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MINI-REVIEW

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Biotechnological production and applications of phytases

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Abstract Phytases decompose phytate, which is the primary storage form of phosphate in plants. More than 10 years ago, the first commercial phytase product became available on the market. It offered to help farmers reduce phosphorus excretion of monogastric animals by replacing inorganic phosphates by microbial phytase in the animal diet. Phytase application can reduce phosphorus excretion by up to 50%, a feat that would contribute significantly toward environmental protection. Furthermore, phytase supplementation leads to improved availability of minerals and trace elements. In addition to its major application in animal nutrition, phytase is also used for processing of human food. Research in this field focuses on better mineral absorption and technical improvement of food processing. All commercial phytase preparations contain microbial enzymes produced by fermentation. A wide variety of phytases were discovered and characterized in the last 10 years. Initial steps to produce phytase in transgenic plants were also undertaken. A crucial role for its commercial success relates to the formulation of the enzyme solution delivered from fermentation. For liquid enzyme products, a long shelf life is achieved by the addition of stabilizing agents. More comfortable for many customers is the use of dry enzyme preparations. Different formulation technologies are used to produce enzyme powders that retain enzyme activity, are stable in application, resistant against high temperatures, dust-free, and easy to handle.

Introduction

Phytase enzyme preparations have a wide range of applications in animal and human nutrition. The first commercial phytase products were launched into market in 1991. Meanwhile the market volume is in the range of 150 Mio Euro. Phytases decompose phytates (*myo*-inositol-1,2,3,4,5,6-hexakisphosphates), the salts of phytic acid (Fig. 1). Phytate is regarded as the primary storage form of both phosphate and inositol in plants (Cosgrove 1966). The phosphorus fraction stored as phytate range from 30% in roots up to 80% in seeds and cereals (Table 1). Phytic acid is a polyanionic chelating agent that forms complexes with several divalent cations of major nutritional importance, e.g., Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, and Mn^{2+} (Harland and Oberleas 1999). The stability of the different salts mainly depends on the type and concentration of cation (Vohra et al. 1965) and pH. Phytic acid can also form complexes with proteins and amino acids at both acidic and alkaline pH (Sebastian et al. 1998).

The term phytase (*myo*-inositol hexakisphosphate phosphohydrolase) describes a class of phosphatases with the in vitro capability to release at least one phosphate from phytate. Despite this definition, up to now, *myo*-inostitol pentakisphosphate (IP5) has yet to be identified as the final product. Usually, the degradation ends with the less phosphorylated *myo*-inostiol phosphates IP3 (Hara et al. 1985; Kerovuo et al. 2000; Quan et al. 2004) or IP (Wyss et al. 1999; Casey and Walsh 2004; Sajidan et al. 2004). The International Union of Pure and Applied Chemistry and the International Union of Biochemistry (IUPAC–IUB) distinguish two classes of phytate degrading enzymes, 3-phytase (EC 3.1.3.8) and 6-phytase (EC 3.1.3.28), initiating the dephosphorylation at the 3 and 6 positions of phytate, respectively.

Phytases are widespread in nature because they can be found in animals, plants, and microorganisms. For example, phytate-degrading enzymes were reported in the blood of calves (McCollum and Hart 1908), birds, reptiles, and fishes (Rapoport et al. 1941), as well as in plants like maize (Huebel and Beck 1996), rice (Hayakawa et al. 1989; Maugenest et al. 1999), wheat (Nagai and Funahashi 1962; Nakano et al. 1999), and soybean (Hamada 1996). However, most of the scientific work has been done on microbial phytases, especially on those originating from filamentous fungi such as *Aspergillus ficuum* (Gibson 1987), *A. fumi-*

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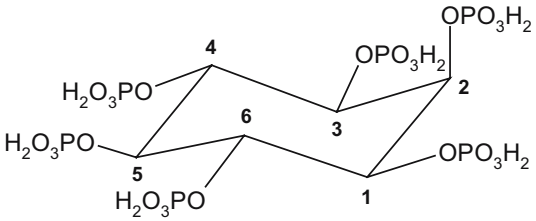

Fig. 1 Structural formula of phytic acid

Table 1 Phytate content of cereals and roots (Ravindran et al. 1995)

	Phytate P [g/100 g dry matter]	Phytate P [% of total P]
Cereals		
Corn	0.24	72
Wheat	0.27	69
Barley	0.27	64
Oats	0.29	67
Sorghum	0.24	66
Rice, unpolished	0.27	77
Roots and tubers		
Cassava	0.04	28
Sweet potato	0.05	24

gatus (Pasamontes et al. 1997) or *Mucor piriformis* (Howson and Davis 1983), *Rhizopus oligosporus* (Casey and Walsh 2004), and *Cladosporium* species (Quan et al. 2004). In the last decade, phytate-degrading enzymes of yeasts (Nakamura et al. 2000) such as *Schwanniomyces occidentalis* (Segueilha et al. 1992), *Pichia anomala* (Vohra and Satyanarayana 2001, 2002), *Arxula adeninivorans* (Sano et al. 1999), gram-negative bacteria such as *Escherichia coli* (Greiner et al. 1993), *Pseudomonas* species (Cho et al. 2003; Kim et al. 2003), *Klebsiella* species (Tambe et al. 1994; Sajidan et al. 2004), and gram-positive bacteria such as various *Bacillus* species (Kerovuo et al. 1998; Kim et al. 1998a; Wang et al. 2001; Tye et al. 2002) were also identified and characterized. The occurrence and the biochemical properties of phytases are reviewed in detail by Oh et al. (2004) and Konietzny and Greiner (2002).

Application in animal nutrition

Phytase is incorporated into commercial poultry, swine, and fish diets to improve the availability of phosphorus, minerals, amino acids, and energy. Phytate accounts for 60–80% of phosphorus found in plant-derived feedstuffs (Table 1). The phytate molecule and thus the nutrients bound to it cannot be absorbed in the digestive tract without enzymatic degradation by phytases. Generally, this degradation can occur in the digestive tract and/or in the feed before consumption (Sebastian et al. 1998). Some cereals such as rye, triticale, wheat, and barley are rich in intrinsic phytase, while other feedstuffs such as corn and oilseed meals contain little or no phytase activity (Eeckhout and De Paepe 1994). Plant phytase is generally active in feeds as shown by Temperton et al. (1965a,b). However, the use of plant phytase in animal feed is limited, because its content is highly variable even within one feedstuff. Moreover, pelleting of feed at temperatures higher than 70°C results in partial inactivation (Pointillart 1988). Additionally, the bioefficacy[1] of cereal phytases was only 40% compared to microbial phytase from *Aspergillus* species (Zimmermann et al. 2002).

Phytase produced by microorganisms in the digestive tract can be very efficient in degrading phytate as demonstrated by the almost complete availability of vegetable phosphorus to ruminants (Rodehutscord 2001). However, the microbial ecosystem in monogastric animals is mainly located in the large intestines and it can be assumed that most of the phosphate released from phytate is not absorbed, but excreted after release by microorganisms. Due to the low availability of phosphorus in plant-derived feedstuffs, diets for nonruminants have been traditionally supplemented with inorganic phosphates. Excessive dietary phosphorus is excreted by animals and thus applied to the soil together with manure. Due to increasing livestock density in many regions, manure has been applied to the soil at rates exceeding plant needs, resulting to accumulation of phosphate in the soil (CAST 2002). This could lead to eutrophication of surface waters, and long-term leaching of phosphate into ground water can be expected (Furrer and Stauffer 1987).

The first commercial phytase product, which became commercially available 10 years ago, offered animal nutritionists the tool to drastically reduce phosphorus excretion of monogastric animals by replacing inorganic phosphates with microbial phytase. Depending on diet, species, and level of phytase supplementation, phosphorus excretion can be reduced between 25 and 50% (Kornegay 1999).

Supplementation of microbial phytase to nonruminant diets also showed effects on other nutrients. The improvement of Ca availability has been shown in many trials (Sebastian et al. 1998). Schoener and Hoppe (2002) demonstrated in a broiler trial with adequate phosphorus supply that enhanced Ca availability is not only based on a direct effect (i.e., cleaving Ca from the phytate complex), but also on an indirect effect accruing from the enhanced phosphorus utilization. Increased availability has also been shown for Mg (Brink et al. 1991), and several trace elements such as Zn (Thiel and Weigand 1992), Cu (Adeola 1999), Fe (Pallauf et al. 1992), and Mn (Mohanna and Nys 1999). Besides improving the availability of minerals and trace elements, microbial phytase is also able to enhance protein digestibility. This was described by Jongbloed et al. (1999) for pigs, Farrell et al. (1993) for broilers, Van der Klis and Versteegh (1991) for laying hens, Yi et al. (1996) for turkeys, as well as Martin and Farrell (1994) for ducks. The protein and amino acid effects of microbial phytase can be explained by the degradation of phytate–protein and phytate–mineral–protein–complexes in plant feedstuffs (Ravindran

[1]Effect of analyzed phytase activity on animal performance feeding phosphorus deficient diets.

et al. 1999). Phytate–protein–complexes may be formed postfeeding in the gut in case phytate has not been hydrolyzed by phytase (Jongbloed et al. 1997). Also, phytate can complex with supplemental free amino acids, which could be partly prevented with phytase (Rutherfurd et al. 1997). Furthermore, phytate is known to inhibit proteolytic enzymes (Caldwell 1992). As phytate can also bind starch and inhibit amylase (Deshpande and Cheryan 1984), it can be hypothesized that activity of phytase is able to increase energy utilization in monogastric animals as well. Ravindran et al. (1999) showed such effects by literature review and via their own trials for poultry. Due to the effects described above and its potential to produce microbial phytase on large scale at low costs, microbial phytase is today widely used in diets for monogastric farm animals. However, it has to be taken into account that microbial phytases of different origin can differ in their bioefficacy per analyzed phytase unit. Differences in bioefficacy are described by Paditz et al. (2004), Klein Holkenborg et al. (2003), and Wendt and Rodehutscord (2004).

Application in human nutrition

Processing and manufacturing of human food is also a possible application field for phytase. Up to now, no phytase product for a relevant food application is on the market. Research in this field focuses on better mineral absorption or technical improvement of food processing.

In cereal and legume-based complementary foods, phytic acid inhibits iron absorption, causing the high prevalence of iron deficiency, e.g., in infants from developing countries, women of fertile age, or vegetarians. In their study, Sandberg et al. (1999) showed that inositol hexaphosphate (IP6) as well as pentaphosphate (IP5) have inhibitory effects on iron absorption. The addition of 10 mg phosphorus as IP5 to white wheat rolls resulted in a 39% reduction in iron absorption. IP3 and IP4 did not reveal such negative effects in isolated forms, but there are indications for a contribution to the negative effects when they are given together with IP5 and IP6. As a conclusion, it was stated that in order to improve iron absorption from cereals and legumes, degradation of inositol phosphates should yield less phosphorylated forms than IP3.

Some food processing methods such as cooking, germination, hydrothermal treatment, fermentation, and soaking are shown to reduce or remove considerable amounts of phytate in legumes (Rehms and Barz 1995; Nout and Rambouts 1990). Use of phytase reduces the phytic acid content in food products, maybe more efficiently. Phytase fully degraded phytic acid during the manufacture of roller-dried complementary foods based on flours from rice, wheat, maize, oat, sorghum, and a wheat–soy flour blend (Hurrell et al. 2003). Phytate degradation was measured as well as the effect on iron absorption. The tested cereal porridges had native phytate levels between 0.12% (wheat porridge) and 0.89% (sorghum porridge). After treatment with phytase, phytic acid content was reduced to ≤0.002%. Iron absorption was significantly increased when the porridges were prepared with water, although the magnitude of the increase differed markedly.

Haros et al. (2001) investigated the possible use of phytase in the process of bread making. Different amounts of fungal phytase were added in whole wheat breads, and it was shown that phytase is an excellent bread-making improver. The main achievement of this activity was the shortened fermentation period without affecting the bread dough pH. An increase in bread volume and an improvement in crumb texture were also observed.

Application in synthesis of lower inositol phosphates

Lower phosphoric esters of *myo*-inositol (mono, bis, tris, and tetrakisphosphates) play a crucial role in transmembrane signaling processes and in calcium mobilization from intracellular store in animal as well as in plant tissues (Michell 1975; Berridge and Irvine 1984; Samanta et al. 1993; Dasgupta et al. 1996; Krystofova et al. 1994). Research interest in this field prompted the need for various inositol phosphate preparations. However, chemical synthesis (for review, see Billington 1993) is difficult. In contrast, an enzymatic synthesis has the advantage of high stereospecifity and mild reaction conditions. The use of phytase has been shown to be very effective in producing different inositol phosphate species. Siren (1986a,b) successfully prepared 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 with the help of phytase derived from *S. cerevisiae*. Also, the use of phytase isolated from *A. niger* was shown to efficiently hydrolyze IP6 to all lower phosphorylated derivates from IP5 to IP2 depending on the amount of enzyme (Dvorakova et al. 2000).

Production of microbial phytases

The first phytase product, which entered the feed market in 1991, was manufactured by Gist Brocades (now DSM) and sold by BASF under the trade name Natuphos. Natuphos is available as powder, granulate, or liquid formulation. Later, other products from different companies appeared, but only a limited number of commercial phytase products are currently available. These first phytases produced on commercial scale were either derived from fungal strains mutated via standard means or by using recombinant DNA technology. Gist Brocades' patent (van Gorcom et al. 1990) describes an *A. ficuum* strain overproducing phytase with at least 50 times increased activity compared to the wild-type strain. At present, all phytase preparations authorized in the EU as feed additives are produced by recombinant strains of filamentous fungi (Table 2). The expressed phytase genes are of fungal origin and originate in two cases from the genus *Aspergillus*.

In most cases, the production of phytases was studied in submerged cultivations. However, there is an increasing number of scientific publications dealing with phytase

Table 2 Phytase preparations authorized in the EU as feed additives

Company	Trademark	Phytase source	Production strain	References
BASF	Natuphos	*Aspergillus niger* var. *ficuum*	*Aspergillus niger*	Simon and Igbasan (2002), Misset (2003), European Union (2004a)
AB Enzymes (former Röhm)	Finase	*Aspergillus awamori*	*Trichoderma reesei*	Simon and Igbasan (2002), Misset (2003), European Union (2004b)
Novozymes	Bio-Feed Phytase	*Peniophora lycii*	*Aspergillus oryzae*	Simon and Igbasan (2002), European Union (2004c,d)

production via solid state cultivation, especially those using filamentous fungi.

Table 3 summarizes published phytase productivities. The underlying phytase activities were all determined on the basis of inorganic phosphate liberation from phytate. Due to obvious differences with respect to cultivation conditions and slight differences with respect to phytase assay conditions, a comprehensive comparison and evaluation of the production strains is difficult. However, one gets an impression (1) of the diversity of phytase productivities (i.e., 4 orders of magnitude) and (2) of those production strains and cultivation conditions resulting in substantially high phytase productivities.

Chen and coworkers (2004) used *P. pastoris* for the heterologous overexpression of the *E. coli* phytase gene *appA*. The *appA* gene was cloned under the control of the AOX1 promotor, which is highly expressed when methanol is the only carbon source. By applying high cell-density cultivation, culture medium replacement prior to methanol induction, and a modified medium composition, extracellular phytase activities of almost 5,000 U ml^{-1} were achieved.

Mayer and coworkers (1999) used *H. polymorpha* strains containing multiple copies of the *A. terreus* phytase gene, two variants of the *A. fumigatus* phytase gene or a consensus phytase gene, respectively. In high cell-density cultivations with glucose as carbon source, very high phytase concentrations in the medium (up to 13.5 g l^{-1}) were obtained.

These two studies deal with both the rational design of a powerful production strain via genetic engineering and the systematic improvement of the cultivation conditions. Mayer and coworkers (1999) even established a downstream processing and performed the scale up of the whole process to 2,000 l.

Production of phytase in transgenic plants

Several attempts were made to use transgenic plants as expression hosts for phytases. Transgenic plants might contain sufficient phytase activity to replace additional supplementation of feed and food with microbial phytases. Alternatively, transgenic plants could be used as bioreactors for the production of phytase as a supplement.

Fungal phytases, like the *A. niger* PhyA, have successfully been expressed in tobacco (Pen et al. 1993; Verwoerd et al. 1995; Ullah et al. 1999), soybean (Li et al. 1997), alfalfa (Gutknecht 1997), wheat (Brinch-Pedersen et al. 2000), and canola (Ponstein et al. 2002). In tobacco, the enzyme was secreted into the apoplast via the default secretion pathway and accumulated to approximately 14% of the total soluble protein (Verwoerd et al. 1995). Purified recombinant phytase expressed from tobacco leaves had the same temperature optimum for phytate hydrolysis, but has been less glycosylated and showed a moderate shift to a more acidic pH optimum (Ullah et al. 1999). Also, the recombinant phytases expressed in soybean and alfalfa had almost the same properties as the endogenous produced fungal phytase, except for the glycosylation pattern (Li et al. 1997; Ullah et al. 2002). The *A. niger* phytase produced in tobacco seeds was functional in releasing phosphate from animal feed under simulated standard conditions and the seeds could be stored for at least 1 year without losing activity (Pen et al. 1993). In feeding trials, phytases that were recombinantly produced in soybean and canola seeds had the same performance as microbial phytases (Denbow et al. 1998; Zhang et al. 2000). However, the thermo tolerance of the *A. niger* phytase would not be high enough to survive the heat encountered in the soybean meal production. In the approach of Gutknecht (1997), the alfalfa plant was used as a bioreactor and not as a valorized animal feedstuff. Most of the transgenically produced phytase was contained in the juice collected after the alfalfa was processed.

Not only fungal phytases were produced in plants. The phytase genes from *E. coli* (*appA*) and the ruminal bacterium *Selenomonas ruminantium* (SrPf6) were expressed in germinated rice seeds. The phytase activity reached up to 1.4 U mg^{-1} of extracted cellular protein, which represented 60 times of the activity of the nontransformant, without any adverse effect on plant development (Hong et al. 2004). The expression of a *B. subtilis* phytase in the cytoplasm of tobacco even increased the number of flowers and fruits (Yip et al. 2003).

Whether or not transgenic plants will be used for production of commercial phytases in the future, either directly for feeding or as a bioreactor, will depend on production costs and on public acceptance of green biotechnology.

Formulation

After production of the enzyme, further processing is necessary. In regards to phytases' main application as feed

Table 3 Published phytase productivities

Phytase source	Production strain[a]	Phytase activity (U ml^{-1})[b]	Phytase concentration (g l^{-1})	Phytase productivity (U l^{-1} h^{-1})[b]	Phytase productivity (mg l^{-1} h^{-1})	Reference
Bacteria						
Bacillus sp.		<1		2		Choi et al. (1999)
Bacillus amyloliquefaciens	*Bacillus subtilis*	2		167		Kim et al. (1999a,b)
Bacillus licheniformis	*Bacillus subtilis*	28				Tye et al. (2002)
Bacillus subtilis		<1		5		Powar and Jagannathan (1982)
Bacillus subtilis		35				Tye et al. (2002)
Citrobacter braakii		1				Kim et al. (2003)
Escherichia coli		105		5830		Miksch et al. (2002)
Escherichia coli		650		7930		Golovan et al. (2000)
Escherichia coli	*Streptomyces lividans*	950		19792		Stahl et al. (2003)
Escherichia coli	*Pichia pastoris*	114				Rodriguez et al. (1999)
Escherichia coli	*Pichia pastoris*	117		2438		Stahl et al. (2003)
Escherichia coli	*Pichia pastoris*	4946		25760		Chen et al. (2004)
Klebsiella sp.		<1		9		Shah and Parekh (1990)
Klebsiella sp.		2		62		Hwang (1999)
Lactobacillus amylovorus		146		4562		Sreeramulu et al. (1996)
Lactobacillus fructivorans		<1		148		De Angelis et al. (2003)
Lactobacillus sanfranciscensis		<1		210		De Angelis et al. (2003)
Megasphaera elsdenii		<1		1		Yanke et al. (1998)
Mitsuokella jalaludinii		13		1078		Lan et al. (2002)
Prevotella ruminicola		<1		4		Yanke et al. (1998)
Pseudomonas mendocina		<1				Richardson and Hadobas (1997)
Pseudomonas putida		<1				Richardson and Hadobas (1997)
Selenomonas ruminatum		<1		59		Yanke et al. (1998)
Weissela confusa		<1		130		De Angelis et al. (2003)
Fungi						
Aspergillus sp.		17		177		Kim et al. (1999a,b)
Aspergillus awamori		200		1190		Martin et al. (2003)
Aspergillus ficuum		15[c]		159[c]		Bogar et al. (2003a)
Aspergillus fumigatus	*Pichia pastoris*	55				Rodriguez et al. (2000a,b)
Aspergillus fumigatus	*Aspergillus awamori*	62		369		Martin et al. (2003)
Aspergillus fumigatus	*Hansenula polymorpha*		7.6		30	Mayer et al. (1999)
Aspergillus niger		7		37		Hong et al. (2001)
Aspergillus niger		8		32		van Hartingsveldt et al. (1993)
Aspergillus niger		108[c]		643[c]		Mandviwala and Khire (2000)
Aspergillus niger		1008[c]		4667[c]		Krishna and Nokes (2001)
Aspergillus niger	*Escherichia coli*		0.2			Phillippy and Mullaney (1997)
Aspergillus niger	*Saccharomyces cerevisiae*	3		186		Han et al. (1999)
Aspergillus niger	*Pichia pastoris*	39	4.2	279	30	Xiong et al. (2004)
Aspergillus niger	*Pichia pastoris*	64		593		Han and Lei (1999)
Aspergillus oryzae		<1		4		Shimizu (1993)
Aspergillus terreus	*Hansenula polymorpha*		4.5		15	Mayer et al. (1999)
Mucor hiemalis		12[c]		160[c]		Bogar et al. (2003b)
Mucor racemosus		26[c]		361[c]		Bogar et al. (2003b)
Rhizopus microsporus		1[c]		18[c]		Bogar et al. (2003b)

Table 3 (continued)

Phytase source	Production strain[a]	Phytase activity (U ml^{-1})[b]	Phytase concentration (g l^{-1})	Phytase productivity (U l^{-1} h^{-1})[b]	Phytase productivity (mg l^{-1} h^{-1})	Reference
Rhizopus oligosporus		5[c]		75[c]		Bogar et al. (2003b)
Rhizopus oligosporus		14[d]		149[d]		Sabu et al. (2002)
Rhizopus oryzae		6[c]		76[c]		Bogar et al. (2003b)
Rhizopus thailandensis		3[c]		38[c]		Bogar et al. (2003b)
Consensus[d]	*Hansenula polymorpha*		13.5		46	Mayer et al. (1999)
Yeasts						
Arxula adininivorans		3		63		Sano et al. (1999)
Fellomyces fuzhouensis		<1		1		Sano et al. (1999)
Pichia anomala		3		63		Vohra and Satyanarayana (2004)
Pichia farinosa		<1		1		Sano et al. (1999)
Rhodotorula gracilis		<1		27		Bindu et al. (1998)
Schwanniomyces occidentalis		<1		8		Lambrechts et al. (1993)
Schwanniomyces occidentalis		<1		9		Sano et al. (1999)
Sporidiobolus johnsonii		<1		1		Sano et al. (1999)
Sporobolimyces sp.		<1		3		Sano et al. (1999)
Sterigmatosporus polymorphum		<1		2		Sano et al. (1999)

[a]In cases where no production strain is listed, phytase source and production system are identical
[b]One Unit is the amount of phytase required to liberate 1 μmol of inorganic phosphate per minute from phytate
[c]Cases where solid state cultivations were performed and hence data are expressed as U g^{-1} and U kg^{-1} h^{-1}, respectively
[d]Consensus phytase (Mayer et al. 1999)

additive, properties such as good stability during storage and application, high bioefficacy, and dust freeness have to be achieved by formulation. Due to easier handling, most feed enzymes on the market are sold as dry formulations.

Preferred manufacturing protocols in the feed industry require that the feed is mixed with steam prior to pelleting. In the subsequent pelleting step, the feed is forced through a die and the resulting strips are cut into suitable pellets. During this process moisture content reaches 12–20%, combined with temperatures in the range of 60–95°C. These conditions are detrimental to most unprotected enzymes. To avoid inactivation of the enzymes, various formulation methods have been developed. The easiest process is to mix the enzyme concentrate with a stabilizer and to spray dry the solution (Barendse et al. 1996). Typical stabilizers are inorganic salts with a bivalent cation, such as $MgSO_4$. The desired enzyme concentration is achieved by downblending the enzyme with a carrier. However, the pelleting stability of products obtained from this technology is limited. Higher stabilities can be achieved by processes presented by Barendse et al. (1998), Jacobsen et al. (1992), Bach et al. (2003), and Ghani and Genencor (2000). Three main procedures can be derived by various combinations of the described technologies. The first procedure is granulation, which consists of a drum, a high shear, or a fluidized bed granulation step in which a carrier (e.g., starch, sugar, or salt) is granulated with the enzyme concentrate. The addition of binders and stabilizers is optional. Afterwards, a coating is applied in a fluidized bed or a mixer, increasing the stability and reducing dust formation of the product. Quite similar to granulation is the process of absorption. Here the enzyme concentrate is sprayed on cores (e.g., sugar cores), which have the ability to absorb the enzyme solution and do not agglomerate with each other (de Lima et al. 1997). Also, here a coating can be applied after drying the enzyme cores. The third technology is extrusion. In a first step, a dough must be produced consisting of a carrier (e.g., starch), a binder, and the enzyme concentrate. This dough is then extrudated. The extrudates can optionally be rounded in a spheronizer. After the particles are dried, a coating could be applied in analogy to the above-mentioned processes. The properties of the products obtained by the different technologies are essentially influenced by the use of stabilizers or other processing aids.

An alternative to the use of dry enzyme formulations is the addition of liquid enzyme formulations postpelleting on the cooled feedstuff pellets. With this method, the enzymes can bypass heat inactivation that would occur during the pelleting process. For liquids the most important property is shelf life stability. Different stabilizers are established and described (Barendse et al. 1993; Brugger et al. 1996). Xylitol and sorbitol belong to the most effective stabilizers regarding shelf life stability. However, one needs specialized equipment to add liquids to the feed after pelleting, which is not available in many feed mills.

Outlook

A major trend in phytase research is the screening for enzymes with higher thermal stability. Until today, only few phytases have been reported with temperature stability or

optima exceeding 70°C. The fungal phytase from *A. fumigatus* was reported to withstand temperatures up to 100°C over a period of 20 min (Pasamontes et al. 1997), but a later report from Ullah et al. (2000) did not confirm these results. A synthetic consensus enzyme, deduced from several fungal phytases and subsequent refinements by site-directed mutagenesis, resulted in unfolding temperatures of up to 90.4°C (Lehmann et al. 2000). Also, phytase from *B. amyloliquefaciens* exhibited optimal activity at 70°C and stability at 90°C during 10 min incubation (Kim et al. 1998a; Park et al. 2003). In pelleting experiments, this enzyme retained >85% activity at temperatures ranging from 60 to 90°C. If such intrinsic thermostable phytases are used for the application in animal nutrition, formulation technologies will change, as thermal stability will be no longer the major task.

Protein engineering also dealt with the pH profile of phytases. The pH range for phytase activity of the *A. niger* phytase (Mullaney et al. 2002) or the *E. coli* phytase (Rodriguez et al. 2000a,b) were broadened at acidic pH by mutagenesis. Furthermore, phytases with various pH optima, ranging from 2.5 (*A. niger* PhyB) to 7.5 (several *Bacillus sp.*), are described in literature (Oh et al. 2004). However, different potential sites of action, like the strong acidic stomach or the crop of poultry with a nearly neutral pH, make the definition of an ideal pH profile regarding the activity and stability of the phytase rather difficult (Lei and Stahl 2001). Most successful feeding trials were performed with acidic phytase. Preliminary data suggests that phytase with neutral pH optimum also show relevant biological activity (BASF, unpublished data). More data for neutral and alkaline phytases are required to evaluate the potential of these enzymes for commercial applications.

A relatively new field in the production of active agents such as enzymes is the use of transgenic animals. In the case of phytase, that would mean the possibility to produce the active enzyme directly in the digestive tract of transgenic monogastric animals. Several past attempts to express a fungal phytase in a transgenic animal ended unsuccessfully (Mullaney et al. 2000). However, the transformation of the bacterial phytase gene *appA* from *E. coli* in a transgenic mouse model resulted in the expression of phytase in the parotid salivary glands. The enzymatically fully active phytase in the salvia reduced fecal phosphorus excretion by 11% (Golovan et al. 2001a). Also, a transgenic pig has been developed that produced the *E. coli* phytase in its saliva with an average of 2,000–3,000 U/ml (Golovan et al. 2001b). These results indicate the potential of transgenic animals, but further developments in this direction might be limited by public acceptance.

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