CHAPTER 29

PHYTASE: SOURCE, STRUCTURE AND APPLICATION

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1. INTRODUCTION

Phytases have been one of the focal enzymes for nutrition, environmental protection, and human health during the past two decades. These enzymes sequentially cleave orthophosphate groups from the inositol core of phytate, the major chemical form of phosphorus in plants. Various phytases have been isolated from plants and microbes, and can be grouped based on their pH optima (alkaline or acid phytases), catalytic mechanisms (histidine acid phosphatases, ß-propeller phytase, cysteine phosphatases or purple acid phosphatases), or stereospecificity of phytate hydrolysis (3- or 6-phytases). Recent phytase research has been driven by the urgent need for improving utilization of phytate-phosphorus in diets for simple-stomached animals to reduce their manure phosphorus excretion to environment. However, potential applications of phytases may extend to release dietary phytate-bound minerals for human nutrition and to develop special inositol phosphates for human health.

2. SOURCES OF PHYTASE

2.1. Microbes

Phytases have been isolated from fungi, yeast, bacteria, and protozoa. Most these enzymes belong to the histidine acid phosphatase or alkaline phytase sub-families, and exhibit considerable variations in kinetics, stereospecificities, and biochemical properties. Several microbial phytases have been commercialized as animal feed supplements.

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2.1.1. Fungal and yeast phytases

Usually classified as 3-phytases, most of phytases isolated from fungi and yeast are histidine acid phosphatases, glycosylated, and active for a wide variety of substrates (Wyss *et al*., 1999a). *Aspergillus niger* PhyA was the first well-characterized and commercialized phytase. Encoded by a 1.4 kb DNA fragment, this enzyme is a monomer with an approximate molecular weight of 80 kDa, a bi-hump pH profile with two optimal pH at 2.5 and 5.0–5.5, an optimal temperature at 55–60 °C, and high affinity for phytic acid (Han *et al*., 1999). *Aspergillus fumigatus* phytase shares a 66% sequence similarity with *A. niger* PhyA phytase, but displays better thermo-tolerance (Pasamontes *et al*., 1997a; Wyss *et al*., 1998). Its thermo-tolerance was related to a great efficiency of refolding after heat denaturation, and can be modulated by specificity of the buffers used in the heat treatment (Rodriguez *et al*., 2000a). The enzyme has a broad range of pH, and is highly active against inositol phosphates with low degree of phosphorylation (Wyss *et al*., 1999a; Rodriguez *et al*., 2000a). However, its specific activity against phytate is low (Tomschy *et al*., 2000). *Peniophora lycii* PhyA phytase has also been commercialized. It is a 6-phytase with an optimal pH at 4.0–4.5 and optimal temperature at 50–55 °C, and has dimeric conformation (Lassen *et al*., 2001). It seems to be susceptible to thermal treatments and proteases (Simon and Igbasan, 2002) or low pH.

Quan *et al*. (2004) have isolated a low molecular weight (32.6 kDa) phytase from the air-borne fungus *Cladosporium* sp. FP-1. The enzyme is not glycosylated, and has an optimal pH at 3.5 and an optimal temperature at 40 °C. It produces inositol tri-phosphate as the final product. Phytases isolated from thermophilic fungi *Myceliophtora thermophila* and *Talaromyces thermophilus* (Mitchell *et al*., 1997; Pasamontes *et al*., 1997b) exhibit a high degree of sequence homology to other fungal phytases from *A. niger, A. terreus* or *A. fumigatus*. Berka *et al*. (1998) isolated a phytase from the thermophilic fungus *Thermomyces lanuginosus* that demonstrated a better thermostability and catalytic efficiency, and a higher transition temperature than that of the *A. niger* phytase. Chadha *et al*. (2004) reported that phytase produced by the thermophilic fungus *Mucor pusillus* was fairly active in a wide pH range of 3 to 7.8.

From a survey on 738 strains of yeast, Nakamura *et al*. (2000) found significant levels of phytase activity in 35 species, with a wide range of optimal pH and temperature. *Arxula adeninivorans* grew well in media containing phytate as the sole source of phosphate, and secreted phytase that had an optimal pH in the range of 4.5–5.0, and optimal temperature around 75 °C (Sano *et al*., 1999). A significant phytase production has also been reported by Quan *et al*. (2002) from the soil-isolated yeast *Candida Krusei* WZ-001. The isolated phytase contained two different subunits with molecular masses of 116 and 31 kDa, had a glycosylation rate of approximately 35%, and exhibited optimal pH and temperature at 4.6 and 40 °C, respectively. Phytase activity has also been detected in *Pichia anomala* (Vohra and Satyanarayana, 2001), *Saccharomyces cerevisiae* (Türk *et al*., 2000), and *Schwanniomyces castellii* (Segueilha *et al*., 1992). These enzymes were active in the acidic pH range, with an optimal temperature at 60–74 °C.

2.1.2. Bacterial phytases

Phytases isolated from bacteria are non-glycosylated histidine acid phosphatases or alkaline phytases with a ß-propeller structure. *Escherichia coli* AppA phytase is a periplasmic protein with a molecular mass of approximately 42 kDa. Because of its acidic optimal pH, strong resistance to pepsin hydrolysis and high specific activity for phytic acid (Wyss *et al*., 1999a), *E. coli* AppA phytase is more effective than *A. niger* phytase in releasing phytate-phosphorus in diets for swine and poultry (Augspurger *et al*., 2003). Meanwhile, a novel *E. coli* phytase gene (*appA2*) has been isolated from pig colon, and expressed in *Pichia pastoris* (Rodriguez *et al*., 1999b). *Bacillus* phytases are monomers with a molecular mass of 38–47 kDa, optimal pH in the neutral range and optimal temperature at 55–70 °C (Kerovuo *et al*., 1998).

Yanke *et al*. (1998) identified several phytase-producing anaerobic bacteria in cattle rumen, and found *Selenomonas* species to be the highest producer followed by a strain of *Mitsuokella multiacidus*. Cho *et al*. (2003) isolated a phytase enzyme from *Pseudomonas syringae* with molecular mass of 45 kDa, specific activity of 649 U/mg protein, pH optimum at 5.5 and temperature optimum at 40 °C. Kim *et al*. (2003) isolated a novel phytase from *Citrobacter braaki* with optimal pH and temperature at 4 and 50 °C, respectively, and higher specificity against phytic acid than other phosphorylated substrates. Phytases have also been isolated from *Obesumbacterium proteus* (Zinin *et al*., 2004), soil bacterium *Klebsiella* spp. ASR1 (Sajidan *et al*., 2004), and several species of the *Bifidobacterium* genera (Haros *et al*., 2005).

2.1.3. Other micro-organisms

Freund *et al*. (1992) have reported the existence of phytase in protozoan *Paramecium tetraurelia*. The enzyme appeared to be a hexamer of 240 kDa with optimal pH of 7.0 and no requirements for divalent cations for activity, and was stereospecific in sequentially removing the phosphates at the 6, 5, 4 and 1 positions (Van der Kaay and Van der Haastert, 1995). Cheng (2005) have sequenced a putative β-propeller phytase gene from the psychrophile *Shewanella oneidensis* MR-1 that showed a 30% peptide sequence identity with that of *Bacillus spp* phytase. The importance of this finding lies, based on the presence of cold-active protein-tyrosinase phosphatases isolated from this species, in the potential application of this new phytase to aquaculture.

2.2. Plants

Most of plant phytases are histidine acid phosphatases, with an optimal pH between 4.5–6.0 and optimal temperature between 38–55 °C. However, there are wide variations of plant phytases in kinetics (K_m: 30 to 300 μM; K_{cat}: 43 to 704 s^{-1}; and specific activity: 43 to 636 U/mg protein). Plant phytases in the histidine acid phosphatase family were generally considered as 6-phytase. However, recent data indicated that some of them (Lupin LP11 and LP12) initiated the hydrolysis of the orthophosphate at the D-3 position of the inositol ring (Greiner *et al*., 2002).

Some plant phytases are found to be alkaline phosphatases, or purple acid phosphatases. The phytase from lilly pollen showed an optimal pH of 8 and an optimal temperature of 55 °C (Jog *et al*., 2005). This enzyme was activated by calcium and inactivated by EDTA, and had a narrow substrate specificity, with D-Ins(1,2,3)P3 as the end product. Hegeman (2001) have isolated a phytase gene from germinating soybean that did not share any sequence similarity to histidine acid phosphatase, but a high degree of sequence similarity to purple acid phosphatase that contains a binuclear Fe(III)-Me(II) center. The enzyme displayed optimal pH at 4.5–5.0 and optimal temperature at 58 °C.

Phytase activity has been detected in cereals, legumes, and oil seeds (Viveros *et al*., 2000) or highly consumed fruits and vegetables like the avocado and scallion leaves (Phillippy and Wyatt, 2001). Certain cereal grains (*i.e.* wheat, spelt, rye, barley, triticale) display high levels of phytase activity that can reach more than 5,000 units/kg. Use of these grains and their by-products as a plant phytase source has been tested in animal feeding (Han *et al*., 1997). Industrial or household processing such as germination, fermentation, and soaking can be employed to make good uses of intrinsic phytase activity present in plant foods (Fredlund *et al*., 2003; Porres *et al*., 2003).

2.3. Animal Tissues

Although phytase activity has been detected in tissues of several animal species (Bitar and Reinhold, 1972), there is no complete molecular characterization of any of animal-derived phytases. Many of these enzymes display an optimal pH in the neutral to alkaline range, with K_m for phytate ranging from 0.03 to 2.6 mM. However, phytase detected in brush border vesicles of poultry by Maenz (1998) showed an optimal pH of 5.5–6.0 and phytase in the hybrid stripped bass showed an optimal pH of 3.5–4.5 (Ellestad *et al*., 2002).

Even though phytases have been isolated from the intestinal brush border membrane (Maenz and Classen,, 1998; Ellestad *et al*., 2002), their practical importance for improving the availability of dietary phytate-phosphorus to simple-stomached animals could be over-shaded by the very affordable supplementation of exogenous phytase in feed. Phytase activity found in the large intestine or rumen is mainly microbial origin (Wise and Gilburt, 1982; Yanke *et al*., 1998).

3. STRUCTURE

The basic structural features of several phytate degrading enzymes have been established as representatives of previously known classes of phosphatase (Mullaney and Ullah, 2003; Chu *et al*., 2004). In others, X-ray crystallographic studies have confirmed they belong to a class with a novel catalytic mechanism (Ha *et al*., 2000). In both instances, the elucidation of the 3-D molecular structure of different phytate-degrading enzymes has enhanced our understanding of the linkage between the molecular structure of the molecule and it's catalytic function. It is now evident

that different phytases have evolved to supply the unique nutritional requirements found in various forms of plant, animal and microbial life. It also appears that there is a direct link between an enzyme's catalytic domain and specific molecular architectural elements. Thus, while specific structural components are essential, other nonessential parts of the molecule may be altered to adapt the catalytic mechanism to various substrates. The precise number of catalytic mechanisms that nature has evolved to hydrolyze phytate will be determined by future research. At this time, four classes of phosphatase enzymes are known to have representatives that can degrade phytic acid: (1) Histidine Acid Phosphatase (HAP), (2) β-Propellar Phytase (BPP), (3) Cysteine Phosphatase (CP) and (4) Purple Acid Phosphatase (PAP). Each one of these has unique structural features due to their distinct catalytic apparatus that allows them to utilize phytic acid as a substrate in various environments.

3.1. Histidine Acid Phosphatase (HAP)

All members of the HAP share a common catalytic mechanism and common active site motif. The N-terminal active site motif is RHGXRXP and the C-terminal motif is HD (Wodzinski and Ullah, 1996). When the amino acid sequence is properly folded, these components position together to form the catalytic site of this class of phosphatases. These distant sequences converge to form a single catalytic center that initiates a two-step reaction that hydrolyzes phosphomonoesters (Van Etten *et al*., 1991).

HAPs are a large group of acid phosphatases that depending on the species can hydrolyze an array of different substrates. Thus, it is important to realize that not all HAPs can effectively degrade phytate. Phytate is a highly negatively charged substrate and in order for any catalytic mechanism to interact with it, the active site must be able to accommodate this feature. Therefore, in order to facilitate substrate binding, the active site is primarily positively charged at acidic pHs in both prokaryotic and eukaryotic HAPs that effectively hydrolyze phytate. Oh *et al*. (2004) has purposed the term Histidine Acid Phytase (HAPhy) to designate HAPs that can effectively hydrolyze phytate. Both prokaryotic and eukaryotic HAPhys are known. The best-characterized prokaryotic HAPhy is *E. coli* phytase (Greiner *et al*., 1993). A 3-D molecular model of its structure is available (Lim *et al*., 2000). In eukaryotes, HAPhys have been cloned in a number of fungal isolates and in maize (Mullaney *et al*., 2000). The most widely studied fungal phytases are from *A. niger* (Fig. 1) and *A. fumigatus*.

An important factor in determining and maintaining the structure of HAPhys is glycosylation. This process, that adds polysaccharides to proteins, confers stability and assist in the correct folding of the enzyme. All the extra cellular fungal phytases that have been characterized to date are glycoproteins. *A. niger* NRRL 3135 PhyA is heavily glycosylated with ten N-glycosylation sites (Ullah and Dischinger, 1993). Another vital structural component in HAPhys are disulfide bridges that perform an important role in maintaining the proper 3-dimensional structure to allow for catalytic activity in phytase (Wang *et al*., 2004; Mullaney, 2005; Kostrewa *et al*., 1997). All ten cysteine residues present in *A. niger* and *A. fumigatus* PhyA

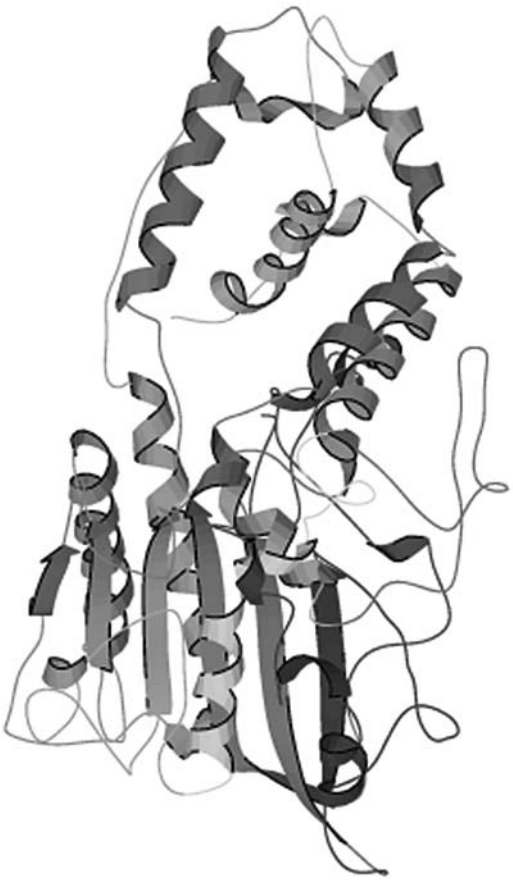

Figure 1. Structure of *Aspergillus (ficuum) niger* NRRL 3135 PhyA (Kostrewa *et al*., 1997), a representative model of histidine acid phosphatases

are involved in the formation of disulfide bridges. All of the eight cysteines in *E. coli* phytase are involved in disulfide bonds (Lim *et al*., 2000). However, in this phytase, significantly enhanced activity was achieved when site-directed mutagenesis was utilized to remove one disulfide bridge (Rodriguez *et al*., 2000b). It was suggested that its removal allowed enhanced domain flexibility and thereby increase the catalytic efficiency of the enzyme.

Structural characterization (Kostrewa *et al*., 1999; Liu *et al*., 2004) and catalytic studies (Wyss *et al*., 1999a) have assigned a vital role to a new site in the enzyme that facilitates its interaction with different substrates. Kostrewa *et al*. (1999a) identified several amino acid residues that constitute a substrate specificity site (SSS) in the *A. niger* PhyA molecule that encircles the enzyme's active site and functions as a "gatekeeper". In the SSS of *A. niger* NRRL 3135 there are two

acidic and four basic amino acid residues: E228, D262, K91, K94, K300 and K301 (Kostrewa *et al*., 1999; Mullaney *et al*., 2000). At pH 2.5 the four basic amino acids; K91, K94, K300 and K301 in the *A. niger* SSS are all positively charged and would attract the phytate molecule. Also, the local electrostatic field of the SSS remains attractive for phytate when the pH is raised to 5.0.

Wyss *et al*. (1999a) has divided all the known microbial HAPhys into two classes based on the substrate specificity. The first class has broad substrate specificity but a low specific activity for phytate, while the second class has narrow substrate specificity and a high specific activity for phytate. It has since been discovered that a correlation exist between amino acid residue 300, a SSS component, and the enzyme's level of specific activity for phytic acid (Mullaney *et al*., 2002). This study also revealed that while amino acid residue 300 varied, residue 301 was strongly conserved as lysine (K). The phytate degrading enzymes cited in Wyss *et al*. (1999a) with high specific activity for phytic have either a basic or acidic amino acid residue at 300, while the phytases with low specific activity have a neutral amino acid at that position. The importance of the lysine residue at that site and the enzyme's high specific activity for phytic acid has been established by site-directed mutagenesis (Mullaney *et al*., 2002).

The importance of the SSS in determining pH optimum and substrate specificity range is evident in a second extracellular *A. niger* phytase, PhyB (Ullah and Cummins, 1987). PhyB has only been reported in the isolates of *A. niger*. PhyB's optimum pH is 2.5 and unlike PhyA displays no ability to hydrolyze phytate at pH 5. While PhyB and PhyA share identical active site characteristics of HAPs, their SSS, are different. Kostrewa *et al*. (1999) identified the SSS of PhyB to be composed of only two acidic amino acids, D75 and E272. This explains why PhyB has a different pH profile than PhyA. At pH 5.0 the acidic amino acids in the PhyB SSS would be negatively charged, while at pH 2.5 they would be uncharged. All negatively charged substrates, such as phytate, would therefore be repelled at the higher pH. Because PhyB's SSS has a more neutral electrostatic field, it can accept a broader variety of phosphomonoesters than PhyA. The highly positive electrostatic field of *A. niger's* PhyA SSS is optimized for the binding of a negatively charged substrate, such as phytate.

This evidence indicates the SSS has a significant role in determining how effectively the enzyme can hydrolyze phytate. By occupying positions adjacent to the catalytic domain, the amino acids in the SSS function as "gatekeeper" in determining which substrates can easily pass and interact with the active site residues. Research is also showing that techniques such as site-directed mutagenesis can be employed to alter the composition of the enzyme's SSS and thus, alter both the enzyme's pH profile and substrate range.

3.2. β–Propeller Phytase (BPPhy)

The molecular structure of a thermostable phytase (TS-phy) from *Bacillus amyloliquefaciens* has been identified (Ha *et al*., 2000). This phytate-degrading

enzyme is not a member of the histidine acid phosphatase class of enzymes, but rather represents an entirely new class of enzyme that displays no obvious homology to any known phosphatase class. Unlike the HAPhys, which are members of a well-studied class of enzymes, the *Bacillus* phytases represent an entirely new class of enzymes and exhibit no homology to any known phosphatases (Kim *et al*., 1998; Kerovuo *et al*., 1998; Ha *et al*., 2000). The name β–Propeller Phytase (BPPhy) was adopted for the group after its molecular structure was determined, which consists mainly of β-sheets and resembles a six bladed propeller (Fig. 2) (Ha *et al*., 2000; Shin *et al*., 2001).

The first reported BPPhys were from *Bacillus* and related bacterial species. All these reported enzymes were similar in that they require Ca^{2+} for both catalytic activity and thermostability. The calcium ions are thought to facilitate the binding of phytate by generating a favorable electrostatic environment. Thus, as in the SSS HAPhys the substrate binding domain of the biocatalyst attracts the substrate. Kinetic studies at pH 7.0–8.0 have established that BPPhys can hydrolyze calcium-phytate at that pH range (Oh *et al*., 2001). Two main components are involved in the catalytic mechanism of BPPhys. The "affinity site" attracts the substrate and an adjacent "cleavage site" to hydrolyze the phosphate group (Shin *et al*., 2001). This model explains BPPhy preference for phytate, since it is necessary for two neighboring phosphate groups to occupy both the cleavage and affinity sites. The enzyme prefers hydrolysis of every second phosphate. This explains why this enzyme alternately removes phosphate groups with the end product being *myo*-inositol triphosphate. Degradation of phytate to IP_3 occurs rapidly, but further hydrolysis is not favored because a neighboring phosphate group is lacking.

Based on its reported narrow substrate range, a requirement for calcium for catalytic activity and IP_3being the predominant product from phytate hydrolysis,

Figure 2. Structure of thermostable *Bacillus* sp phytase (Ha *et al*., 2000), a representative model of β-propeller phytase

Oh *et al*. (2004) have proposed that plant alkaline phytases may share a similar catalytic mechanism with BPPhys. Like BPPhys the activity of several plant alkaline phytases, such as lily (*Lilium longiflorum*) pollen (Scott and Loewus, 1986) and a number of legumes (Scott, 1991), is enhanced by calcium. However, none of the plant alkaline phytase genes have been cloned and no sequence data exist to determine if and which of them are BPPhys.

3.3. Cysteine Phosphatase (CP)

It had long been suspected that the presence of certain micro-organisms in the rumen was the reason ruminants could utilize phytic acid and monogastric animals could not. A survey of anaerobic ruminal bacteria has recently revealed phytase activity in one isolate, *Selenomonas ruminantium* (Yanke *et al*., 1999). The isolation of this micro-organism and the characterization of its unique enzyme have in turn yielded another phytate degrading class of enzymes. Initial characterization established that the enzyme was monomeric, approximately 46 kDa in size, had an optimal pH range of 4.0–5.5, an optimal temperature of 50–55 °C and was inhibited by cations of iron and several other metals. Subsequent studies of this enzyme reveal that it is neither a HAPhy nor a BPPhy (Chu *et al*., 2004). Its structure and proposed catalytic mechanism suggest it is a member of the cysteine phosphatase (CP) superfamily. Its deduced amino acid sequence contains the active site motif HCXXGXXR(T/S) and other substantial similarities with protein tyrosine phosphatase (PTP), a member of the CP group. The active site forms a loop that functions as a substrate binding pocket unique to PTPs (Fig. 3). The depth of this pocket is important because it appears to determine the substrate specificity (Denu and Dixon, 1998). Consistent with this model *S. ruminantium* phytase, the cysteine phytase (CPhy), has a wider and deeper pocket than PTP and thus is able to accommodate the fully phosphorylated inositol group of phytic acid (Chu *et al*., 2004). This, plus the presence of a favorable electrostatic environment, allows it to accommodate phytate as a substrate while other members of this group of enzymes lack this capability.

The current model suggests that the initial binding of phytate to the CPhy active site pocket is facilitated by the negatively charged substrate. The hydrolysis of phosphate groups proceeds sequentially with the end product being inositol 2-monophosphate (Chu *et al*., 2004). The inhibitory effect of iron and other metal cations (Cu^{2+}, Zn^{2+} and Hg^{2+}) has been attributed to their complexing with the substrate, but the stimulatory effect of lead cations remains unexplained (Yanke *et al*., 1999).

3.4. Purple Acid Phosphatase (PAP)

All members of purple acid phosphatases (PAP) metallophosphoesterases class contain a unique set of seven metal-liganding amino acid residues. These seven metal-liganding residues (D, D, Y, N, H, H, H) are contained in a shared a pattern

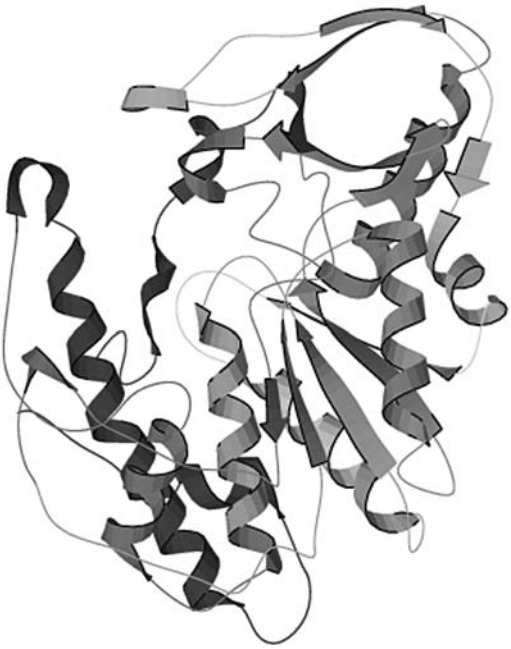

Figure 3. Structure of *Selenomonas ruminantium* phytase (Chu *et al*., 2004), a representative model of cysteine phosphatases

of five common consensus motifs ($DxG/GDx_2Y/GNH(E, D)/Vx_2H/GHxH$) (Schenk *et al*., 2000). This is a large class of phosphtases with known representatives in plants, mammals, fungi and bacteria (Schenk *et al*., 2000; Olczak *et al*., 2003). As in the HAPs and CPs, not all of these enzymes effectively utilized phytate as a substrate. A binuclear metallic center containing two irons is found in animal PAPs, while in plant PAPs the second iron ion is replaced by either a zinc or manganese ion (Olczak *et al*., 2003). The plant PAPs are further divided into two classes: small monomeric proteins (around 35 kDa) and large homodimeric proteins (about 55 kDa). The first binuclear metal-containing hydrolase identified as a phytase was reported in the cotyledons of a germinating soybean (*Glycine max* L. Merr.) seedling (Hegeman and Grabau, 2001). Sequence analysis of GmPhy and other plant PAP phytases (PAPhy) reveal they are similar to the homodimeric plant PAPs.

The adaptation of PAPs in plants to degrade phytate may be a unique case. A fungal PAP, *A. niger* (Apase6) has been isolated and cloned (Ullah and Cummins, 1988; Mullaney *et al*., 1995), but it does not effectively utilize phytate as a substrate. When the active site of A. *niger* Apase 6 and the soybean PAPhys are compared they both contain the conserved active site motif (Mullaney and Ullah, 1998). This indicates the plant PAPhys may have evolved to fill this role as a phytase. However, unlike HAPhys, BPPhys and CPhys, no X-ray crystallography study has

been performed on PAP phytases and no information on the adaptation of the PAPhy active site to phytate as a substrate is available.

Table 1 summarizes the structural division of phytate degrading enzymes based on mechanistic enzymology. This classification scheme furthers the model developed by Oh *et al*. (2004) by incorporating two classes of phytases, PAPhys and CPhys not included in their system. While there are numbers of HAPs, PAPs and CPs that cannot degrade phytic acid effectively, members of each class do share a common enzymatic pathway with other members that do hydrolyze phytate efficiently. How each of these different catalytic mechanisms has evolved to hydrolyze phytate is now being elucidated. This classification system also enables uncharacterized phytases to be assigned based on unique characteristics (calcium requirement, pH profile, end product, *etc*.) associated with each enzyme class. This system also allows for new groups of phytases to be added and existing groups subdivided when desirable.

Table 1. Structural Classes of Phytate Degrading Enzymes

Enzyme Family	Unique Structural Feature	Catalytic mechanism/Adaptation to hydrolyzes Phytate	Examples
Histidine Acid Phosphatase	N-terminal RHGXRXP C-terminal HD consensus motif	N-terminal H forms a phosphohistidine intermediate, C-terminal acts as proton donor/Substrate specificity site residues positively charged	*A. niger* *P. Lycii* *E. coli* *Zea mays* L.
β Propeller Phytase	Six-bladed β propeller shaped molecule	Mechanism consist of an affinity site and a cleavage site. Affinity sites binds phosphate group while other site attacks adjacent phosphate group./Dual site favors IP_6, $IP_{5,\ or}$ IP_4 as substrate.	*Bacillus* sp *X. oryzae*
Cysteine Phosphatase	P loop structure contains HCXXGXXR(T/S) consensus motif	Protein tyrosine phosphatase mechanism cleaves phosphate groups/Deeper active site pocket accommodates phytate	*S. ruminatium*
Purple Acid Phosphatase	Consensus motif: DXG/GDXXY/GNH (E,D) /VXXH/GHXH	Metalloenzymes, phylogenetically linked to large plant PAP/unknown	*Glycine max* *M. truncatula*

4. APPLICATIONS OF PHYTASE

Since the first commercial phytase product Natuphos® was launched in 1991, the market volume has reached ca. 150 million euros and will likely expand with new applications. The main application is still as a feed supplement to improve P bioavailability in plant feed-stuffs via the enzyme-mediated hydrolysis of phytate. Most importantly, the improved utilization of the phosphate deposits in the feed results in a substantial reduction in the phosphate content in animal manure and hence decreases of phosphate load on the environment in areas of intensive animal agriculture. High dietary P bioavailability reduces the need for supplemental inorganic phosphorus such as mono- and dicalcium-phosphate (MCP, DCP). Because of the strong economic growth in China and India along with the oil price hike, the supply and cost of MCP and DCP has become a practical issue. Furthermore, inorganic phosphate is non-renewable resource, and it has been estimated that the easily-accessible phosphate on earth will be depleted in 50 years. Thus, phytase is an effective tool for natural resource management of phosphorus on a global scale.

The ban of dietary supplementation of meat and bone meal, as a cheap source of feed phosphorus, in Europe to prevent possible cross-species transfer of diseases such as BSE, has led to a profound change in the feed P management. This has given phytase a new socio-economic impact as a cost effective alternative to ensure animals to obtain adequate available P from the plant-based diets. Being the major storage form of P in seeds, plant phytate was produced in 2000 at a global yield > 51 million metric tons. This amount accounts for approximately 65% of the elemental P sold world wide as fertilizers (Lott *et al*., 2000). Apparently, phytase can turn the plant phytate into a very valuable resource of P by improving its bioavailability for animal nutrition. Denmark and the Netherlands have imposed regulations to promote the use of microbial phytases.

4.1. As a Feed Supplement

Supplemental dietary phytases have been shown to effectively improve phytate-P utilization to simple-stomached animals under various dietary conditions. In a maize based diet with little intrinsic phytase activity, the improvement derived by the supplemental phytase is generally greater than that in a barley/wheat-based diet with a significant phytase activity. In pigs, supplemental microbial phytase at 750 units/kg increased P bioavailability in maize from 18 to 56%, in wheat from 62 to 74%, and in triticale from 52 to 67% (Dungelhoef *et al*., 1994). Based on large bodies of literature, Ravidran (1995) and Maenz (2001) concluded that supplementation with microbial phytase to diets for poultry and pigs enhanced phytate-P utilization by 20 to 45%. Consequently, fecal phosphorus excretion is reduced by 42% in weanling pigs (Lei *et al*., 1993a). The P equivalency of microbial phytase for 1 g of non-phytate P in broilers is equivalent to 650 to 750 FTU phytase $\times$ kg^{1} diet (Kornegay *et al*., 1996; Schoner *et al*., 1991). In pigs, 1 g of inorganic P is equivalent to 500 FTU $\times$ kg^{-1} microbial phytase (Yi *et al*., 1996).

Phytic acid is a strong anti-nutritional factor because of the ability of phytic acid and the lower myo-inositol phosphates to form complexes with divalent metals. Thus, supplemental phytase has been shown to improve bioavailabilities of minerals (Lei *et al*., 1993a, b). Jondreville *et al*. (2005) reported that 500 units of Natuphos® replaced 30 mg of Zn as sulphate given in a maize-soya-bean meal based diet. However, increasing Ca levels from 0.4 to 0.8% in low P maize-soybean meal diets significantly reduced the efficacy of microbial phytase in weanling pigs (Lei *et al*., 1994). Effects of supplemental microbial phytases on digestibility of starch, protein, and amino acids have been reported, but inconsistent.

4.2. Potential in Human Nutrition

As mentioned above, ingesting high levels of dietary phytate severely impedes the absorption of important trace elements such as iron and zinc in digestive tracts. Partially due to this anti-nutritional effect of phytate, approximately two to three billion people, primarily in the developing world, afflict deficiencies of these nutrients. There are two ways to reduce dietary phytate intake and its negative effects. One is to develop low-phytate crops via impairing the phytic acid biosynthesis by disruption of the inositol polyphosphate kinases or other mutations (Brinch-Pedersen *et al*., 2002; Stevenson-Paulik *et al*., 2005). Although this approach has led to success for the primary goal, the low-phytate maize and soybean have shown reductions in yield and seed germination (Pilu *et al*., 2003; Oltmans *et al*., 2005). This undesirable changes may not be completely surprising as recent evidence shows that phytate is required as a cofactor for RNA editing (Macbeth *et al*., 2005), in addition to its previously recognized roles in storing P and energy for germination.

Supplementing phytogenic or extrinsic phytases into human foods is another, perhaps more effective, way to reduce the negative effect of phytate on mineral utilization. Both sources of phytases were effective in reducing bread phytate and improving iron availability (Porres *et al*., 2001). The phytase-mediated dephytinization of infant formulas, infant cereals or complementary foods has been studied, and the effectiveness in improving trace element nutrition is greater if the protein supply is from legume seeds instead of milk (Hurrell, 2004). Treatments with phytase, or in combination with other processing, have been assayed in cereal and legumes (Maklinder *et al*., 1995; Porres *et al*., 2005) for developing food ingredients with high nutritional and functional value.

There are issues to be clarified before implementing a phytase strategy in human nutrition. Although activation of the intrinsic phytase, *i.e.* during baking of whole wheat bread can decrease the phytate content by 50 to 60%, some research has indicated that the reduction must reach $> 77\%$ to show impacts on iron and zinc absorption in humans (for review see Sandberg and Andlid, 2002). Meanwhile, certain levels of dietary inositol phosphates may be health-beneficial with possible functions in antioxidation, anti-tumorigenesis, reducing serum lipids and cholesterol levels, preventing renal calculi via mineral-binding, and in diabetes

(Burgess and Gao, 2002; Jenab and Thompson, 2002). It will be a challenge to minimize the negative effect of phytate on iron and zinc nutrition without losing its potential health benefits.

4.3. Novel Industrial Uses

In consideration of the potential health values of certain inositol phosphates, phytase may be used in cost-effective bioreactors for large scale production of these compounds. Successful attempts to immobilize phytase on a variety of matrices have been made. Similarly, Quan *et al*. (2003) have attempted the immobilization of a phytase-producing *Candida crusei* cells on calcium alginate gel-beads for the preparation of specific *myo*-inositol phosphates. Variation in the composition of end products resulted from a change in the flow rate of phytic acid solution (5mM) through the bioreactor.

Fujita *et al*. (2001) have tested a mutant strain of *A. oryzae* with high phytase activity for sake brewing. When compared to the wild-type strain, alcohol fermentation was promoted in the high phytase producing strain with a subsequent increase in the yield of alcohol production. Haros *et al*. (2001) have used exogenous microbial phytase as a novel bread making additive to improve several baking and physical parameters like proofing time (24% reduction) width/height ratio of bread slices (5% reduction), specific volume (21% increase), and crumb firmness (28.3% reduction). A novel industrial use of phytase has been proposed for efficient separation of soybean β-conglycinin and glycinin by Saito *et al*. (2001). Although commercial use remains to be tested, the potential role of thermophilic phytases as powerful additives in the pulp and paper industry has been suggested (Madhavan *et al*., 2004). Phytase could act synergistically with xylanase in crude multi-enzyme preparation from xylanase-producing micro-organisms like *Streptomyces cupidosporus* (Maheswari, 2000) that are used for the treatment of pulp.

Enzyme immobilization has been employed by Vieira and Nogeira (2004) for the development of a flow injection spectrophotometric procedure to determine the amount of ortho-phosphate, phytate and total phosphorus in cereal samples, and by Mak *et al*. (2004) for the development of novel biosensors to measure phytic acid and phytase activity.

5. CURRENT RESEARCH INTERESTS

5.1. Improving Heat Stability

Most phytases from plants and micro-organisms start to lose activity around 55–60 °C (Phillippy, 2002). For example, the T_m of *A. niger phy*A phytase is 63.3 °C and the denaturation is associated with an irreversible conformational change with loss of 70% to 80% of the activity. Apparently, the limited thermotolerance is the major constraint for the application of phytase in both feed and food industry because most animal feed are pelleted at 80–90 °C in order to eliminate

Salmonella infections, and human staples are processed by boiling or baking. Therefore, intensive research and development have been directed toward screening of thermophiplilic and hyperthermophilic organisms for thermotolerant enzymes, mutagenesis of mesophilic enzymes to increase their thermostability, and design of formulations of chemical coating for protecting the enzymes from heat-denaturation. Initial success has resulted from rational design to generate a consensus phytase with a T_m of 89.3 °C (Lehmann *et al*., 2000a). The initial enzyme originating from *A. fumigatus* is not a genuine heat stable enzyme, as it posses a low T_m (62.5 °C) but can refold into a fully active conformation after cooling (Wyss *et al*., 1998).

Dozens of exogenous agents have been reported to stabilize phytase during pelleting (see Phillippy, 2002 for review). However, endogenous seed components also have a protective effect on phytase activity. Skoglund *et al*. (1997) found that the intrinsic phytase in a barley-pea-rapeseed pig diet was unaffected after pelleting at 81 °C. When transgenic rice expressing the *A. fumigatus* phytase was boiled for 20 min, only 8% of the initial phytase activity was retained (Lucca *et al*., 2001). This residual activity was much lower than that (59%) of the commercial preparation of the fungal enzyme boiled for the same period of time, indicating that the *in planta* expression may interfere with refolding of the enzyme or provide an environment unfavourable to refolding. Expression and accumulation of the consensus phytase 10-thermo-[3]-Q50T-K91A and the *A. fumigatus* phytase in the protein storage vacuoles of transgenic wheat has recently revealed that with this inter-cellular deposition, heat stability based on high unfolding temperature of the consensus is superior to high refolding capacity of the *A. fumigatus* enzyme (Brinch-Pedersen *et al*., 2006).

5.2. Shifting pH-Activity Profiles

Since many of the known phytases have pH optima that are not within the pH range of the stomach (the main site of phytate-hydrolysis), approaches have been taken to improve pH-activity profiles. Those include modification of ionizable groups directly involved in substrate specificity or catalysis, replacement of amino acid residues in direct contact with residues located in the active or substrate specificity site by means of hydrogen bonds or salt bridges, or alteration of distant charge interactions by modification of the surface charge of the enzyme. Using these strategies, Tomschy *et al*. (2002) improved the activity of *A. fumigatus* phytase and a consensus phytase at low pH.

To improve the catalytic properties of the thermostable consensus-1 phytase, Lehmann *et al*. (2000b) replaced all the divergent amino acid residues present in the active site of the consensus phytase by those of *A. niger NRRL 3135* phytase. The new phytase termed consensus-7 phytase featured a major shift in the catalytic properties that were similar to those of *A. niger NRRL 3135* phytase, thus demonstrating the feasibility of rational transfer of favorable catalytic properties. However, the active site residues transfer caused a decrease in the unfolding temperature of consensus-7 phytase compared with consensus-1 phytase.

5.3. Enhancing Acid and Protease Resistance

The low pH in the stomach (2–5) of pigs and poultry, in the crop of poultry (4–5) and the neutral pH in the small intestine (6.5–7.5) provide challenges for the stability of feed phytases (Simon and Igbasan, 2002; Phillippy, 2002). Konietzny (2002) concluded that most microbial phytases are more pH stable than plant phytases as stability of most plant phytases was reduced significantly at $4 < pH > 7.5$, whereas most microbial enzymes are relatively stable at $3 < pH > 8.0$.

An effective phytase needs to be resistant to proteolysis in the animal digestive tracts. Major differences in the resistance to pancreatic and pepsin proteases are present among various phytases. Phillippy (1999) reported that intrinsic wheat phytase is more susceptible to inactivation by pancreatin and pepsin than *A. niger* phytase. In contrast, the *A. niger* phytase is less resistant to degradation by pepsin than recombinant *E. coli* phytase (r-AppA) (Rodriguez *et al*., 1999a). In another comparison of *Bacillus subtilis* and *E. coli* with four recombinant fungal phytases, the fungal phytases were most susceptible to inactivation by pancreatin and pepsin, *Bacillus subtilis* was stable to pancreatin whereas *E. coli* was stable to both pancreatin and pepsin (Igbasan *et al.*, 2000).

Commercial phytases also need to resist degradation during production and storage. When the phytases from *A. fumigatus* and *Emericella nidulans* were expressed in *A. niger*, they were cleaved by proteases present in the culture supernatant (Wyss *et al*., 1999b). The cleavage had no effect on the activity of *E. nidulans* phytase, but significantly reduced the activity of *A. fumigatus* phytase activity. Site-directed mutagenesis at the protease sensitive sites of the *A. fumigatus* and *E. nidulans* phytases yielded mutants with significant reduced susceptibility to proteolytic degradation.

5.4. Searching for Efficient Production Systems

Phytase production has been attempted in several fungal species by either submerged or solid state fermentation. Fungal species from the *Aspergillus* genera are widely employed for phytase production (Wyss *et al*., 1999a, b; Martin *et al*., 2003), although other mesophilic fungi like *Mucor racemosus* and *Rhizopus oligosporus* (Sabu *et al*., 2002; Bogar *et al*., 2003), or the thermophilic fungi *T. aurantiacus* (Madhavan *et al*., 2004) have also been employed.

Methylotrophic yeast such as *P. pastoris* or *Hansenula polymorpha* exhibit great potential for producing high levels of *A. niger, E. coli*, and *A. fumigatus* phytases (Mayer *et al*., 1999; Rodriguez *et al*., 1999a, b, 2000a, b). The phytase production can be greatly enhanced by optimizing culture conditions, restriction of oxygen supply during passage, stabilization and screening process, and changes in codon usage of the phytase gene and modification of signal peptide (Mayer *et al*., 1999; Stöckmann *et al*., 2003).

To circumvent the inconvenience of intracellular expression, Miksch *et al*. (2002) have tested extracellular expression of *E. coli* phytase in bacteria using a secretion

system based on the controlled expression of the *kil* gene. Arndt *et al*. (2005) have further developed a controller based on glucose detection from the culture media and implementation of a kalman filter in order to maximize extracellular phytase expression by *E. coli* and minimize acetate production that inhibits cell growth. Kerovuo *et al*. (2000) have developed a novel *Bacillus* expression system for efficient extracellular phytase production, whereas Gerlach *et al*. (2004) have attempted optimal expression of *E. coli* phytase in *Bacillus subtilis* bearing induced *Tat*-dependent transport system components (*TatAd/TatCd*) using the PhoD-specific export signals. Introduction of the *appA* signal peptide cleavage site into the fusion protein resulted in efficient processing of the recombinant enzyme, and expression was further improved by the use of a protease deficient *Bacillus subtilis wprA* strain.

In searching for alternative efficient bacterial expression systems, Lan *et al*. (2002) have studied the effect of different components of culture media and culture conditions on phytase production by the rumen bacteria *Mitsuokella jalaludinii* in fed-batch fermentation. Dharmsthiti *et al*. (2005) have successfully attempted *E. coli* appA phytase production in *Pseudomonas putida* based on the effective secretory system, less limitations in codon usage, and capability of growth using a wide variety of substrates. Effective extracellular *E. coli AppA* phytase expression has also been reported by Stahl *et al*. (2003) in *Streptomyces lividans*. Recently, Yin *et al*. (2005) have described a novel phytase expression system based on silkworm larvae with a yield of 7,710 units per ml hemolymph.

5.5. Developing Phytase Transgenic Plants and Animals

Phytase transgenic pigs, named EnviroPig, have been generated by overexpressing the *E. coli* AppA phytase in their saliva, and have shown reductions in fecal P output by up to 75% (Golovan *et al*., 2001). The transgene phytase strategy has also been implemented in several crops (see Brinch-Pedersen *et al*., 2002 for review). The phytase genes of choice in these plant studies include those from *A. niger, S. ruminantium*, *E. coli* and *A. fumigatus* or synthetic ones.

While the first studies were based on constitutive expression driven by the cauliflower mosaic virus (CaMV) 35S and maize ubiquitin promoters, subsequent studies have largely used seed specific expression promoters such as the wheat HMW glutenine 1DX5, the oilseed rape cruciferin and the rice glutelin promoters. Germination-specific expression directed by the specific α-amylase α*amy*8 promoter, was used in rice. To ensure guidance into the ER for glycosylation and export to the appoplast, signal peptides from tobacco extensin, barley α-amylase, *Brassica napus* cruceferin, barley β-glucanase, rice α-amylase and soybean VSPβ were tested. Recent studies in wheat have unraveled unequivocally that the heterologous phytase is deposited in the vacuole albeit that the transformation constructs were designed for secretion to the appoplast (Brinch-Pedersen *et al*., 2006). A microarray study of transgenic wheat has shown no unintended side effects on the wheat transcriptome by the expressing of an *A. fumigatus* phytase (Gregersen *et al*., 2005).

Phytase transgenic seeds of soybean (Denbow *et al*., 1998), oilseed rape (Zhang *et al*., 2000a, b) and tobacco (Pen *et al*., 1993) have been tested in feeding trials with broilers, pigs, and rats. Without any unfavorable feeding effects, these phytase transgenic seeds have improved P utilization and reduced manure P excretion, comparably with supplemental microbial phytases.

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