Phytase, a New Life for an "Old" Enzyme

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Abstract

Phytases are phosphohydrolytic enzymes that initiate stepwise removal of phosphate from phytate. Simple-stomached species such as swine, poultry, and fish require extrinsic phytase to digest phytate, the major form of phosphorus in plant-based feeds. Consequently, this enzyme is supplemented in these species' diets to decrease their phosphorus excretion, and it has emerged as one of the most effective and lucrative feed additives. This chapter provides a comprehensive review of the evolving course of phytase science and technology. It gives realistic estimates of the versatile roles of phytase in animal feeding, environmental protection, rock phosphorus preservation, human nutrition and health, and industrial applications. It elaborates on new biotechnology and existing issues related to developing novel microbial phytases as well as phytase-transgenic plants and animals. And it targets critical and integrated analyses on the global impact, novel application, and future demand of phytase in promoting animal agriculture, human health, and societal sustainability.

INTRODUCTION

Overview of Phytase History

Phytase (*myo*-inositol hexakisphosphate phosphohydrolase) catalyzes the stepwise removal of phosphates from phytic acid (*myo*-inositol hexakisphosphate) or its salt phytate. Notably, the first phytase was reported in 1907 (1), only four years after the first scientific reference to phytic acid appeared in literature (2). Although research on this enzyme persisted for over a hundred years, it has grown exponentially during the past two to three decades. The scientific and practical significances of phytase are best attested by its recent distinction as one of the ten most important discoveries in swine production in the past century (3). Meanwhile, its remarkable impact has also been recognized in several past and current reviews (4–20). Therefore, this chapter is not intended to revisit those already well-documented early achievements. Instead, our reflections focus on critical events that created the current phytase market and scientific milestones that supported technology for the global commercialization of the enzyme.

The turn of this century saw a significant landmark in phytase research with the discovery that not all phytases share identical catalytic mechanisms. Although all phytases enable phosphate monoester hydrolysis of phytic acid (21), the diversity of these enzymes allows a variety of chemical means to accomplish this task. The first and most extensively studied group of phytases belongs to the class of histidine acid phosphatases (HAPs) (22). The other three groups of phytase are classified as β-propeller phosphatase (BPP; also referred to as alkaline phytase) (23), purple acid phosphatase (PAP; metaloenzymes) (24), and protein tyrosine phosphatase [PTP; also referred to as dual-specificity phosphatase (DSP) or cysteine phytase] (25). Subsequently, a commonly accepted phytase nomenclature has been proposed to delineate the corresponding three-dimensional structures and catalytic mechanisms: histidine acid phytase (HAPhy), β-propeller phytase (BPPhy), purple acid phytase (PAPhy), and protein tyrosine phytase (PTPhy), respectively (7, 18) (**Figure 1**). This array of distinct catalytic mechanisms expands the potential applications of phytases and the range of chemical environments in which they could function. **Table 1** summarizes the currently known distribution of different types of phytases.

Emerging Changes and Challenges in Animal Agriculture

The latter part of the past century witnessed drastic increases in the size of swine and poultry facilities for better efficiency and lower production cost (49). Concurrently, fast-growing or high-producing breeds of swine and poultry were developed and raised to utilize a large portion of soybean meal and other plant-based feeds in their rations (50). The cost advantage of soybean meal over fish meal or other animal-based protein sources also shifted the fish-feeding programs. Because phytate-phosphorus in these plant feeds is not readily available, increasing amounts of inorganic phosphate supplementation were required to meet the phosphorus requirements of these simple-stomached species. The unutilized feed phytate-phosphorus was largely excreted by these animals, which rendered phosphorus in their excreta too high to be applied to cropland. The above-mentioned integration of large swine and poultry production units further aggravated the problem and generated public concern over runoff from phosphorus-rich animal excreta. The adverse environmental consequences of high-phosphorus animal excreta, including fish kills, have long been recognized (51). During the 1990s, the increased frequency of fish kills on the east coast of the United States provoked public alarm. News accounts about an "ambush-predator," the dinoflagellate *Pfiesteria piscicida* (52), prompted governmental investigations and even legislation mandating the use of phytase as one measure to reduce phosphorus concentrations in animal excreta.

Phytase: enzyme that initiates hydrolysis of phosphate from phytate, producing phosphoric acid, inositol phosphate, or inositol

Phytic acid: *myo*-inositol-1,2,3,4,5,6-hexakisphosphate; represents 1–1.5% of weight and 60–80% of total phosphorus in seeds

Phytate: salt of phytic acid

Phosphatase: hydrolytic enzyme that releases phosphate from its phosphorylated substrates

Histidine acid phytase (HAPhy): active at acidic pH, contains RH[GN]xRx[PAS] and HD motifs

β-propeller phytase (BPPhy): active at alkaline pH, requiring Ca^{2+} for activity and thermostability; also known as alkaline phytase

Purple acid phytase (PAPhy): metaloenzymes containing dinuclear Fe^{3+} and Fe^{3+}, Zn^{2+}, Mg^{2+} or Mn^{2+} catalytic center; low activity

Protein tyrosine phytase (PTPhy): active at acidic pH; contains catalytic cysteine; also known as dual-specificity phytase or cysteine phytase
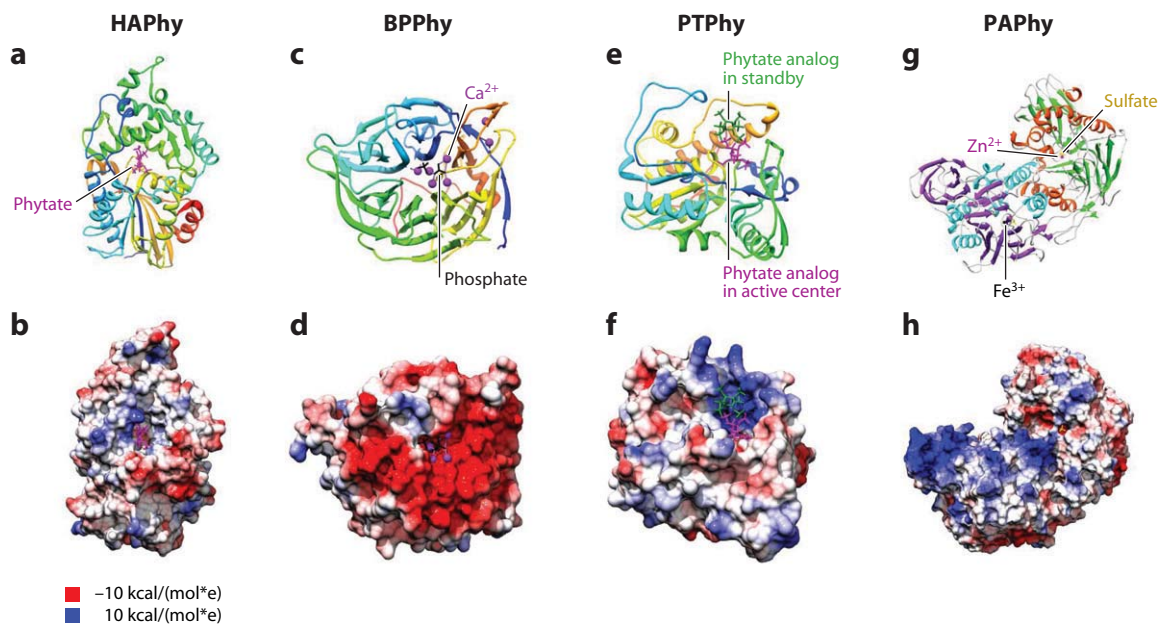
Figure 1

Crystal structures of representatives of each of the four structural classes of phytases. Secondary structure (*first row*) and surface colored by electrostatic potential (*second row*) are displayed. Coulombic surface coloring (Chimera) was used to calculate electrostatic potentials with charges of –10 kcal/(mol*e) (*red*) and 10 kcal/(mol*e) (*blue*) using a dielectric constant of 4.0. Images are not shown to scale. (*a,b*: 1DKQ) Histidine acid phytase (HAPhy), *Escherichia coli* AppA in complex with phytate (*magenta*) in the active center. (*c,d*: 1H6L) β-propeller phytase (BPPhy), *Bacillus amyloliquefaciens* phytase in complex with Ca^{2+} (*magenta*) and phosphate (*black*). Four Ca^{2+} ions are involved in catalysis and creation of a favorable electrostatic potential; three stabilize the enzyme. (*e,f*: 1U25, 1U26) Protein tyrosine phytase (PTPhy), *Selenomonas ruminantium* phytase in complex with *myo*-inositol hexasulfate (phytate analog) in the standby position (*green*) and in the active center (*magenta*). The inositol hexasulfate has five axial and one equitorial sulfates, in contrast to the more general five equitorial and one axial phosphates of phytate seen in crystal structures of HAPhy. (*g,h*: 2QFR) Purple acid phytase (PAPhy), *Phaseolus vulgaris* (kidney bean) PAP (no PAPhy structure available) in complex with sulfate (*yellow*). PAPhy are generally homodimeric and contain two metal ions involved in catalysis and creation of a favorable electrostatic potential. Shown here are Fe^{3+} (*black*) and Zn^{2+} (*magenta*). (*g*) One chain is colored cyan and purple (active site on reverse-side of the molecule), the other orange and green. Images created with Chimera (26) using Protein Data Bank accessions as indicated.

Development of Microbial Phytase as a Feed Supplement

In 1962, International Minerals & Chemicals (IMC) made initial efforts (4) to develop a commercial phytase. In those efforts, over 2,000 microorganisms were screened for phytase production. Although IMC supplied the animal-feed industry with inorganic (rock) phosphates, it foresaw the potential to market a phytase that would hydrolyze phytic acid in soybean and other plant meals. That prediction was indeed forward-looking, because terrestrial inorganic phosphorus deposits are limited and eventually will be mined out. The project was terminated in 1968 when the company failed to identify an organism that produced phytase activity sufficiently high to be commercially viable. However, IMC's pioneering attempt provided a very valuable isolate of *Aspergillus (ficuum) niger* NRRL 3135 (ATCC 66876), which enabled the later identification of the first marketed phytase in the 1990s after the initial characterization by Irving & Cosgrove (53).

Table 1 Presently known distribution of representatives of the different catalytic classes of phytate degrading enzymes[a]

Type	Taxa		Exemplary protein	NCBI accession number	Amino acids[b]	MW kDa[b]	Isoelectric point[b,c]	pH optima	Temp. optimum (°C)	Specific activity (units/mg)	V_{max} (units/mg)	T_m (°C)	K_m (µM)	k_{cat} (s⁻¹)	References[e]
HAPhy	Bacteria	Gram-positive	*Selenomonas ruminantium* Agp	YP_005432715	413[d]	46	(7.9)	Predicted phytase					—	—	This work[e]
		Gram-negative	*Escherichia coli* AppA	M58708	410	47	(6.1)	4.5	55	1,700	—	63.7	—	—	(27–29)
	Fungi	Ascomycetes Filamentous	*Aspergillus niger* PhyA	CAA78904	448	49	4.8–5.2 (4.8)	2.0, 5–5.5	65	—	120	66.3	34	170	(30–32)
		Ascomycetes Yeast	*Candida krusei* WZ-001	—	—	—	5.5	4.6	40	1,210	—	inactivated >50	30	—	(33)
		Basidiomycetes	*Peniophora lycii* PhyA (mushroom)	CAC48195	410	45	3.6 (4.4)	4–4.5	50–55	1,080	—	60	—	—	(34)
	Plants	Monocots	*Zea mays* Phy S11 (corn)	CAA11390	369[f]	80 (dimer)	(5.4)	4.8	55	2.3	—	—	117	—	(35, 36)
		Dicots	*Arabidopsis thaliana*	AAB60740	449	51	(8.6)	Predicted phytase							(37)
	Animals		Avian (*Gallus gallus*) MINPP (chicken)	NP_989975	430	48	(8.0)	5	—	—	0.7	—	140	—	(38)
BBPhy	Bacteria	Gram-positive	*Bacillus amyloliquefaciens* DS11	O66037	356[d]	39	(4.9)	7–8	70	—	—	inactivated >70	138	17	(39, 40)
		Gram-negative	*Sphingomonas sp.* SKA58	EAT09404	336[d]	35	(4.7)	Predicted phytase							(41)
		Cyanobacteria	*Nostoc sp.* PCC 7120	NP_488278	1,821	193	(4.2)	Predicted phytase							(41)
PAPhy	Fungi	Ascomycetes Filamentous	*Aspergillus niger* pH 6.0 optimum acid phosphatase (APase)	AAB31768	583	64	(5.1)	—	—	—	—	—	315	2.6	(42)
	Plants	Monocots	*Triticum aestivum* (wheat) phytase	AX298209	520[d]	58	7.4 (6.1)	6.0	45	137	—	—	—	—	(43)
		Dicots	*Glycine max* (soybean) GmPhy	AAK49438	519[d]	59	(5.1)	4.5–5	58	—	—	inactivated >60	61	—	(24)
PTPhy	Bacteria	Gram-positive	*Clostridium acetobutylicum* ATCC 824	NP_149178	319	36	(9.7)	Predicted phytase							(41)
		Gram-negative	*Selenomonas ruminantium* PhyAsr	AAQ13669	319	37	(8.4)	4.5–5.5	50–55	—	—	inactivated >60	42.5	264	(44, 45)
Unknown	Protozoa		*Paramecium tetraurelia* 51s	—	—	240 (hexamer)	—	7.0	—	10	—	—	250	—	(46)

[a]Only proteins confirmed to possess phytase activity or those predicted to be phytases based on sequence homology to known phytases were included. This table is not an exhaustive list and may exclude other enzymes. Highlighted proteins are commercialized (**Table 2**). Mention of a phytase does not constitute an endorsement by the authors or their institutes. Values represent those of the purified protein and do not reflect properties conveyed by proprietary product formulations such as coatings. Caution should be used when comparing values owing to effects of assay conditions (31). It is best to compare values from experiments done in parallel, so as to compare across the same assay conditions.

[b]Mature polypeptide only.

[c]Isoelectric point determined by isoelectric focusing. Value in parentheses predicted by ProtParam (47).

[d]Amino acids constituting the signal peptide as predicted by SignalP (48) were removed from the NCBI entry for calculation purposes.

[e]Based on BLASTP searches using *A. niger* PhyA (CAA78904), *E. coli* AppA (M58708), *E. coli* AppA (AAA23426), and avian MINPP (NP_989975) as queries against the nonredundant sequences of gram-positive bacteria in NCBI on May 10–11, 2012. Because it most closely resembled *E. coli* Agp, it likely has greater activity for glucose-1-phosphate than for phytate.

[f]First 18 amino acids removed according to N-terminal sequencing of the protein (35).

Application of recombinant DNA technology in the 1980s led to another milestone in the development of phytase as an animal-feed additive. The *A. niger* NRRL 3135 strain served as an excellent candidate to test this new technology's ability to yield a phytase acceptable in the marketplace. Dr. Rudy Wodzinski, a former member of the IMC phytase research team, assisted the Agricultural Research Service of the US Department of Agriculture in the 1980s after learning of its interest in exploiting recombinant DNA technology for agriculture-related projects. With his initiative, a phytase research project was started by the USDA in 1984. This federally funded research rendered the very first cloning of a partial sequence of a phytase gene (*phyA*) by Mullaney and coworkers (54), which in turn guided the later cloning of the full sequence of the gene and its overexpression (55) to produce the first commercialized phytase.

Although Natuphos®, the first globally commercialized phytase, was launched in 1991, development of the phytase market during the following 15 years was slow before *Escherichia coli* pH 2.5 acid phosphatases (AppA and AppA2) were identified as more effective phytases than the fungal PhyA (29, 56). This significant milestone arose from speculation by Greiner et al. (57) that the *E. coli* AppA was a putative phytase based on its peptide-sequence homology with a protein fragment of a partially purified phytase, P2, also produced by *E. coli*. Rodriguez and coworkers (29) actually cloned the *appA* gene and, after overproducing the enzyme in a *Pichia pastoris* yeast system, provided the first direct evidence that the AppA enzyme was indeed more a phytase than an acid phosphatase. Consequently, a wave of research on bacterial phytases has led to the development of a new generation of phytases (**Table 2**), which in many aspects are superior to the first generation of fungal phytases as a feed additive. At the first international phytase summit in 2010, the current global phytase market was estimated to account for more than 60% of the total feed enzyme market and to be worth $350 million annually. The current inclusion rate of phytase in all diets for swine and poultry is approximately 70%.

PhyA: phytase isolated from *Aspergillus (ficuum) niger* NRRL 3135

AppA: phytase isolated from *Escherichia coli*; with acidic pH optimum, higher catalytic efficiency, and pepsin resistance

AppA2: phytase isolated from a strain of *Escherichia coli* in pig colon, with properties similar to those of AppA

Phytase activity unit: the amount of enzyme required to release 1 μmol of phosphoric acid per minute under certain pH, temperature, and buffer conditions

APPLICATION OF PHYTASE AND RELATED ISSUES

Nutritional Values of Phytase in Animal Feeds

The most widely used phytases in animal feeds are HAPs. Dietary phytase is added mainly to release feed phytate-phosphorus so that inorganic phosphorus supplementation is unnecessary for meeting the phosphorus requirements of the target animals. Thus, inorganic phosphorus equivalences of phytases and their determinants in different diets for various species have been studied and reviewed intensively (8–10, 15–17, 19). Briefly, an estimated 300–600 phytase activity units/kg of diets releases approximately 0.8 g of digestible phosphorus and may replace either 1.0 or 1.3 g/kg of phosphorus from mono- and dicalcium phosphate, respectively (60–62). Meanwhile, supplemental phytase improves calcium, zinc, and iron utilization by animals (63–66). More specifically, phytase supplementation consistently enhances calcium digestibility from 60–70% in control diets to 70–80% in experimental diets. However, the digestibility response of copper or manganese is less consistent, and the ability of phytase to improve amino acid availability has been controversial (16); some studies report responses (67), whereas others fail to see changes (68). The benefit of phytase for amino acid availability to pigs, if any, remains small (68). Selle & Ravindran (17) reported an average of 1–2% improvement in apparent ileal digestibility in the pig. In poultry, the amino acid response was also inconsistent, and the energy response was less than 100 kcal/kg of diet (0.36 MJ/kg). This was the average of 17 studies with an average phytase inclusion rate of 662 units/kg diet (range 400 to 1200 units). Most of the diets were wheat/sorghum blends, with a few corn/soy diets as well.

Table 2 Some commercially available microbial phytases[a]

Product	Company	Protein origin	Type	Subtype[b]	Expression	NCBI accession number	Amino acids[c]	MW kDa[c]	Isoelectric point[c,d]	pH optima	Temp. optimum (°C)	Specific activity (units/mg)	V_{max} (units/mg)	T_m (°C)	K_m (µM)	k_{cat} (s^{-1})	References
Allzyme® SSF	Alltech	Aspergillus niger	HAPhy	3	Aspergillus niger, nonrecombinant[e]	A. niger naturally secretes PhyA, PhyB, and pH 6.0–optimum acid phosphatase.											
Finase® EC	AB Vista	Escherichia coli AppA	HAPhy	6	Trichoderma reesei	M58708	410	47	(6.1)	4.5	55	1,700	—	63.7	—	—	(27–29)
Finase® P/L	AB Vista	Aspergillus niger PhyB	HAPhy	3	Trichoderma reesei	L20567	460[f]	51	(4.6)	2.5	—	—	—	> 80	103	628	(42, 58)
Natuphos®	BASF	Aspergillus niger PhyA	HAPhy	3	Aspergillus niger	CAA78904	448	49	4.8–5.2 (4.8)	2.0, 5–5.5	65	—	120	66.3	34	170	(30–32)
OptiPhos®	Enzyvia (JBS United)	Escherichia coli AppA2	HAPhy	6	Pichia pastoris	AAR87658	410[g]	45	(5.8)	3,4, 5.0	58	—	1,070	62.1	74	840	(31, 59)
Phyzyme® XP	Dupont Industrial Biosciences	Escherichia coli AppA	HAPhy	6	Schizosaccharomyces pombe (ATCC.5233)	M58708	410	47	(6.1)	4.5	55	1,700	—	63.7	—	—	(27–29)
Quantum™	AB Vista	Escherichia coli PhySX[h]	HAPhy	6	Pichia pastoris DSM 15927	DD141515[i]	410[g]	45	(5.5)	4.5		1,700	—	75.7	—	—	(27)
Ronozyme® P (Bio-Feed® phytase, ZY® Phytase)	Novozyme and DSM	Peniophora lycii PhyA	HAPhy	6	Aspergillus oryzae DSM 14223	CAC48195	410	45	3.6 (4.4)	4–4.5	50–55	1,080	—	60	—	—	(34)

aThis table is not an exhaustive list and may omit other available enzymes. Mention of a trademark or proprietary product does not constitute an endorsement by the authors or their institutes. Values represent those of the purified protein and do not reflect properties conveyed by proprietary product formulations such as coatings. Caution should be used when comparing values owing to effects of assay conditions (31). It is best to compare values from experiments done in parallel, so as to compare across the same assay conditions.

bCorresponding carbon number of the first phosphate hydrolyzed.

cMature polypeptide only.

dIsoelectric point determined by isoelectric focusing. Value in parentheses predicted by ProtParam (47).

eProduces additional naturally secreted enzymes that are synergistic with phytase.

fFirst 19 amino acids constituting the signal peptide were removed from NCBI entry.

gFirst three amino acids (AFA) of NCBI entry "mature peptide" excluded according to mature peptide of E. coli AppA.

hFirst commercialized engineered phytase with eight amino acids changed by site-directed mutagenesis relative to E. coli AppA (21). AB Vista acquired license from Syngenta in 2008.

iAmino acid sequence same as M58708. Sequence does not include the eight mutations.

Many experiments have shown the superior feed efficacy of *E. coli* phytases compared to that of fungal products (69), despite discrepant reports (70). Whereas Jones et al. (71) found similar phosphorus release curves for two of the *E. coli*–derived enzymes, Kerr et al. (72) noted differences in efficacy among two fungal and two *E. coli* phytases. Differences in assay conditions, diets, and ages of animals, as well as inherent enzymatic properties, may in part account for efficacy variations of commonly used phytases. Over the past decade, new phytase enzymes have been introduced with improved properties, functions, and utilities. These innovations are evidenced as higher activity concentrates in both liquid and granular forms as well as heat-stable products that are more resistant to the heat inactivation associated with feed pelleting (13, 73).

Environmental Benefit and Economic Consideration of Feed Phytase

Although the phytase market represents more than 60% of the current total feed-enzyme market (15), it developed slowly until 2007–2008. This was because in the first 15 years after introduction, use of phytase provided no cost benefit to the use of inorganic phosphorous. Only the Netherlands and the Delmarva peninsula of the United States mandated phytase supplementation as a means to reduce phosphorus pollution of animal waste. Without legislative enforcement, adaptation of phytase was dictated by the market price of inorganic phosphorus. Prior to 2007, when the cost of inorganic phosphorus sources such as dicalcium phosphate was $200–$250/ton, replacing it with phytase did not result in any net savings. However, the current price of dicalcium phosphate is up to $1,200/ton owing to hikes in energy cost and use of phosphorus fertilizer. This increase translates to a cost change from $2 to $12/ton for supplementing diets with 1% dicalcium phosphate. Apparently, this potential sixfold increase in inorganic phosphorus cost has made phytase much more cost effective. Practically, typical dietary phytase supplementation at 500–1,000 units/kg of feed virtually replaces the routine addition of 1% dicalcium phosphate or 0.18% phosphorus to a diet. The average cost of supplemental phytase at 1,000 units/kg of feed is approximately $0.5–$2/ton of feed. Certainly, caution should be given to compare phytase activity units between different products (72). In addition, responses may be observed at high levels (> 2,500 units/kg) (74).

As pigs excrete very little phosphorus (typically < 100 mg/day) through the urine (75), fecal loss represents the main utilization inefficiency of this essential nutrient in feeds. In young pigs (10–50 kg of body weight) fed typical corn-soybean meal–based diets with 0.6% total phosphorus, fecal phosphorus is approximately 2% of the dry matter content. Supplemental phytase can decrease fecal phosphorus to less than 1% (76, 77). Digestibility of feed phosphorus is typically in the 40–50% range and can be improved to 60–80% depending on the dose of phytase. The 50% increase in feed phosphorus digestibility, concurrent with the 50% decrease in fecal phosphorus concentration, by supplemental dietary phytase can be of tremendous environmental significance as 60 million pigs are produced per annum in the United States, and each pig excretes 1.23 kg of phosphorus in the full life cycle. Similar benefits can also be expected from the application of phytase in poultry and fish feeding.

Issues Related to Feed Application of Phytase

Because phytase has been marketed primarily as an animal-feed additive, many of the concerns over the safety of genetically modified crops or animals that are directly consumed by humans did not arise initially. With the advent of the phytase-transgenic Enviropig® (76) and phytase-transgenic rice (78), however, a degree of public concern has developed. Analysis of the

Thermostability: resistance of a protein to heat denaturation; attributed to high melting temperature or protein refolding after cooling

public's attitude reveals conflicting views of the value of these genetically modified organisms for food. Industry has focused on the economic gain from these innovations, whereas consumers see rather small benefit over the seeming risk (79). This is in contrast to the public's greater acceptance of genetically modified organisms for medical applications, probably owing to greater apparent individual gains, personal interactions with physicians, and available safety notices on possible side effects (80). Also likely contributing is the consumers' ability to choose to use genetically modified organisms for medical applications as compared to the apparent absence of choice (lack of appropriate labeling) to consume genetically modified agricultural products.

One environmental concern regarding the use of phytase to reduce manure phosphorus excretion is a possible concomitant increase of free or soluble phosphorus (81). Although dietary phytase supplementation results in substantial reductions in total phosphorus excretion, a slight increase of soluble phosphorus in the excreta is possible. A precise prescription of appropriate phytase inclusion in diets is needed to release the actually required phosphorus for the animals to avoid or minimize this side effect (9).

Allergic responses to powdered phytase exposure in workers have been known for some time (5) and are linked to specific immunoglobulin E immune reactions. More recently, occupational asthma (82) and hypersensitivity pneumonitis (83) also have been associated with phytase exposure in the animal-feed industry. These possible deleterious effects of powdered phytase require the practice of adequate health and safety measures. As phytase technology expands to plants and animals, these health concerns may extend beyond the animal-feed industry. In fact, a study was conducted to determine if allergenic proteins increased in phytase-transgenic rice (84), and another experiment addressed the need to determine the allergenicity of pork from the Enviropig® (85).

Phytase-Transgenic Plants as an Alternative Technology

Several phytase-transgenic plants (86) have been created by overexpressing HAPhy (87) and BPPhy (88) genes in an array of crop hosts. These studies principally focused on one of three major objectives. The first major objective was to devise an alternate means of commercially producing phytase. Two examples of this are studies on phytase-transgenic tobacco (89) and alfalfa (87). In the latter study, the *A. niger* HAPhy was engineered for localization in the alfalfa's apoplast. The majority of the recombinant phytase would thus be in the extracted juice, a by-product, when the herbage is processed. The concern with using transgenic plants to produce phytase for feed application is the heat inactivation of phytase during pelleting. The second major objective was to express a transgenic phytase in plants to reduce phytic acid in seeds and grains used for animal and human consumption. In humans, this approach may help to ameliorate global iron and zinc deficiencies resulting from the chelation of these trace elements by phytate in staple foods (78). The objective parallels breeding programs to produce cultivars with lower phytic acid levels, and the transgenic rice study (78) additionally incorporated other engineered proteins or enhancers to improve its iron bioavailability to humans. These included a phytoferritin for increasing iron content of the rice endosperm and a cysteine-rich peptide for improving iron absorption in the gut. However, the previously reported, thermostable *Aspergillus fumigatus* phytase (90) did not retain activity after cooking (20). In fact, discrepancies in the thermostability of the native and recombinant *A. fumigatus* phytase were noted (86). The third major objective was to improve utilization of phytate from the soil by plants. Despite plentiful phytate in many soil types, it is not utilized effectively as a source of phosphorus by plants because root exudates contain little phytase activity. If overexpression of phytase in plant roots could be developed to exploit this soil phytate

as a source of phosphorus, the continuous need for phosphorus fertilizers in agriculture would be mitigated. Richardson et al. (91) engineered *Arabidopsis* to express the *A. niger* NRRL 3135 *phyA* gene so its plant roots might secrete the transgenic phytase into the rhizosphere. Their study resulted in a transgenic line with improved phosphorus nutrition and growth compared with a control when phytate was the sole phosphorus source.

Several factors should be considered in adapting a phytase to optimally hydrolyze phytate in the rhizosphere around plant roots. The isoelectric point (pI) of phytase seems to be an important factor for this hydrolysis (92). The lower pI (3.6) of *Peniophora lycii* phytase allowed it to remain more soluble in the soil environment and thus more effective in hydrolyzing phytate than *A. niger* phytase with a pI of 5.0. Compared with the crystal structure of the *E. coli* AppA, the *Klebsiella* sp. ASR1 PhyK displays a stiffer structure around the active site that results in a broader substrate spectrum and is better suited to meet the requirements of a plant rhizosphere enzyme (93). This plant-associated *Klebsiella* PhyK is an extracellular HAPhy that in nature scavenges for phosphorus from phytate. Its rigid catalytic pocket results in a conformation always suitable for phytate binding. This feature may derive from an evolutionary selection that favors enzymes that accommodate a range of substrates and liberate phosphate at a constant rate. Tang et al. (94) compared the performance of three classes of phytases (HAPhy, BPPhy, and PAPhy) in a root/ rhizosphere environment to assimilate precipitated phytate, and they suggested that tolerance of these enzymes to organic acids was a determining factor. Under phosphorus stress, plant roots secrete citrate and malate to facilitate phosphorus acquisition. Therefore, genetic engineering of plants to acquire more phosphorus from soil phytate might be achieved by enhancing both phytase secretion and organic acid excretion.

Phytase-Transgenic Animals as an Alternative Technology

The best-known phytase-transgenic animal is the Enviropig® (76). These pigs were transformed to overexpress the *E. coli appA* gene product in their salivary gland. Success of this novel technology was confirmed by a 75% reduction in fecal phosphorus excretion in these animals. Other reported phytase-transgenic animals include fish, such as the Japanese medaka (*Oryzias latipes*) (95), and mice (96). A modified avian multiple inositol polyphosphate phosphatase phytate–degrading enzyme was overproduced in a chicken cell line (38). This study suggested a possible development of poultry varieties with improved phytate digestion, although public acceptance of transgenic food animals still remains low.

Low-Phytate Crops as an Alternative Technology

Considerable success has been achieved in developing low-phytate soybean and grains, such as maize, barley, rice, and wheat, by employment of low phytic acid mutants (97). The effectiveness of these low-phytate crops in improving animal and human nutrition of phosphorus and other elements has been well documented (98–103). Thus, widespread adoption of these low–phytate level cultivars would provide an additional means to alleviate dietary and environmental problems emanating from excessive seed phytate. However, phytate is involved in regulation of plant metabolism and physiology (104), which seemingly would make phytate essential for normal seed germination, plant growth, and disease resistance. Potential adverse effects of removing substantial amounts of phytate in plants may be alleviated but could not be avoided completely (104). For example, low–phytic acid crops have lower yields and higher stress susceptibility than the native wild types (105).

NEW BIOTECHNOLOGY OF MICROBIAL PHYTASES

The three alternative phytase technologies described above—transgenic plants, transgenic animals, and low-phytate crops—represent sustainable approaches for coping with the indigestion of feed phytate-phosphorus by simple-stomached animals. However, these remarkable scientific progresses have met with limited applications owing to poor consumer acceptance, industry hesitation, and technological constraint. Chemical degradation of feed phytate before feeding was considered, but the procedure could be deleterious to feed quality (106). Inoculation into gastrointestinal tracts of animals with phytase-producing microbes was also proposed, but it raised concern over colonization stability and fecal contamination of the environment with the microbes. Consequently, supplementing dietary microbial phytases that are effective in the stomach, but are inactivated in the lower gut (107), becomes not only practically convenient but also the most feasible solution to the problems associated with feed phytate-phosphorus in animal production.

Concept of Ideal Phytase

Lei & Stahl (8) proposed that an ideal phytase possess at least three characteristics: the ability to effectively hydrolyze phytate-phosphorus in the upper digestive tract of the animal, resilience to the 65–80°C temperatures of feed pelleting, and cheap production costs. Because no single phytase can be ideal for all applications, they further suggested the need for an array of application-specific phytases to match the phytate hydrolysis conditions in the functional sites of phytase in swine, poultry, and fish. The quest for ideal phytases has taken two routes: identifying novel wild-type (WT) phytases in nature and engineering desired characteristics in known phytases.

Identification of Novel Phytases

Before the advent of recombinant DNA technology, early searches for novel phytases focused on the identification of microbes that produce high phytase activity. Strategies included screening phytase activity by hydrolysis of insoluble phytate in agar plates or growth on phytate as the sole phosphorus source. The most notable among early searches for phytases was that by IMC (see above), which resulted in the identification of *A. niger (A. ficuum)* PhyA (108). This enzyme belongs to HAPhy and was first characterized by Irving & Cosgrove (53) to have a bimodal pH profile with optima at 2.0 and 5.5. The subsequent advent of recombinant DNA technology enabled the cloning and overexpression of PhyA in *A. niger*, which led to its commercialization (54, 55). Other reviews have cataloged additional, earlier-identified phytases (6, 109).

Technological development in the overproduction of target proteins or enzymes in heterologous expression systems has shifted the initial search for novel phytases toward enzymatic properties including catalytic efficiency; pH profile; thermostability; and resistances to proteolysis, acid, and heat. Subsequent screens for phytase-producing microbes resulted in identification and characterization of the second-generation commercialized phytases (29, 57). The *E. coli* phytases AppA and AppA2, just like PhyA, belong to the HAP family and are manifested with desired pH profiles, without a dip at pH (3.5) similar to that of the stomach in young pigs (110). These two bacterial enzymes also have high activity and great proteolytic resistance (29, 31, 57). However, the *E. coli* phytases were less heat-stable than their fungal counterparts. The other commercialized phytases are PhyB from *A. niger* and *P. lycii* phytase, both of which are also HAPhys (34, 111). Besides its presence in bacteria and fungi, including yeast, HAPhy, referred to as multiple inositol polyphosphate phosphatase, has also been found in plants and animals (35, 37, 38).

The other three major groups of phytases have not been commercialized to our knowledge. They may be classified by pH optimum (acid versus neutral/alkaline), carbon site of first hydrolysis of phytic acid (position 1 or 3 versus 6), and structure. PAPhys are generally found in plants like soybean and have lower activity than HAPhys (24, 112). They have also been identified in fungi, like PhyC from *A. niger* (113). The third group of phytases is the PTPhy, of which the most notable is from *Selenomonas ruminantium* (44). This phytase has an optimal temperature similar to that of HAPhy, optimal activity at pH 4–5.5, and catalytic efficiency similar to that of fungal HAPhy but lower than that of bacterial HAPhy (44, 45). The family of BPPhy, like those from *Bacillus subtilis* and *B. amyloliquefaciens* (39, 114), has been characterized (18). This class of phytase was first identified from *B. subtilis* (114, 115). Its structure shows a dependence on Ca^{2+}, which creates a favorable electrostatic environment for the binding of phytate, and they dephosphorylate phytate to *myo*-inositol-3-phosphate (116, 117). Compared with HAPhy, this *Bacillus* phytase displays a more basic pH profile, greater thermostability, and higher activity for the calcium-phytate complex (118). The enzyme phytase is also resistant to papain, pancreatin, and trypsin but is susceptible to pepsin, presumably because of protein denaturation at low pH (119). This combination of characteristics makes BPPhy suitable for functioning in the intestine. The possibility of remnant BPPhy activity in the feces and subsequent contribution to eutrophication must be addressed. BPPhy may also find use in laying hens owing to their high dietary calcium concentration, which exerts an adverse effect on HAPhy (62). Its sole presence in aquatic bacteria (120) may imply an advantage in aquaculture (121). Effectiveness of a *Bacillus* phytase has been shown in carp (118) and in poultry (13). However, more feeding trials should be conducted to test whether BBPhy susceptibility to pepsin allows it sufficient activity to bypass stomach digestion. Furthermore, even at its optimal pH, BBPhy still has far less activity than HAPhy.

Peer-reviewed publications on the search for novel phytases for biotechnological application have increased in the past decade. A useful strategy employed in these attempts is to search for phytases in extremophiles, organisms living in environments with extremes of heat (expected to be thermostable) and cold (expected to have low temperature activity). For example, phytases with low optimal temperatures were isolated from microorganisms in glaciers (122). Phytases have also been isolated from thermophilic fungi (123). There were reports of high-activity phytases (124) as well, although the claim might be confounded by potential differences in assay conditions (31). Comprehensive summaries of newly identified phytases and their biochemical characteristics are available in recent reviews (11–13). Lim et al. (41) used a bioinformatics approach to analyze the diversity of phytase genes based on publicly available sequences. They found that BPPhy-like sequences existed in plants, soil, and aquatic bacteria; PTPhy-like and HAPhy-like sequences appeared in plant pathogenic and enteric bacteria; and PTPhy-like sequences were additionally present in free-living bacteria. Some bacteria contained sequences for two or three types of phytases. For example, *Pseudomonas syringae* and *Xanthomonas campestri* contained HAPhy, PTPhy, and BPPhy genes. The γ-proteobacteria appeared to be the highest-reported group to carry phytase-like genes. In addition to their presence in plants, PAPhy-like sequences were also found in a restricted set of bacteria and cyanobacteria (125) despite no relevant phytase activity being verified for these sequences.

Protein Engineering of Effective Phytases

Owing to the advent of recombinant DNA technology, protein engineering has become an approach concomitant with the search for novel phytases in developing effective enzymes for field application. There are two broad strategies, random mutagenesis and rational design, along with the combination of these two methods as a semirational design. A flow diagram depicting

Gene site saturation mutagenesis (GSSM): library-creation method that produces every possible amino acid substitution at every residue throughout an entire gene

Site-directed mutagenesis (SDM): method to produce targeted amino acid substitution of rational design

protein-engineering strategies was presented in a recent review (11). Random mutagenesis is independent of structural or primary sequence data availability. Specific procedures of this strategy include error-prone polymerase chain reaction (PCR) through error-prone polymerases, imbalances in deoxyribonucleotide triphosphates, or excess Mn^{2+}; gene site saturation mutagenesis (GSSM) (27, 126); and DNA shuffling of analogous regions between homologous proteins. All of these strategies are coupled with functional screening to identify mutants possessing improved characteristics.

Rational design is generally information rich and is carried out through site-directed mutagenesis (SDM). Desired amino acid–residue substitutions are designed based on structural and sequence information and are predicted to have certain effects on the protein's functionality. Methods include substitution of amino acid residues from homologous proteins of thermophiles, incorporation of prolines or disulfide bridges, and building of salt bridges. Rational design often is conducted on a protein-by-protein basis after careful structural analysis. Semirational design uses structural or sequence information to enhance the effectiveness of random mutagenesis or to make changes without explicit rationale. For example, select mutations of unknown function may be made based on sequence alignments (127), or sequences may be aligned to produce a consensus sequence (128).

Function-Targeted Enhancements

Phytase engineering has been used to improve enzymatic properties, such as thermostability, pH-activity profile, and protease resistance, that determine its efficacy in field application. Because of its commercial importance, HAPhy has been the most-studied target of phytase engineering, and thermostability enhancements in this enzyme have been the primary aim. These engineering efforts have produced *A. niger* PhyA, *E. coli* AppA and AppA2, and *A. fumigatus* phytase variants with one or more improved characteristics of thermostability, pH-activity profile, catalytic efficiency, and protease or gastric fluid resistance. The creation of consensus phytases with a desired thermostability and pH profile highlights the success of phytase and enzyme engineering. Meanwhile, efforts have been devoted to add pepsin resistance or thermostability in BPPhy.

Thermostability. Error-prone PCR and a 96-well plate screen based on residual activity after incubation at 85°C for 15 min were used to increase the thermostability of *E. coli* AppA2 (58). The best mutants resulted in an increase in melting temperature of 6–7°C owing to increased local hydrogen bonding and slight improvements in catalytic efficiency. However, a subsequent study aimed at the sequential addition of the identified point mutations failed to further increase the thermostability of AppA2 (129). GSSM of *E. coli* AppA was conducted to theoretically create a library of all possible single-site mutations in the entire protein using a fluorescent model substrate after a heat treatment (27). This approach is somewhat advantageous over error-prone PCR, which has a limited repertoire of residue changes owing to statistically unlikely mutations. The resultant Phy9X, comprising eight point mutations, showed a 12°C increase in melting temperature over the WT. This phytase variant also displayed a 3.5-fold increase in the half-life of the enzyme in simulated gastric fluid without change in specific activity. Currently, Phy9X is being marketed as Quantum™ Phytase (**Table 2**).

Rational design was successfully applied to increase thermostability of *A. niger* PhyA on the basis of residue substitutions from the homologous Afp phytase produced by the thermophilic *A. fumigatus* (32, 130). Loss-of-function mutations in Afp decreased thermostability, whereas the gain-of-function mutations in PhyA increased hydrogen bonding and ionic interactions, which lead to a melting temperature 7°C higher than that of WT PhyA (melting temperature = 66.3°C).

Specific activity of the thermostable PhyA was unaltered compared with its native form. Functional-targeted enhancements of phytase have been achieved successfully by the de novo synthesis of consensus phytase from identified phytase sequences via semirational design (128, 131, 132). The first consensus phytase was created from the alignment of 13 fungal phytases, which resulted in a melting temperature of 78°C that was 15–22°C over that of the parent phytases (131). Consensus phytase-1 showed comparable activities with *A. fumigatus* phytase (30–36 units/mg). The further-improved consensus phytase-10-thermo[5] Q27T, K68A, was made based on alignments with six additional fungal-phytase sequences and removal of destabilizing mutations as identified by SDM. The advanced consensus phytase had a melting temperature of 90.4°C that showed a 21–42°C increase over parental phytases but retained good activity (128, 132). This incredible increase in thermostability was attributed to the combined effect of many moderately stabilizing mutations (which individually increased the melting temperature < 3°C each). The feeding efficacy and dose-dependent action of the consensus phytase have been verified in a pig experiment (133).

Other strategies for phytase stabilization have also been used, such as increasing the number of N-linked (attached to nitrogen) glycosylation sites, which curiously increased thermostability owing to inadvertent loss of a disulfide bridge (134), and swapping secondary structural elements (135, 136). Meanwhile, BPPhy engineering was conducted to add a disulfide bridge based on sequence comparisons. However, this modification resulted in no increase in thermostability (137). Substitution of several types of residues with Pro and of Gly with Ala successfully increased thermostability without detrimental effects on activity (138). A structure-guided consensus approach has also been used to increase the stability of a *Bacillus* phytase (139).

pH-activity profile. The pH profile of phytases has also garnered much attention, because the stomach is the primary functional site of dietary-supplemented phytases (107, 110, 140). The gastric pH of weanling swine often is near pH 3.5, whereas that of older swine is more acidic (140). In poultry, supplemental phytases may exert a function in the crop where the pH is near 5.5 (141). However, in the crop the degradation of phytate may be hindered by undigested or partially digested feed. The pH optima of both *A. fumigatus* and consensus phytase have been decreased by substitution with corresponding residues from *A. niger* NRRL 3135 (132, 142).

To improve functions of *A. niger* PhyA phytase in gastric conditions, its activity nadir at pH 3.5 was corrected by rational design based on charge interactions of polar and ionic residues in the active site (143). Though the improved pH profile of some mutants was paired with decreased specific activity at pH 3.5, mutant E228K displayed an improved pH profile and over twice the catalytic efficiency of WT at pH 3.5, as well as enhanced efficiency at pH 5.5. In feeding trials with young swine, mutant E228K was further shown to be more effective at releasing dietary phytate-phosphorus than WT (143). These improvements in *A. niger* PhyA pH profile were compatible with mutations that lead to increased thermostability based on the already-described substitution of residues from *A. fumigatus* Afp (32, 144). Further stabilizing mutations identified in error-prone PCR-random mutagenesis were also added into the enzyme, which resulted in combined improvements in thermostability and pH profile. This demonstrates that rational design (thermostability and pH profile) and random mutagenesis (thermostability) can be used complementarily.

Specific activity. In absolute terms, the specific activity or catalytic efficiency is more important than the pH profile. Akin to the use of *A. fumigatus* Afp as a model to improve the thermostability of *A. niger* PhyA, *A. terreus* CBS phytase has served as a model to increase the activity of Afp (145). Thus, both strategies would produce a thermostable and active enzyme. When Afp Q27 was mutated to the corresponding Leu of *A. terreus* CBS phytase, the variant showed an increase in

activity at pH 5.0 from 27 to 92 units/mg of protein, as compared with 196 units/mg of protein for the CBS phytase. This resulted from decreased hydrogen bonding with the product, which facilitated the rate-limiting step of product release. However, unlike the previously mentioned case, in which the thermostability of *A. niger* PhyA was increased without affecting the specific activity, the increased activity of *A. fumigatus* Afp came at the cost of a melting temperature decreased by 6.5°C. However, when Afp Q27 was mutated to Thr, the variant had an activity of 75 units/mg of protein and the same melting temperature as WT. This mutant was further improved by the addition of six mutations from consensus phytase-10 to yield a melting temperature increased by 4.5°C, and four mutations from consensus phytase-1 and S126N that reduced protease susceptibility to yield *A. fumigatus* phytase 13073[hythermo] (30, 131, 132). Adding positively charged residues near the active site of a *Bacillus* phytase elevated its activity and acid resistance (146). Similar approaches have used substitutions from *A. niger* NRRL 3135 PhyA to yield consensus phytase-7 by improving the activity and pH profile of consensus phytase-1 as well as the mutation R297Q to increase the activity of *A. niger* T213 by threefold (147).

Alternative Approaches to Protein Engineering

Approaches other than protein engineering have been used to improve thermostability of commercialized phytases. One commonly used strategy is to apply coating to phytase granules (i.e., Ronozyme® CT, or Coated Thermotolerant), although some research suggests that phytase thermotolerance is dictated more by intrinsic protein properties than by coating (148). Addition of stabilizing additives and enzyme oligomerization has also been used to increase stability (149). A second strategy is the use of liquid phytase products sprayed onto postpelleted, cooled feed. This alternate process seemingly may obviate the need for high inherent thermostability of phytase to resist heat inactivation at pelleting. However, stability of phytase during storage and transportation can be neither bypassed nor ignored.

Systems and Efficiency of Phytase Production

Expression of phytase by native *A. niger* in solid-state fermentation systems has been applied for the commercial production of Allzyme® SSF (Alltech, **Table 2**), a natural mixture of enzymes that includes phytase. However, most phytases are produced by overexpressing the selected genes in either native or heterologous hosts. Native overexpression has been used for the production of PhyA by *A. niger* (Natuphos®, **Table 2**). Heterologous expression has been performed in fungi, yeast, bacteria, and plants, as reviewed previously (8). Commercialized phytases are produced mainly in fungi and yeast hosts (**Table 2**). Bacterial expression is limited by formation of inclusion bodies or by the lack of adequate posttranslational modification. Plant expression is complicated by the need for downstream processing of the overproduced enzyme or the loss of phytase activity at feed pelleting.

Traditional and biotechnological methods are applied to increase phytase protein expression. As reviewed previously (150), the former include submerged fermentation, solid-state fermentation, and manipulated medium ingredients and inoculum. The latter has been employed to increase phytase production in heterologous hosts. Codon usage varies between the donor of the original phytase gene and the heterologous expression host, causing tRNA levels for uncommon codons to limit translation or protein production in the host. Thus, codon optimization of the gene to match the codon preference of the host improves phytase expression (151). In the case of *P. pastoris*, increasing gene copy numbers integrated into the host genome enhanced phytase expression (152). The choice of the promoter and leader signal peptide sequence for extracellular

expression affected the protein expression (153). The yeast α-signal sequence was optimized by including a consensus Kozak sequence and by minimizing interfering secondary RNA structure to produce the MF4I leader sequence, which led to a 14.5-fold increase in protein expression (154). Cooverexpression of chaperones and alternating fermentation temperatures also led to expression improvements in yeast (155).

Unanswered Questions of Phytase Biotechnology

In developing the concept of an ideal phytase, Lei & Stahl (8) suggested tailoring phytases for specific needs. In spite of the growing list of novel and engineered phytases, transition for commercialization has been limited. For example, the remarkable laboratory success in creating consensus phytase has not resulted in development of new commercial products in spite of its development by industry over a decade ago. Some areas of investigation, such as the development of low-temperature active phytases for aquaculture, remain largely unexplored. Another open area of research is the engineering of protein-refolding pathways. Although the ability of *A. fumigatus* phytase to refold completely after heat denaturation at 90°C has been known for some time (58), continuing work has failed to address the contribution of protein refolding versus high-denaturation temperature to protein thermostability. Finally, an acceptable chimeric enzyme possessing the thermostability to withstand feed pelleting as well as other desired characteristics has yet to be created.

GLOBAL IMPACT AND FUTURE DIRECTION

Past Success, Current Challenges, and Future Direction of Phytase

The heightened environmental need to reduce manure phosphorus excretion by simple-stomached food-producing species has driven current phytase research and application. Initial success has been documented in controlled animal experiments and in open, large-scale ecological studies. Impressively, phytase supplementation in animal feed has been found to contribute to the reduction of total phosphorus concentrations over the past decade in eight of ten rivers that are dominated by diffuse pollution in Quebec (Canada) (156). Although it took 20 years after the first commercial launch of phytase in 1991 for the phytase market to reach approximately $350 million per year, several factors likely will expand phytase application to much greater values.

First, decreasing the phosphate load on the environment in areas of intensive animal agriculture is still a long way off (157). As in the above-mentioned Quebec study, seven of the ten rivers tested displayed median P concentrations at least two times greater than the Quebec water-quality guideline for protection of rivers against eutrophication. As a result, many countries and states have issued special laws to incorporate phytase into animal diets. Second, supplies of two main traditional sources of feed-phosphorus supplements, inorganic phosphorus such as mono- and dicalcium-phosphate and meat and bone meals, are becoming either limited or prohibited. Because of the growing demands in China, India, and other countries with strong economic growth, the hiking oil price, and the large portion of feed in the total energy cost of the animal-production system (158), supplementation of inorganic phosphorus has become more expensive and less sustainable or cost effective. As indicated above, the price for dicalcium phosphate has increased from $200, prior to 2007, to $1,200/ton. Moreover, the feed application of inorganic phosphate at the current rate will accelerate the projected depletion of this nonrenewable resource over the next 50 years. The European ban of dietary supplementation of meat and bone meal as a cheap source of feed phosphorus, to prevent possible cross-species transfer of diseases such as mad cow disease, has

cut off a major source of feed-phosphorus supply. Third, biofuels and other industrial productions of various plants will provide voluminous amounts of high-phytate biomass as new feed sources (159). In fact, the global yield of plant phytate is estimated at > 51 million metric tons per year, which accounts for approximately 65% of the elemental phosphorus sold as fertilizers worldwide in 2000 (160). Therefore, a greater demand for phytase in the future will enable this enzyme to exert a larger socioeconomic impact than for animal agriculture alone.

Next-Generation Phytase Tailored for Species and Diets

Although the nutritional values and environmental benefits of the first generation of fungal phytases and the second generation of bacterial phytases are well established for different species, no single available phytase can perform at its maximal efficacy in all species. This is because each phytase possesses unique properties, such as pH and temperature optima and resistance to heat, proteolysis, acidity, and metals. Meanwhile, the conditions of these parameters in the stomach—the main functional site of phytase (107, 110)—of different species or at different ages vary considerably. A striking example is the difference in body temperature between terrestrial animals (37–38°C) and aquatic animals (16°C) (19), which results in a need for three to four times the amount of phytase supplementation in fish feed compared with that in poultry or swine. As another example, layer hen diets often contain calcium as high as 4%, a level that is much greater than that in diets for broilers and pigs and that adversely impacts phytase efficacy (63). Because no single available phytase is ideal for or compatible to digestive-system conditions in all species, multiple enzymes tailored for specific species or animal types should be developed to make phytase a mature and competitive commodity.

Although phytase undoubtedly has been the most consistent and effective feed enzyme during the past two decades, combined dietary additions of phytase with other enzymes such as proteases and nonstarch polysaccharide enzymes have become more common in production. However, previous screening and characterization of phytases for feed application was mainly, if not exclusively, based on the sole incorporation of the enzyme in diets for various animals. Therefore, existing and new phytases need to be tested for synergism and compatibility with other feed enzymes (15, 161) to achieve desired overall responses. Although phytase resistances to heat, acid, and proteolysis are of practical significance, possible effects of excessive, residual supplemental phytases in the lower gut and feces should not be ignored completely.

Novel and Unexpected Functions of Phytase

The best-studied function of phytase is phytate-phosphorus release, or inorganic phosphorus replacement equivalence (162). Whereas parallel benefits of phytase to utilizations of calcium, iron, zinc, and other metals are seen often, impacts on amino acid digestibility or energy efficiency are inconsistent or equivocal (14). Notably, recent animal experiments have moved into molecular, biochemical, and metabolic impacts or regulation of phytase in vivo. Woyengo et al. (163) found that Na-dependent glucose transporter 1 gene expression in small intestines of piglets was decreased by phytic acid but rescued by dietary phytase. Józefiak et al. (164) demonstrated that multicarbohydrase and phytase supplementations improved growth performance and liver insulin-receptor sensitivity in broilers fed diets containing full-fat rapeseed. Lei and colleagues determined the effect of supplemental phytase on the intestinal microbiota of pigs (X.G. Lei, unpublished data). In addition, effects of phytase (dephytinization) on muscle protein expression (165) and signaling by the phytase-mediated hydrolysis of inositol phosphates (166) have been tested to reveal novel functions of phytase beyond its well-illustrated nutritional and environmental roles.

New Applications of Phytase

Applications of phytase in human foods can equal, if not exceed, those in animal feeds. This is because phytate chelates essential minerals, including iron, zinc, and calcium, and contributes to or aggravates deficiencies of these nutrients in approximately two to three billion people around the world. In fact, the effectiveness of phytase or phytase-producing bacteria (167) has been illustrated in human interventional or efficacy trials (168); in improved bread iron availability (169); and in dephytinization of infant formulas, infant cereals, and complementary foods (170). However, several issues have limited wide implementation of phytase in human nutrition. The first issue is the consumer perception of recombinant phytase and the lack of readily available native phytase. The second is the debate over comparing ingestion of phytase with a direct consumption of low-phytate crops via impairing phytic acid biosynthesis through disruption of the inositol polyphosphate kinases or other mutations (171). The third issue is the potential health risk of losing the antioxidant roles of phytate and other forms of inositol phosphates in food and gastrointestinal tracts (172).

The application of phytase in human health and medicine may represent an exciting new avenue. Pagano et al. (173) demonstrated the nonphosphorus-related benefit of high–dietary phytase supplementation for bone development in young pigs. Because pigs are an excellent model for humans, it would be interesting to test whether pig results can be translated into humans. For instance, phytase could be used alone or in combination with other reagents, such as strontium, to treat or prevent osteoporosis. A recent human study suggested a potential role for zinc and/or phytase in enhancing the efficacy of botulinum toxin in the treatment of cosmetic facial rhytids, benign essential blepharospasm, and hemifacial spasm (174). Because potential health values of certain inositol phosphates are well known, phytase and phytase-producing cells are immobilized on a variety of matrices as cost-effective bioreactors for large-scale production of these compounds (175).

There is a great potential for industrial applications of phytase in food processing (176) and biofuel production (177). There are many successful attempts to use phytase in brewing to improve alcohol production (178); in bread making to improve proofing time, width/height ratio of bread slices, specific volume, and crumb firmness (179); in dephytination of soy milk (180); and in separation of soybean β-conglycinin and glycinin (181). Meanwhile, thermostable phytases, along with appropriate xylanases, have been suggested as powerful additives in the pulp and paper industries (182, 183).

SUMMARY POINTS

1. Phytic acid and its salt form, phytate, represent 60–80% of total phosphorus in plant feeds. Phytase catalyzes the hydrolysis of phosphorus from phytate. The poor digestion of phytate-phosphorus in feeds by phytase-deficient, simple-stomached food animals causes three major problems. These are: the environmental pollution from manure phosphorus, the need for dietary supplementation of inorganic phosphorus, and the depletion of rock phosphorus deposits.

2. Emerging changes and challenges in animal agriculture during the past two decades have driven basic research and technology development of phytic acid and phytase to the current heightened stage, although both were discovered a century ago. The world phytase market has experienced a fast expansion since 2007–2008 owing to the drastic increase in the price of feed inorganic phosphorus and the regulation of manure phosphorus disposal.

3. Development of commercial phytases as a feed additive was initiated by a feed mineral company half a century ago. The quest has been signified by several scientific milestones: isolation of the PhyA-producing fungal strain and the subsequent characterization of the strain and the enzyme; cloning of the PhyA gene and its overexpression in the native host; and identification and overproduction of bacterial AppA and AppA2 as a superior and new generation of phytases.

4. Nutritional values of phytases in replacing inorganic phosphorus supplementation and in improving bioavailabilities of calcium, iron, and zinc are well documented, whereas effects of phytases on utilizations of amino acids or feed energy are inconsistent or controversial.

5. Despite technical and perceptional constraints related to these innovations, there are three alternative phytase technologies: phytase-transgenic plants, phytase-transgenic animals, and low-phytate crops. Three issues have been raised in the feed application of microbial phytases: consumers' acceptance of genetically modified products, potential increases of soluble phosphorus in animal excreta, and allergic reactions to phytase-powder exposure.

6. Both traditional and biotechnological approaches have been applied successfully to search for novel phytases and to overproduce them efficiently. Protein engineering using structure-based rational design and directed evolution has resulted in functional-target enhancements of fungal and bacterial phytases with commercial values.

7. The increasing public awareness of environmental protection, along with a concurrent price rise and supply shortage of conventional feed phosphorus sources, will lead to a greater demand for phytase in the future. Phytase will exert larger and broader socioeconomic impacts when next generations of the enzyme, tailored for species and diets, are developed; when novel functions of phytase in metabolism and health are revealed; and when new human and industrial applications of phytase are explored.

FUTURE ISSUES

1. Although no single phytase can perform effectively under various conditions of digestive systems and dietary compositions, currently available commercial phytases are used indistinctively in diets for all purposes. Therefore, next-generation phytases should be developed for utilities tailored for specific species and diets.

2. Although dietary additions of phytase combined with other enzymes, such as proteases and nonstarch polysaccharide enzymes, have become more common in production, current estimates of phytase efficacy and optimal incorporation rates have been based on the sole supplementation of the enzyme in diets for various animals. It will be necessary to test the compatibility and interaction of supplemental phytase with other types of feed enzymes for best overall responses as well as to reestimate phytase efficacy in the presences of those enzymes for precise dietary prescription.

3. Substantial loss of phytase activity during feed pelleting arguably remains the most limiting factor for its feed application. Although phytase protein engineering for enhanced heat resilience and chemical coating of the enzyme for a direct protection against heat inactivation are undertaken independently to tackle the problem,

a combined approach of these two strategies should be considered to achieve potential complementary and additive improvements in this regard.

4. The nutritional value and environmental significance of phytase in releasing feed phytate-phosphorus are well documented, but research on potential metabolic impacts of various intermediate inositol phosphates derived from the phytate hydrolysis has been scarce. The novel roles of these intermediate compounds in gut microbiotia, nutrient uptake and transport, signal transduction, hormone regulation, and cellular metabolism should be studied.

DISCLOSURE STATEMENT

Three authors (X.G.L., E.M., and A.H.U.) hold patents on phytase that are owned by Cornell University and USDA.

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