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Review

Phytase enzymology, applications, and biotechnology

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Abstract

Phytases are phosphohydrolases that initiate the step-wise removal of phosphate from phytate. These enzymes have been widely used in animal feeding to improve phosphorus nutrition and to reduce phosphorus pollution of animal waste. The potential of phytases in improving human nutrition of essential trace minerals in plant-derived foods is being explored. This review covers the basic biochemistry and application of phytases, and emphasizes the emerging biotechnology used for developing new effective phytases with improved properties.

Introduction

During ripening, cereal and legume seeds accumulate a substantial amount of phytic acid (*myo*-inositol-1,2,3,4,5,6-hexakis dihydrogen phosphate) (Honke *et al.* 1998). As a result, most of these seeds and their co-products contain 1–2% phytic acid that represent $>60\%$ of their total phosphorus (Reddy *et al.* 1982). Presumably, a large portion, if not all, of phytic acid in seeds is in the form of salts called phytate. Although phytate serves as the major source of energy and phosphorus for seed germination, the bound phosphorus is poorly available to simple-stomached animals. Thus, inorganic phosphorus, a non-renewable and expensive mineral, is supplemented in diets for swine, poultry, and fish to meet their nutrient requirement for phosphorus. Meanwhile, the unutilized phytate phosphorus from plant feeds is excreted, becoming an environmental pollutant in areas of intensive animal agriculture. Excessive phosphorus in soil runs off to lakes and the sea, causing eutrophication and stimulating growth of aquatic organisms that may produce neurotoxins injurious to humans. Furthermore, the negatively charged phytic acid chelates with positively charged divalent cations (Figure 1), rendering a poor absorption of the bound metals in small intestine (Cheryan 1980). This is partially attributed to the wide-spreading human nutritional deficiencies of calcium, iron, and zinc in developing countries where the staple foods are plant origin (Ferguson *et al.* 1989, Manary *et al.* 2002). As a whole, challenges in the above three areas of animal nutrition, environmental protection, and human health have prompted the fast emerging of phytase science and biotechnology.

Nomenclature of phytase

Phytases are *meso*-inositol hexaphosphate phosphohydrolases that catalyze the stepwise phosphate splitting of phytic acid (IP_6) or phytate to lower inositol phosphate esters (IP_5-IP_1) and inorganic phosphate (Figure 1). A number of phytase genes and proteins have been identified from plants and microbes including bacteria, yeast, and fungi. The first and probably the best characterized phytase is *Aspergillus niger* PhyA that is encoded by a 1.4 kb DNA fragment and has a molecular mass of 80 kDa, with 10 *N*-glycosylation sites (Han & Lei 1999, Van Hartingveldt *et al.* 1993). Average molecular masses of bacterial

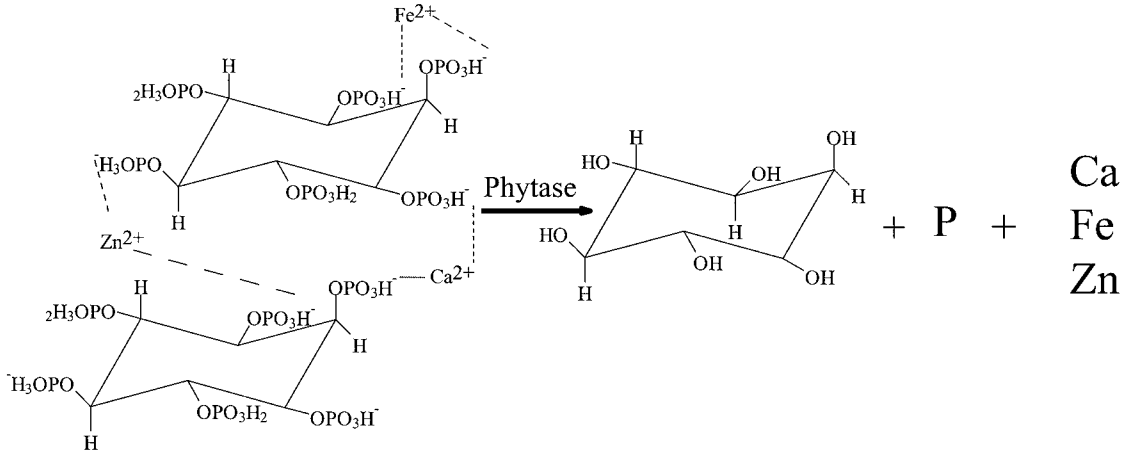

Fig. 1. Phytate hydrolysis by phytase into inositol, phosphate, and other divalent elements. Phytate is *myo*-inositol-1,2,3,4,5,6-hexakis dihydrogen phosphate that contains approx. 14 to 28% phosphorus and 12–20% calcium. Phytate also chelates trace elements of iron and zinc (1 to 2%) between phosphate groups within a single phytate molecule or between two phytate molecules. Phytase is the only known enzyme that can initiate the phosphate hydrolysis at carbon 1, 3 or 6 in the inositol ring of phytate. The removal of phosphate group by phytase results in releasing of calcium, iron, zinc, and other metals.

phytases are smaller than those of fungal phytases (40–55 vs. 80–120 kDa), mainly due to glycosylation differences (Choi *et al.* 2001, Golovan *et al.* 2000, Han & Lei 1999, Kerovuo *et al.* 1998, Rodriguez *et al.* 2000b, Ullah *et al.* 2000, Van Hartingveldt *et al.* 1993). The molecular masses of plant phytases isolated from corn, wheat, lupine, oat, and barley range from 47 to 76 kDa (Greiner 2002, Greiner *et al.* 2000a,b, Greiner & Larsson Alminger 1999, Maugenest *et al.* 1997).

Phytases can be divided into two groups based on the initiation site of phosphate hydrolysis in the carbon ring of inositol. Microbial phytases, especially those of fungal origin (E.C. 3.1.3.8), often split the phosphate group at the C_1 or C_3 (carbon) of the inositol ring (D-and L-configuration), and are called 3-phytases. Plant phytases (E.C. 3.1.3.26) act preferentially at the C_6 carbon, and are called 6-phytase. However, phytases from *Escherichia coli* (Greiner *et al.* 1993) or *Peniophora lycii* and basidiomycete fungi (Lassen *et al.* 2001) are exceptions. Catalytically, most of phytases belong to the family of histidine acid phosphatases that is characterized by a conserved active site hepta-peptide motif RHGXRXP and the catalytically active dipeptide HD. This group of enzymes catalyze the phytic acid hydrolysis in two steps: a nucleophilic attack from the histidine in the active site of the enzyme to the scissile phosphoester bond of phytic acid (Ostanin *et al.* 1992, Van Etten 1982, Vincent *et al.* 1992) and protonation of the leaving group by the aspartic acid residue of the HD motif (Ostanin & Van Etten 1993). However, exceptions are the phytases isolated from different *Bacillus* sp. whose gene sequences are not homologous to any of the histidine acid phosphatases in the data bank or do not display the active site hepta-peptide motif RHGXRXP or the catalytically active dipeptide HD. Those enzymes have a six-bladed folding scaffold for phytase activity and two distinct features: metal-assisted increase in thermostability and metal-mediated activation of activity (Ha *et al.* 2000, Kerovuo *et al.* 1998, 2000, Kim *et al.* 1998). Meanwhile, a soybean phytase is a purple acid phosphatase with a dinuclear Fe-Fe or Fe-Zn center in the active site (Hegeman & Grabau 2001). All phytases have pronounced stereospecificity and a strong preference for equatorial phosphate groups, while they are virtually unable to cleave axial phosphate groups.

Characteristics of phytase

Phytase activity is usually measured by the amount of inorganic phosphate released per min from a selected substrate under certain pH and temperature. Just like other enzymes, phytase activity or function is affected by the inherent properties of the enzyme and the action conditions. The following properties of phytase are of practical significance:

Substrate specificity

There has been intensive research on the substrate specificity and affinity of different phytases (Greiner *et al.* 2002, Ullah & Phillippy 1994, Wyss *et al.* 1999a). Microbial phytases (*A. niger, E. coli, Bacillus* sp.) seem to have a high affinity to phytic acid, whereas plant phytases and some fungal enzymes such as the one from *A. fumigatus* have a broader substrate specificity (Wyss *et al.* 1999a) and are capable of degrading the lower inositol phosphates. Although most phytases are able to degrade phytic acid to the monophosphate ester of inositol as the final product (Greiner *et al.* 2002, Ullah & Phillippy 1994, Wyss *et al.* 1999a), phytases from *Bacillus* sp. hydrolyze preferentially every second phosphate over the adjacent ones and degrade the phytic acid molecule to inositol triphosphate [IP_3 either as IP_3(2,4,6) or IP_3(1,3,5)] as the final product (Kerovuo *et al.* 2000).

pH and temperature optima

Most isolated phytases have their pH optima in the range of 4.5–6. But, phytases from *Bacillus* sp. have neutral or alkaline pH optima (Choi *et al.* 2001, Kim *et al.* 1998). The pH profile of *A. niger* phytase (*phyA*) is featured by two pH optima at 2.5 and 5.5, respectively, and a dip in activity between these two points (Han & Lei 1999). Mullaney *et al.* (2002) used site-directed mutagenesis to remove the drop of activity in the pH range 3–5 for maintaining a high specific activity at the stomach pH. The temperature optima of most plant and microbial phytases range from 45 to 60 °C. These relatively high optimal temperatures preclude a full activity of phytases at the stomach temperatures of swine or poultry (37–40 °C), and result in even poorer performance of phytases in fish.

Thermostability

Because commercial feeds are often pelleted, a process using high temperature (60–80 °C) and steam, all feed enzymes need to be heat stable to avoid substantial activity loss during this process. Thermostability of any given phytase, just like other proteins, is decided by its ability to resist heat denaturation such as in the case of hyperthermophilic organisms and(or) its ability to refold appropriately into the native-like, fully active conformation after heat denaturation (Wyss *et al.* 1998). This later premise may be affected by environmental conditions such as buffer specificity (Rodriguez *et al.* 2000a). A major drawback of thermostable enzymes is their usually low specific activity at ambient temperatures, which is associated to a great rigidity and thus decreased flexibility of the protein. Chemical coating of phytases has been used to improve their heat stability, but the coating may somewhat compromise the release and function of the enzymes in stomach. Spraying liquid phytase preparations to feed after pelleting can by-pass the activity loss by pelleting, but increases labor and equipment costs. As discussed below, there are biotechnological means for improving the thermostability of phytases.

Proteolysis resistance

An effective phytase needs to have a strong resistance to hydrolytic breakdown by digestive proteinases in the digestive tract. Fungal and bacterial phytases show different sensitivities to pepsin and trypsin (Kerovuo *et al.* 1998, Rodriguez *et al.* 1999), and the latter seem to have a higher resistance to proteolytic degradation than the former (Igbasan *et al.* 2000). The protease-sensitive sites of phytases, normally in the exposed loops at the surface of the molecules, may be blocked or modified using site-directed mutagenesis (Wyss *et al.* 1999b).

Current and potential applications of phytase

Up to now, phytase has been mainly, if not solely, used as a feed supplement in diets largely for swine and poultry, and to some extent for fish. Numerous laboratory experiments and field trials have shown that 500 to 1000 units of phytase can replace approx. 1 g inorganic phosphorus supplementation and reduce total phosphorus excretion by 30–50% (Kemme *et al.* 1997, Lei *et al.* 1993b, Liu *et al.* 1997, Yi *et al.* 1996). Thus, the benefits of phytase are two-fold: saving the expensive and non-renewable inorganic phosphorus resource by reducing the need for its inclusion in animal diets and protecting the environment from pollution of excessive manure phosphorus runoff.

Several dietary factors may reduce or enhance phytase-feeding efficacy. High levels of dietary calcium or calcium:phosphorus ratios reduce the effectiveness of phytase (Lei *et al.* 1994, Sandberg *et al.* 1993). Moderate to high levels of inorganic phosphorus may inhibit the full function of phytase. Supplemental organic acids such as citric acid or

lactic acid enhance phytase efficacy (Han *et al.* 1998, Jongbloed *et al.* 2000, Maenz *et al.* 1999). Hydroxylated cholecalciferol compounds improve dietary phosphorus and zinc utilization by chicks in an additive manner with phytase (Biehl *et al.* 1995). Supplementing different phytases in combination have not shown any benefit over the singular additions. However, adding phytase with other hydrolytic enzymes seems to produce a synergism (Zyla *et al.* 1995).

There is a great potential of phytase in improving mineral nutrition of humans. A large portion of world population ingest a high level of phytate from the plant staple foods, suffering from iron and zinc deficiencies (Bentley *et al.* 1997, Ohri-Vachaspati & Swindale 1999, Tatala *et al.* 1998). In addition, infant soy formula and other soy protein products also contain high levels of phytate (Lönnerdal *et al.* 1988, 1999). Effectiveness of supplemental phytases in reducing the phytate content of legume-derived food products (Fredrikson *et al.* 2001, Greiner & Konietzny 1999), and improving nutritional availability of zinc and iron from plant-based diets to animals have been documented (Lei *et al.* 1993a, Stahl *et al.* 1999). Ability of phytases to release iron from phytate in wheat or bread has been shown *in vitro* (Porres *et al.* 2001) and in human studies (Sandberg *et al.* 1996). Future research is needed to determine the optimal dose and appropriate delivery of phytase to human foods. In addition, phytases can be used to prepare novel specific phytic acid-derivatives (Greiner & Konietzny 1996) or improve soil fertilization and nutrient uptake by plants (Hayes *et al.* 2000, Richardson *et al.* 2001).

Biotechnology of phytase

If the heightened environmental awareness of phosphorus pollution originating from animal waste created the needs for phytase, biotechnology has led its fast development to the current stage. Although phytase was initially shown to hydrolyze phytate-phosphorus in diets for chicks 30 years ago (Nelson *et al.* 1971), the commercial application had not been feasible for many years, due to the low activity yield and the anticipated high cost of the conventional phytase fermentation system. With the development of heterologous microbial expression systems, large amounts of the enzyme could be produced for animal feed use at relatively low costs.

Microbial expression systems

Submerged or solid-state fermentation of phytase-overexpressing filamentous fungi (i.e., *Aspergillus* species) produces good yields of phytase at low cost (Pandey *et al.* 2001). Recently, a great research effort has been made towards the use of methylotrophic yeast (*Pichia pastoris, Hansenula polymorpha*) (Han & Lei 1999, Rodriguez *et al.* 2000a,b, Wyss *et al.* 1999a,b). Phytase has also been expressed in *Stretptomyces lividans* (Stahl *et al.* 2003) and *Lactobacillus plantarum* (Kerovuo & Tynkkynen 2000). The latter expression system offers a possibility of combining phytase with the beneficial probiotic lactic acid bacteria. A fungal phytase has been successfully expressed in seeds of soybean or alfalfa (Li *et al.* 1997, Ullah *et al.* 2002).

Transgenic plants and animals

Transgenic rice has been developed to over-express genes encoding for phytase from *Aspergillus fumigatus*, ferritin from *Phaseolus vulgaris*, and a cysteine-rich metallothionein-like protein to improve rice iron bioavailability to humans. The plant has been crossed with a recently developed β-carotene producing rice line (Lucca *et al.* 2001). Meanwhile, transgenic mice and pigs have been generated by overexpressing phytase in their salivary glands (Golovan *et al.* 2001a,b).

Protein engineering

Although properties of phytases vary, there is no single wild-type enzyme that is perfect or ideal for the field application. Theoretically, an 'ideal' phytase should be catalytically efficient, proteolysis-resistant, thermostable, and cheap (Lei & Stahl 2001). In reality, phytase of this good may never be found or generated. However, single or multiple traits of phytases have been successfully improved by genetic manipulations. Site-directed mutagenesis, based on crystal structure of phytases (Kostrewa *et al.* 1997, 1999, Lim *et al.* 2000), has been used to improve the specific activity of the heat-stable *A. fumigatus* phytase (Tomschy *et al.* 2000), thermostability of *E. coli* AppA phytase (Rodriguez *et al.* 2000b), and pH profile of *A. niger* PhyA phytase (Mullaney *et al.* 2002). Based on the conserved sequences of multiple homologous fungal phytases, a series of experimental consensus phytases with good thermostability and catalytic efficiency have been synthesized (Lehmann *et al.* 2000a). Exchanging the active sites between phytases (Lehmann

et al. 2000b) or designing structure-based chimeric enzymes (Jermutus *et al.* 2001) has been successfully applied to improve phytase thermostability, specific activity, and pH optimum.

Issues related to phytase

Despite all benefits of phytase, there are concerns over its application that warrants further research. As a strong chelator of iron and zinc, phytate in plant foods actually can serve as an antioxidant to reduce free radical formation mediated by these metals. Indeed, we have shown that pigs fed phytase for 4 months became more prone to high-iron-induced lipid peroxidation in colon than the control pigs (Porres *et al.* 1999). As food-producing animals live for a relatively short period of time and do not normally receive high levels of dietary iron, feeding phytase or low-phytic acid (Sand *et al.* 2003, Veum *et al.* 2001) ingredients does not likely cause any health problem. However, low-phytic acid grain may have potential adverse effect on human health, in particular in those with high iron stores caused by high dietary intakes of highly available iron from animal products or high dietary intakes of fruits that greatly enhance the absorption of non-heme iron (Fleming *et al.* 2002). Thus, adequate caution should be given in promoting that low-phytic acid grain strategy from the animal production point to a broad application. The other concern is whether supplemental phytases hydrolyze phytate-phosphorus from digesta more than the animals can absorb, releasing more free phosphorus to the environment than that without phytase (Dao 2003). In fact, total soluble phosphorus excretion is substantially reduced in animals fed phytase although their relative percentage of soluble phosphorus in the total excreta phosphorus is marginally increased (Xavier *et al.* 2003). Meanwhile, appropriate phytase doses may be titrated with phosphorus needs and dietary conditions (Kemme *et al.* 1997) of animals. For workers handling phytase, inhalation exposure may produce immune responses such as work-related asthmatic and other respiratory symptoms (Doekes *et al.* 1999). The hypersensitivity symptoms can be avoided by improving local exhaust systems and wearing of all protective clothing and masks with P2 filters (Baur *et al.* 2002).

Concluding remarks

As phytase is increasingly used worldwide, science and technology related to the enzyme have evolved to a new exciting field at a fast pace. Clearly, supplemental phytases improve dietary phytate-phosphorus utilization by food-producing animals, and reduce environmental pollution of phosphorus from animal waste in areas of intensive animal production. Potentials of phytase in improving human nutrition and health and in developing specific phytic acid- or inositol-derived products have increasingly been gained attention and will expand as a new direction of phytase. Biotechnology has been and will continue to be an exceptionally effective tool for developing and improving phytase enzymes and their delivery systems.

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