 1996). The phytate-degrading enzymes from *B. subtilis* (Kerovuo *et al.*, 1998) and *B. amyloliquefaciens* (Kim *et al.*, 1998b) appear not to have cysteine residues.

Fluoride, a well known inhibitor of different acid phosphatase, was also found to be a strong competitive inhibitor of several acid bacterial, fungal and plant phytate-degrading enzymes. The reported inhibitor constants range from 0.1 to

0.5 mM. In contrast, the alkaline phytate-degrading enzymes from *B. subtilis* (Powar & Jagannathan, 1982), *B. amyloliquefaciens* (Kim *et al.*, 1998a), and lily pollen (Baldi *et al.*, 1988) did not show any reduction in activity in the presence of fluoride. Furthermore, the hydrolysis product orthophosphate was recognized as a competitive inhibitor of enzymatic phytate degradation. Molybdate, wolframate and vanadate are also known to inhibit phytate-degrading enzymes. It has been suggested that these transition metal oxoanions exert their inhibitory effects by forming complexes that resemble the trigonal bipyramidal geometry of the transition state (Zhang *et al.*, 1997).

Besides the hydrolysis product phosphate, the substrate phytate was also reported to act as an inhibitor of many phytate-degrading enzymes. The lowest phytate concentration necessary to inhibit phytate-degrading activity ranges from 300 μM for the maize root enzyme (Hübel & Beck, 1996) up to 20 mM for the soybean enzyme (Gibson & Ullah, 1988). With high substrate concentrations, the charge because of the phosphate groups may affect the local environment of the catalytic domain of the enzyme. This might inhibit conversation of the enzyme-substrate complex to enzyme and product, although inhibition because of the formation of poorly soluble protein-phytate complexes cannot be ruled out.

Substrate specificity and kinetic parameters

Phytate-degrading enzymes usually show a broad substrate specificity (Table 4). Adenosine monophosphate (AMP), Adenosine diphosphate (ADP), Adenosine triphosphate (ATP), guanosine mono phosphate (GMP), guanosine triphosphate (GTP), nicotinamide–adenine dinucleotide phosphate (NADP), p-nitrophenyl phosphate, phenyl phosphate, 1-naphthyl phosphate, 2-naphthylphosphate, fructose 1,6-diphosphate, fructose 6-phosphate, glucose 1-phosphate, glucose 6-phosphate, galactose 1-phosphate, α-glycerophosphate, β-glycerophosphate, pyridoxalphosphate, o-phospho-L-serine, and pyrophosphate are frequently hydrolysed. Only a few phytases have been described as highly specific for phytate such as the alkaline phytate-degrading enzymes from *B. subtilis* (Powar & Jagannathan, 1982; Shimizu, 1992), *B. amyloliquefaciens* (Kim *et al.*, 1998a), lily pollen (Baldi *et al.*, 1988) and cattail pollen (Hara *et al.*, 1985). The acid phytate-degrading enzymes from *E. coli* (Greiner *et al.*, 1993), *A. niger*, *A. terreus* (Wyss *et al.*, 1999a) have also been reported to be rather specific for phytate. With the exception of the phytate-degrading enzymes from *Emericella nidulans* and *Myceliophthora thermophila* (Pasamontes *et al.*, 1997a) all phytate-degrading enzymes hitherto studied follow Michaelis–Menten kinetics.

In general, phytate-degrading enzymes from microbial sources exhibit the highest turnover number with phytate, whereas the corresponding plant enzymes yield the highest relative rates of hydrolysis with pyrophosphate and ATP. However, most of the phytate-degrading enzymes characterized so far showed the highest affinity to phytate among all phosphorylated compounds studied. The K_M values of the phytate-degrading enzymes studied range from < 10 to 650 μM (Table 4). Relatively low K_M values have been reported for the phytate-degrading enzymes from *A. niger* (10–40 μM), *A. terreus* (11–23 μM), *A. fumigatus* (< 10 μM), *Schwanniomyces castellii* (38 μM), *K. aerogenes* (62 μM), cattail pollen (17 μM), maize root (24–43 μM), tomato root (38 μM), oat (30 μM), wheat bran (PHY1: 48 μM, PHY2: 77 μM), barley (P1: 72 μM), soybean (48–61 μM), and lupine (L11: 80 μM).

The catalytic constants for the degradation of phytate by phytate-degrading enzymes reported so far range from < 10 (soybean, maize) to 1744 s^{-1} (*E. coli*) (Table 4). The kinetic efficiency of an enzyme is validated by means of the k_{cat}/K_M values for a given substrate. The phytate-degrading enzyme from *E. coli* has a k_{cat}/K_M value of 1.34×10^7 M^{-1} s^{-1} (Golovan *et al.*, 2000), which is the highest value reported for a phytate-degrading enzyme. The formerly reported turnover number of 6209 s^{-1} and k_{cat}/K_M value of 4.78×10^7 M^{-1} s^{-1} for the phytate-degrading enzyme from *E. coli* (Greiner *et al.*, 1993) was overestimated because of a low estimation of enzyme concentration by the Bradford assay used for protein estimation (Golovan *et al.*, 2000).

Although the relative rates of hydrolysis of some phosphorylated compounds such as pyrophosphate and ATP by the phytate-degrading enzymes from plants are significantly higher than those of phytate, these plant enzymes gave the

highest k_{cat}/K_M values with phytate. Thus, the kinetic parameters suggest phytate to be the likely substrate for the phytate-degrading enzyme from plants under physiological conditions.

From studies on microbial phytate-degrading enzymes it was concluded that phytate-degrading enzymes with a broad substrate specificity readily degrade phytate to *myo*-inositol monophosphate with no major accumulation of intermediates, whereas phytate-degrading enzymes with narrow substrate specificity resulted in *myo*-inositol tris- and bisphosphate accumulation during phytate degradation coupled with a progressive rate of phosphate release, which suggest that lower *myo*-inositol phosphates are worse substrates than phytate (Wyss *et al*., 1999a). Furthermore, it was suggested that the difference in the substrate specificity of the two classes of phytate-degrading enzymes reflects to a considerable extent a selective difference in the specific activities with phytate, because phytate-degrading enzymes with broad substrate specificity inherently had rather low specific activities with phytate as substrate, while the phytate-degrading enzymes with narrow substrate specificity had higher specific activities. The plant phytate-degrading enzymes and those of *B. subtilis* and *B. amyloliquefaciens* do not fit into this classification. The plant enzymes have a very broad substrate specificity and a rather high specific activity, whereas the *Bacillus* enzymes appear to be very specific for phytate, but have an apparently low specific activity. Furthermore, an accumulation of *myo*-inositol tetrakis-, tris- and bisphosphates has been reported during phytate degradation by phytate-degrading enzymes from plants coupled with a progressive rate of phosphate release (Konietzny *et al*., 1995; Greiner *et al*., 1998; Greiner *et al*., 2001b).

Enzymatic phytate degradation

Phytate-degrading enzymes catalyse the stepwise hydrolysis of *myo*-inositol hexakisphosphate to orthophosphate and lower *myo*-inositol phosphates. The reaction intermediates are released from the enzymes and serve as substrates for further hydrolysis. The only exception was reported to be the D/L-Ins(1,2,3,4,5)P5-intermediate generated by the phytate-degrading enzyme of *B. subtilis*, which was suggested to remain tightly bound to the active-site of the phytate-degrading enzyme until the phosphates both in 6- and 4-position are removed (Kerovuo *et al*., 2000b). The resulting Ins(1,2,3,5)P4 is then released from the phytate-degrading enzyme from *B. subtilis*.

The different phosphate residues of phytate may be released by phytate-degrading enzymes at different rates and in different order. In general, a marked decrease in hydrolysis rate was observed during enzymatic phytate degradation. This results in a pronounced accumulation of *myo*-inositol tetrakis-, tris- and/or bisphosphates in the reaction mixture. Considerable amounts of *myo*-inostiol tris- and bisphosphates accumulated during phytate degradation by the phytate-degrading enzymes from *A. niger* and *A. terreus* (Wyss *et al*., 1999a), whereas a pronounced accumulation of *myo*-inositol tetrakis- and trisphosphates was observed with the enzymes from *E. coli* (Greiner *et al*., 1993) and rye (Greiner *et al*., 1998). The decrease in the rate of phosphate release might be the result of product inhibition by phosphate or a lower hydrolysis rate of the lower *myo*-inositol phosphates. Probably both factors play a role, but information about kinetic parameters of the different lower *myo*-inositol phosphates are still lacking, as most of the reaction intermediates are not available in pure form and sufficient quantities.

One possibility to make pure breakdown products of phytate available in sufficient quantities is the use of an immobilized enzyme-based bio-reactor followed by anion-exchange chromatography of the hydrolysis mixtures. The amount of the desired hydrolysis product could be controlled by the number of passages of the *myo*-inositol hexakisphosphate solution through the bio-reactor. A few studies have been made on the application and properties of immobilized phytate-degrading enzymes (Ullah & Cummins, 1987; Greiner & Konietzny, 1996; Liu *et al*., 1999). The pH dependence of the phytate-degrading activity was not influenced by immobilization, whereas stability against heat treatment was enhanced as a consequence of immobilization. In addition, the immobilized phytate-degrading enzymes exhibited good optional and storage stability over a period of several months.

Two classes of phytate-degrading enzymes are recognized by the International Union of Pure and Applied Chemistry and the International Union of

Biochemistry (IUPAC-IUB); 3-Phytase (EC 3.1.3.8) initially removes orthophosphate from the D-3 position of phytate, whereas 6-phytase (EC 3.1.3.26) preferentially initiate the phytate dephosphorylation at the L-6 (D-4) position of the *myo*-inositol ring. Phytate-degrading enzymes from micro-organisms are considered to be 3-phytases (EC 3.1.3.8), whereas seeds of higher plants are said to contain 6-phytases (EC 3.1.3.26). The phytate-degrading enzymes from rye, barley, spelt, oat, wheat bran, rice and mung bean fit into this general consideration, because the major *myo*-inositol pentakisphosphate generated by these enzymes has been identified as D-Ins(1,2,3,5,6)P5 [identical with L-Ins(1,2,3,4,5)P5] (Maiti *et al*., 1974; Hayakawa *et al*., 1990; Greiner & Larsson Alminger, 2001). The phytate-degrading enzymes from *S. cerevisiae* (Greiner *et al*., 2001a), *Pseudomonas* (Cosgrove, 1970), *K. terrigena* (Greiner *et al*., 1997), and *A. niger* (Irving & Cosgrove, 1972) generate D-Ins(1,2,4,5,6)P5 as the major *myo*-inositol pentakisphosphate. Thus, according to the general consideration these enzymes are 3-phytases. However, this is not a general rule, as exemplified by indications of 3-phytase activity in soybean seeds and 6-phytase activity in *Paramecium* (van der Kaay & van Haastert, 1995) and *E. coli* (Greiner *et al*., 2000a). Especially in plant seeds phytate-degrading enzymes are found in multiple forms and those multiple forms may even exhibit different stereospecificity of *myo*-inositol hexakisphosphate dephosphorylation as reported recently for the phytate-degrading enzymes from lupines (Greiner, 2001). The phytate-degrading enzymes L11 and L12 have been identified as 3-phytases, as they generate D-Ins(1,2,4,5,6)P5 as the major *myo*-inositol pentakisphosphate, whereas L2 has been identified as a 6-phytase generating mainly D-Ins(1,2,3,4,5)P5.

To bring a clarification into biochemical pathway interpretation, the current rule is to number the *myo*-inositol phosphates in the D configuration (counter clockwise). Thus the above mentioned phytate-degrading enzymes of plant origin (with the exception of the phytate-degrading enzymes L11 and L12 from lupines) have to be classified as 4-phytases. This is exceptionally important to distinguish the former from the phytate-degrading enzymes from *E. coli* and *Paramecium*, which generate D-Ins(1,2,3,4,5)P5 [identical with L-Ins(1,2,3,5,6)P5] as the major *myo*-inositol pentakisphosphate and have therefore to be classified as 6-phytases.

The alkaline phytate-degrading enzyme discovered and isolated from lily pollen was shown to start the hydrolysis of phytate at the D-5 position and could therefore be classified as 5-phytase (Barrientos *et al*., 1994). Thus it could be suggested that all six possibilities of initiating phytate dephosphorylation are realized in nature, although the existence of a 1-phytase has not been proven until now.

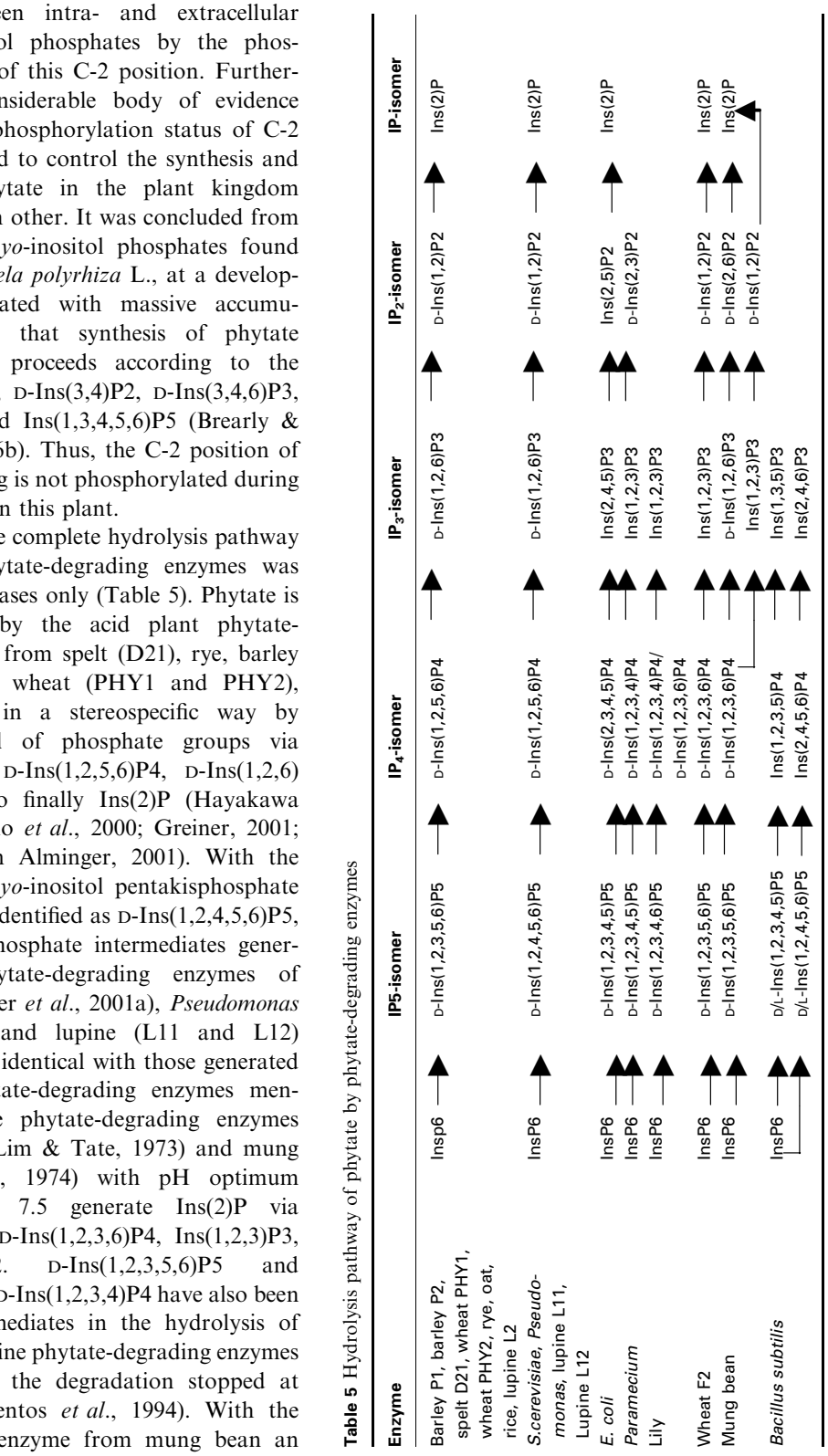
Independent of their bacterial, fungal or plant origin, the acid phytate-degrading enzymes studied so far in respect to phytate degradation (*A. terreus, A. niger*, *Emericella nidulans*, *Myceliophthora thermophila*, *S. cerevisiae*, *Pseudomonas*, *E. coli*, rice, rye, barley P1, barley P2, oat, wheat PHY1, wheat PHY2, wheat F2, lupine L11, lupine L12, lupine L2, mung bean) release five of the six phosphate groups of phytate, and the final degradation product was identified as Ins(2)P (Cosgrove, 1970; Lim & Tate, 1973; Maiti *et al*., 1974; Hayakawa *et al*., 1990; Wyss *et al*., 1999b; Greiner *et al*., 2000a, 2001a; Nakano *et al*., 2000; Greiner, 2001; Greiner & Larsson Alminger, 2001). This indicates that all those phytate-degrading enzymes have a strong preference for equatorial phosphate groups, whereas they are virtually unable to cleave the axial phosphate group. Only in rare cases traces of free *myo*-inositol were detected. On the other hand, the alkaline phytate-degrading enzymes from cattail (Hara *et al*., 1985), lily pollen (Barrientos *et al*., 1994) and *B. subtilis* (Kerovuo *et al*., 2000b) are not capable of accepting a *myo*-inositol phosphate with three or fewer phosphate residues as a substrate. Thus, a *myo*-inositol trisphosphate isomer is the end product of phytate degradation by these alkaline phytate-degrading enzymes. The final product of phytate degradation by the lily enzyme was reported to be Ins(1,2,3)P3, whereas the *B. subtilis* enzyme was reported to generate Ins(1,3,5)P3 and Ins(2,4,6)P3. Thus, the enzyme from *B. subtilis* is the first phytate-degrading enzyme capable of removing the axial phosphate group at the C-2 position of the *myo*-inositol ring.

As intracellular occurring lower *myo*-inositol phosphates are not phosphorylated at the C-2 position of the *myo*-inositol ring, the cell may

discriminate between intra- and extracellular formed *myo*-inositol phosphates by the phosphorylation status of this C-2 position. Furthermore, a not inconsiderable body of evidence supports that the phosphorylation status of C-2 position is also used to control the synthesis and breakdown of phytate in the plant kingdom independent of each other. It was concluded from the identities of *myo*-inositol phosphates found in the plant *Spirodela polyrhiza* L., at a development stage associated with massive accumulation of phytate, that synthesis of phytate from *myo*-inositol proceeds according to the sequence D-Ins(3)P, D-Ins(3,4)P2, D-Ins(3,4,6)P3, D-Ins(3,4,5,6)P4 and Ins(1,3,4,5,6)P5 (Brearly & Hanke, 1996a, 1996b). Thus, the C-2 position of the *myo*-inositol ring is not phosphorylated during phytate formation in this plant.

Elucidation of the complete hydrolysis pathway of phytate by phytate-degrading enzymes was performed in rare cases only (Table 5). Phytate is dephosphorylated by the acid plant phytate-degrading enzymes from spelt (D21), rye, barley (P1 and P2), oat, wheat (PHY1 and PHY2), rice, lupine (L2) in a stereospecific way by sequential removal of phosphate groups via D-Ins(1,2,3,5,6)P5, D-Ins(1,2,5,6)P4, D-Ins(1,2,6)P3, D-Ins(1,2)P2 to finally Ins(2)P (Hayakawa *et al*., 1990; Nakano *et al*., 2000; Greiner, 2001; Greiner & Larsson Alminger, 2001). With the exception of the *myo*-inositol pentakisphosphate isomer, which was identified as D-Ins(1,2,4,5,6)P5, the *myo*-inositol phosphate intermediates generated by the phytate-degrading enzymes of *S. cerevisiae* (Greiner *et al*., 2001a), *Pseudomonas* (Cosgrove, 1970) and lupine (L11 and L12) (Greiner, 2001) are identical with those generated by the plant phytate-degrading enzymes mentioned above. The phytate-degrading enzymes from wheat (F2) (Lim & Tate, 1973) and mung bean (Maiti *et al*., 1974) with pH optimum between 7.0 and 7.5 generate Ins(2)P via D-Ins(1,2,3,5,6)P5, D-Ins(1,2,3,6)P4, Ins(1,2,3)P3, and D-Ins(1,2)P2. D-Ins(1,2,3,5,6)P5 and D-Ins(1,2,3,6)P4 or D-Ins(1,2,3,4)P4 have also been identified as intermediates in the hydrolysis of phytate by the alkaline phytate-degrading enzymes of lily pollen, but the degradation stopped at Ins(1,2,3)P3 (Barrientos *et al*., 1994). With the phytate-degrading enzyme from mung bean an

Table 5 Hydrolysis pathway of phytate by phytate-degrading enzymes



alternative pathway from D-Ins(1,2,3,6)P4 to Ins(2)P via D-Ins(1,2,6)P3 and D-Ins(2,6)P2 was observed. Even if the microbial phytases from *Paramecium* (van der Kaay & van Haastert, 1995) and *E. coli* (Greiner *et al*., 2000a) are both 6-phytases, the dephosphorylation routes are different. The only *myo*-inositol phosphate intermediate both hydrolysis pathways have in common is the *myo*-inositol pentakisphosphate isomer D-Ins(1,2,3,4,5)P5. The *E. coli* enzyme generates Ins(2)P via D-Ins(2,3,4,5)P4, D-Ins(2,4,5)P3, and Ins(2,5)P2, whereas the enzyme from *Paramecium* generates D-Ins(2,3)P2 as the end product via D-Ins(1,2,3,4)P4, and D-Ins(1,2,3)P3. The very slow degradation of the *myo*-inositol trisphosphate intermediate by the phytate-degrading enzyme from *Paramecium* and its missing capability to degrade the *myo*-inositol bisphosphate intermediate might be the result of the conditions used and it is very probable that the *Paramecium* enzyme also generates Ins(2)P as the final product of phytate hydrolysis as reported for all other acid phytate-degrading enzymes.

A novel mode of phytate degradation was proposed for the phytate-degrading enzyme from *B. subtilis* (Kerovuo *et al*., 2000b). It was reported to exhibit hybrid 3-/6-phytate-degrading activity. The enzyme hydrolyses only three phosphate residues from phytate and it seems to prefer the hydrolysis of every second phosphate over that of adjacent ones. Furthermore, it is very likely that the enzyme has two alternative pathways for the hydrolysis of phytate, resulting in two different *myo*-inositol trisphosphate end products: Ins(2,4,6)P3 via D/L-Ins(1,2,4,5,6)P5 and Ins(2,4,5,6)P4 and Ins(1,3,5)P3 via D/L-Ins(1,2,3,4,5)P5 and Ins(1,2,3,5)P4. A plausible explanation for the formation of two different end products and for the cleavage of the axial phosphate residue at the C-2 position of the *myo*-inositol ring is that the enzyme could twist the *myo*-inositol ring of the bound substrate into a more planar conformation. Thus the concept of axial and equatorial phosphates would be diminished.

Final remarks

The increasing economic pressures currently being placed upon animal producers demand more-efficient utilization of low-grade feed. Recent market trends have clearly shown that hydrolytic enzymes have emerged as feed supplements in order to improve the digestion and absorption of poorly available nutrients from the animal diet. The use of fungal phytase as a feed supplement has proven effective in alleviating the negative effects of phytate in livestock diets. The phytate-degrading enzyme from the fungus *A. niger* is widely used in Europe as a commercial feed supplement. Numerous feeding studies with poultry, swine and fish have demonstrated the efficacy of phytate-degrading enzyme supplementation for improving phosphorus and mineral availability. Especially, phytate-degrading enzymes from microorganisms offer technical and economical feasibility for their production and application. Because of the interest in the use of microbial phytate-degrading enzymes in feed applications, highly efficient and cost-effective processes for their production by recombinant microorganisms have been developed. Recently, high levels of phytate-degrading activity accumulating in the fermentation medium has been described by using economically competitive expression/secretion systems for the yeasts *Hansenula polymorpha* (Mayer *et al*., 1999) and *Pichia pastoris* (Yao *et al*., 1998).

Depending on the application, an enzyme in which there is commercial interest should fulfil a series of predefined quality criteria. In the case of phytate-degrading enzymes, which are added to animal feed, these criteria include high specific activity, broad substrate specificity, a broad pH optimum, and good stability during storage, feed pelleting and passage through the digestive tract (Wyss *et al*., 1999b). Thermostability is a particularly important issue as feed pelleting is commonly performed at temperatures between 65 and 95 °C. Naturally occurring phytate-degrading enzymes having the required level of thermostability for application in animal feeding have not been found in nature thus far. The poor thermostability of the phytate-degrading enzymes is therefore still a major concern for animal feed applications. Therefore, it comes as no surprise that isolation and characterisation, and engineering of thermostable enzymes, as well as the search for the determinants of thermostability, are hot spots of current research. Different strategies can be used to obtain a thermostable enzymes with the desired catalytic activity. These strategies

include screening of thermophilic and hyperthermophylic organisms for the catalytic activity and substrate specificity of interest; mutagenesis of a mesophilic enzyme in order to increase its thermostability and mutagenesis of a known thermostable enzyme that catalyses a closely related reaction, with the aim of modifying its substrate specificity.

Optimization of catalytic properties has been approached in the past mostly on a trial-and-error basis by random mutagenesis. Most recently, technological advances have paved the way for several direct evolution approaches, i.e. rapid, iterative processes of mutation and/or recombination of genes and selection or screening of improved protein variants. By using a novel consensus approach a fully synthetic phytate-degrading enzyme was generated, that was 15–26 °C more thermostable than the 13 parent fungal phytate-degrading enzymes used in its design (Lehmann *et al*., 2000). Furthermore, by replacing a considerable part of the active site of the generated enzyme with the corresponding residues of the phytate-degrading enzyme of *A. niger* NRRL 3135 a shift in catalytic properties was observed, demonstrating that rational transfer of favourable catalytic properties from one phytate-degrading enzyme to another is possible by using this approach.

In vitro feed experiments suggest that phytate-degrading enzymes with broad substrate specificity are better suited for animal nutrition purposes than phytate-degrading enzymes with narrow substrate specificity (Wyss *et al*., 1999a). Unfortunately, broad substrate specificity currently must be paid for dearly in that it is coupled with low specific activity. In future it will be a challenge to combine high specific phytate-degrading activity with broad substrate specificity. An increase in specific activity of the phytate-degrading enzyme of *A. terreus* while preserving broad substrate specificity and high activity over a broad pH range was achieved by site-directed mutagenesis (Tomschy *et al*., 2000).

Supplementation of animal feed might be not profitable for the average livestock producer, as the added expense of feed supplement can be significant. Reduction of phytate levels or increase in phytate-degrading activity in the plant seed itself are alternative strategies for improving nutrient management in animal production. Maize and barley mutants were generated, whose seeds had normal levels of total phosphorus but greatly reduced levels of phytate (Rasmussen & Hatzack, 2000; Raboy *et al*., 2000). The use of those low phytate grain genotypes in feeds might help to reduce animal waste phosphorus. Increased phytate-degrading activity in the plant seed was achieved by heterologous expression of phytate-degrading enzymes in the plant seeds using transformation of plant host with an expression vector containing the gene of a phytate-degrading enzyme from *Aspergillus* ssp. (Pen *et al*., 1993; Li *et al*., 1997a; Ullah *et al*., 1999; Brinch-Pedersen *et al*., 2000). It was shown that only limited amounts of transgenic seeds are required in compound feeds to ensure proper degradation of phytate.

Furthermore, it is evident that the inability of plants to utilize phosphorus from soil phytate is associated with a lack of extracellular phytate-degrading activity (Hayes *et al*., 2000). Thus, an opportunity exists for using gene technology to improve the ability of plants to utilize phytate phosphorus. Extracellular secretion of the phytate-degrading enzyme from *A. niger* from plant roots was shown to enable the plants to obtain phosphorus from phytate (Richardson *et al*., 2001). A more effective utilization of phosphorus from soil and fertilizer sources would be particularly beneficial to agriculture throughout the world.

A different strategy to overcome the problems using phytate-degrading enzymes as a feed additive such as cost, inactivation at the high temperatures required for pelleting feed, and loss of activity during storage, might be to add those enzymes to the repertoire of digestive enzymes produced endogenously by swine and poultry. Swine were generated with a gene from *E. coli* for the production of a phytate-degrading enzyme in the saliva (Golovan *et al*., 2001). It was shown that provision of salivary phytate-degrading activity enables essentially complete digestion of dietary phytate, largely relieving the requirement for inorganic phosphate supplementation, and reduces faecal phosphorus output by up to 75%. This reduction even exceeds the 40% reduction reported for pigs fed expensive phytase supplements.

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