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MINI-REVIEW

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Biotechnological development of effective phytases for mineral nutrition and environmental protection

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Abstract Phytases are hydrolytic enzymes that initiate the release of phosphate from phytate (*myo*-inositol hexakisphosphate), the major phosphorus (P) form in animal feeds of plant origin. These enzymes can be supplemented in diets for food animals to improve P nutrition and to reduce P pollution of animal excreta. This mini-review provides a synopsis of the concept of "ideal phytase" and the biotechnological approaches for developing such an enzyme. Examples of *Escherichia coli* AppA and *Aspergillus fumigatus* PhyA are presented to illustrate how new phytases are identified from microorganisms and developed by genetic engineering based on the gene sequences and protein structures of these enzymes. We also discuss the characteristics of different heterologous phytase expression systems, including those of plants, bacteria, fungi, and yeast.

Introduction

During the last 15 years, phytases have attracted considerable attention from both scientists and entrepreneurs in the areas of nutrition, environmental protection, and biotechnology. These hydrolytic enzymes are able to initiate the release of phosphate from phytate (*myo*-inositol hexakisphosphate), the major phosphorus (P) form in the cereal grains and oilseeds that are commonly used in diets for food animals (Reddy et al. 1982). Because simple-stomached swine and poultry have little phytase activity in their digestive tract (Bitar and Reinhold 1972), these animals are unable to utilize phytate-P (Nelson et al. 1971) and thus require dietary supplementation of inorganic P, an expensive and non-renewable mineral. The unutilized dietary phytate-P is excreted by these animals, causing P pollution in areas of intensive animal production (Sweeten 1992). In addition, phytate can chelate calcium, zinc, iron, and magnesium, and reduces utilization of these nutrients (O'Dell and Savage 1960; Oberleas 1973; Lei et al. 1993a). Figure 1 shows that phytases can release not only phosphate, but also the chelated divalent metals from phytate.

Undoubtedly, increasing public concern regarding the environmental impact of high P levels in animal excreta has driven the biotechnological development of phytase and its application in animal nutrition. Numerous experiments have shown the effectiveness of supplemental microbial phytases in improving utilization of phytate-P and the phytate-bound minerals by swine and poultry (see a review by Lei and Stahl 2000). Nutritionally, inorganic P supplementation in the diets for swine and poultry can be obviated by including adequate amounts of phytase, along with appropriate manipulation of other dietary factors (Han et al. 1997). As a result, the P excretion of these animals may be reduced by up to 50% (Lei et al. 1993b, c). Nevertheless, the cost and thermo-tolerance constraints of the current commercial phytases have

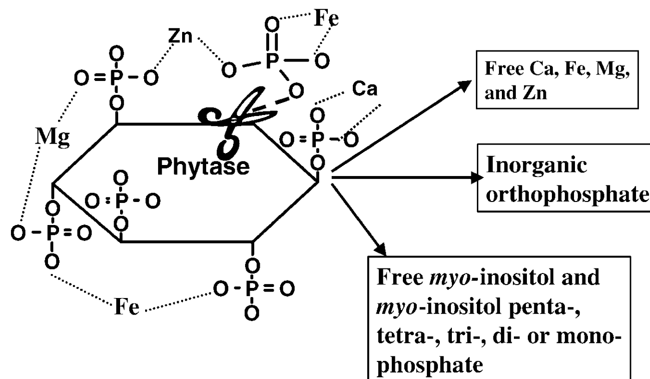

Fig. 1 Schematic illustration of phytase function. Phytate represents the salt of phytic acid (*myo*-inositol hexakisphosphate) and divalent metals such as Ca, Fe, Mg and Zn. Phytase, showing as a pair of scissors, initiates the removal of phosphate groups from phytate at the carbon ring positions 1 or 3 (3-phytase) or 6 (6-phytase), producing free inorganic phosphate, metals, and *myo*-inositol or its intermediate metabolites

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precluded widespread use of these enzymes in animal feeds.

What is an "ideal phytase"?

Although acceptance of any new phytase by the animal industry depends on many factors, at least three biological characteristics seem to be necessary for an "ideal phytase": effective in releasing phytate-P in the digestive tract, stable to resist inactivation by heat from feed processing and storage, and cheap to produce. The ability of any given phytase to hydrolyze phytate-P in the digestive tract is determined by its enzymatic properties, such as catalytic efficiency, substrate specificity, temperature and pH optima, and resistance to proteolysis. As the stomach is the main functional site of supplemental phytase, a phytase with a low or acidic pH optimum and high resistance to pepsin is certainly desirable. Because dietary ingredients for swine and poultry are often processed through a pelleting machine at 65–80°C with steam, an ideal phytase should be able to withstand the high temperature and steam encountered during this pelleting, although phytase inclusion using an after-spray apparatus for pelleted diets and/or chemical coating of phytase may help bypass or overcome the heat destruction of the enzyme that would otherwise occur during pelleting. Likewise, an enzyme that can tolerate long-term storage or transport at ambient temperatures is undisputedly attractive. Finally, a phytase will not be competitive if it cannot be produced in high yield and purity by a relatively inexpensive system. However, it is important to realize that any single phytase may never be "ideal" for all species or in all cases. For example, the stomach pH in finishing pigs is much more acidic than that of weanling pigs (Radcliffe et al. 1998). Thus, phytase with a pH optimum on the low side (e.g., <3) will perform better in the former than in the latter. For poultry, an enzyme would be beneficial if it is active at the nearly neutral pH of the crop (pH 6.5, Riley and Austic 1984) as well as at the acidic pH of the stomach. Phytases used for aquacultural animals (Ramseyer et al. 1999), due to their low body temperature, require a lower temperature optimum than that for swine or poultry. Thereby, an array of phytases will be needed for different target applications.

How do we develop effective phytases?

Two approaches have been taken to develop effective phytases: identifying new native phytase proteins from microorganisms or plants, and genetically modifying these cloned phytases. Up to now, approximately a dozen fungal phytases have been characterized. Among these enzymes, *Aspergillus niger (ficuum)* PhyA and *Peniophora lycii* PhyA have been commercialized. Recently, Mullaney, Daly, and Ullah (2000b) reviewed the identification and biochemical characterization of these fungal phytases. Meanwhile, several new phytases that vary widely in their biochemical properties have been identified from bacteria (Table 1) and plants (Phillippy 1998; Maugenest et al. 1999). Figure 2 depicts the range of pH

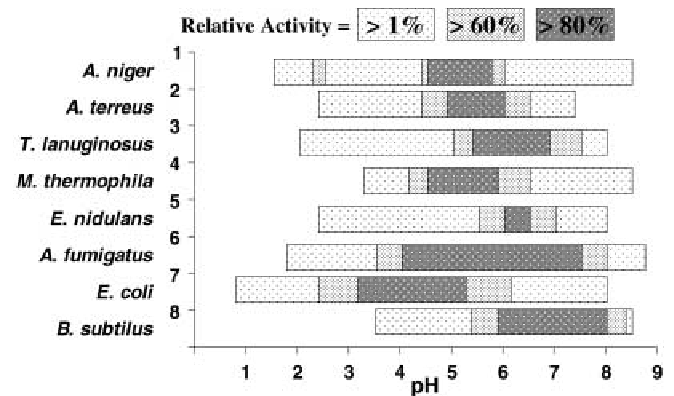

Fig. 2 Comparison of effective pH range among various phytases. Values are compiled from different laboratories as listed below to give a general overview. Direct comparisons should not be taken without considering the assay conditions used by various groups. (1) van Hardingsveldt et al. (1993) and Wyss et al. (1999a); (2) Wyss et al. (1999a); (3) Berka et al. (1998); (4) Wyss et al. (1999a); (5) Wyss et al. (1999a); (6)Pasamontes et al. (1997a), Wyss et al. (1999a), and Rodriguez et al. (2000a); (7) Greiner et al. (1993), Rodriguez et al. (1999a), Wyss et al. (1999a), and Golovan et al. (2000); (8) Kerovuo et al. (1998) and Kim et al. (1998a, b)

Table 1 Newly characterized and expressed bacterial phytases[a]

Origin	Expression host	M_r (kDa)	pI	pH optimum	Temperature optimum (°C)	Reference
Bacillus sp. DS11		44	5.3	7.0	70	Kim et al. (1998a)
Bacillus sp. DS11	*E. coli* BL21 (DE3)	44			70	Kim et al. (1998b)
Bacillus subtilis		43		7.0	55	Kerovuo et al. (1998)
Enterobacter sp. 4				7.0–7.5	50	Yoon et al. (1996)
Escherichia coli		42	6.0	4.5	55	Greiner et al. (1993)
Escherichia coli	*E. coli* BL21 (DE3)	45	6.3–6.5	4.5	60	Golovan et al. (2000)
Escherichia coli	*E. coli* BL21	39–47	7.5	4.5		Wyss et al. (1999a)
Escherichia coli	*Pichia pastoris*	51–56		3.5	60	Rodriguez et al. (1999a)
Klebsiella sp. strain PG-2		112		6.0	37	Shah and Parekh (1990)
Klebsiella terrigena		40		5.0	58	Greiner et al. (1997)

profiles of microbial phytases. Although caution should be given when comparing properties of phytases measured by different groups, due to different assay conditions, the distinct pattern of individual phytases regarding effective pH range, thermostability, and substrate specificity does give us potential targets for improvement. In the following discussion, we will use published data on *Escherichia coli* AppA and *A. fumigatus* PhyA, two new phytases that we have studied in our own laboratory during the past few years, to illustrate how biotechnological approaches can be used to develop improved phytase variants.

Escherichia coli AppA

This enzyme was initially characterized as a pH 2.5 acid phosphatase (Dassa et al. 1982) and was suggested by Greiner et al. (1993) to be the phytase subsequently isolated from *E. coli*. It is a non-glycosylated, periplasmic protein with a molecular mass of approximately 42 kDa. We successfully expressed the *appA* gene (from ATCC) in two yeast hosts (Han and Lei 1997; Rodriguez et al. 1999a) and found that the recombinant enzyme was indeed a phytase and effective in releasing phytate-P and phytate-bound iron in corn-soy diets for young pigs (Stahl et al. 1999, 2000). Later, two other groups reported similar observations (Wyss et al. 1999b; Golovan et al. 2000). Meanwhile, we also cloned and expressed another *E. coli* phytase gene, *appA2*, from a pig colon isolate. Despite 95% sequence homology with *appA*, there are distinct biochemical differences between the two recombinant proteins (Rodriguez et al. 1999a).

Using an efficient *Pichia* yeast expression system, we obtained a large quantity (up to 2–3 g/l) of AppA in relatively high purity (>80%) for biochemical characterization (Rodriguez et al. 1999a, b). This enzyme displayed several favorable characteristics: an acidic pH optimum close to the physiological pH of the stomach of pigs and chickens, higher affinity to sodium phytate than *p*-nitrophenyl phosphate, greater resistance to pepsin than the commercially available *A. niger* PhyA, and stronger catalytic efficiency for phytic acid than that of other known phytases. In a series of animal feeding trials, this enzyme proved to be as effective as, if not more than, *A. niger* PhyA in releasing phytate-P in diets for young pigs (Stahl et al. 1999, 2000). These biochemical properties of AppA were similar, but not identical to those of the native enzyme (Greiner et al. 1993) or the overexpressed protein in *E. coli* systems (Wyss et al. 1999b; Golovan et al. 2000).

The excellent potential of AppA phytase in animal nutrition has tempted us to improve its thermostability by engineering additional *N*-glycosylation sites into the peptide (Rodriguez et al. 2000b). Because the *appA* gene has only three, instead of ten putative *N*-glycosylation sites as in the *A. niger phyA* (Dassa et al. 1982), we hypothesized that enhancing *N*-glycosylation would improve the thermostability of the enzyme, as in the case of *A. niger* PhyA expressed in *Pichia pastoris* (Han and Lei 1999) or *Saccharomyces cerevisiae* (Han et al. 1999). In the absence of a crystal structure of AppA when we started the experiment, we used the crystal structure of rat acid phosphatase (Schneider et al. 1993) to estimate the solvent-accessible surface of all of the amino acids in AppA based on sequence alignment of several phytases and phosphatases (Kabsch and Sander 1983; Luthy et al. 1996; Smith and Smith 1997), under the assumption that the overall structure of rat acid phosphatase and AppA would be conserved. Based on the solvent accessibility and the ease of engineering an *N*-glycosylation motif using a single nucleotide mutation, we selected five out of 31 potential sites. Three variants were expressed in *P. pastoris* as active phytase. The two mutants with four additional *N*-glycosylation sites showed higher *N*-glycosylation (48 and 89%) than the wild-type control (14%). The other mutant (C200 N/D207 N/S211 N) had *N*-glycosylation level similar to that of the control, despite two additional engineered *N*-glycosylation sites. However, this mutant, but not the other two that had higher levels of glycosylation, retained significantly higher activity after being heated at 65–90°C than the controls. This mutant also displayed improved catalytic efficiency and affinity to phytate. Thus, the impact of *N*-glycosylation on the thermostability of AppA phytase is not simply predictable, as shown by Wyss et al. (1999b). Based on the recently released AppA phytase crystal structure (Lim et al. 2000), the substitution C200 N in mutant C200 N/D207 N/S211 N seems to eliminate the disulfide bond between the G helix and the GH loop in the α-domain of the protein, which in turn modulates the domain flexibility and thereby the catalytic efficiency and thermostability of the enzyme.

Aspergillus fumigatus PhyA

The desire of the animal industry for a heat-tolerant phytase with less activity loss during feed pelleting than the current commercial phytases has generated some interest in *A. fumigatus* PhyA (Pasamontes et al. 1997b). When the purified enzyme was heated at 100°C for 20 min, it retained 90% of its initial activity (Pasamontes et al. 1997b), whereas the *A. niger* PhyA retained only approximately 30% of its initial activity when heated at 70°C (Wyss et al. 1998). The gene shares 66% sequence similarity with *A. niger phyA* (Passmontes et al. 1997b) and contains seven putative *N*-glycosylation sites. Estimates of the molecular mass of the protein are quite variable, ranging from 60 to 100 kDa (Pasamontes et al. 1997b; Rodriguez et al. 2000a; Ullah et al. 2000). Besides its thermo-tolerance, another favorable trait of the *A. fumigatus* PhyA as reported by Pasamontes et al. (1997b) and Wyss et al. (1999b) is its broad effective pH range, with a pH optimum at 5 and >80% of its optimal activity between pH 4 and 7.5. We expressed the *A. fumigatus phyA* gene in *P. pastoris* and demonstrated that thermotolerance of the expressed phytase was modulated by the

specificity of the buffers used in the heat treatment (Rodriguez et al. 2000a). Using the same expressed enzyme, Ullah et al. (2000) could not detect a thermo-tolerance superior to that of the *A. niger* PhyA. In contrast, they observed extremely instability of the enzyme at 0 or 4°C. Mullaney et al. (2000a) found a correlation between low optimal pH (4.0) and a higher optimal temperature (70°C) among four *A. fumigatus* PhyA enzymes prepared from different isolates, but could not identify any primary sequence difference among these enzymes. Thus, thermostability or thermo-tolerance of the *A. fumigatus* PhyA or any given phytase is highly conditional, and caution should be given in comparing data from different groups. Despite a significant difference in the ability to refold between *A. niger* and *A. fumigatus* PhyA enzymes in test tubes, both retained similar residual activities after being pelleted in broiler diets at 75°C (Wyss et al. 1998). This serves as a reminder that enzymatic data obtained from a purified enzyme in a specified buffer may not hold true under practical conditions.

However, all groups have found that the specific activity of the native *A. fumigatus* PhyA is too low to be suited for animal feed industry use. Based on amino acid sequence alignments among several PhyA enzymes, Tomschy et al. (2000a) hypothesized that amino acid residue 27 might be highly correlated with the seven-fold difference in specific activity between the native *A. fumigatus* and *Aspergillus terreus* enzymes. The former has a glutamine instead of a leucine as in the latter at residue 27. When they substituted leucine with glutamine at that residue in *A. terreus* PhyA, specific activity was reduced from 196 to 35 U/mg. When they made the opposite switch in the *A. fumigatus* PhyA, the specific activity was increased from 26.5 to 92.1 U/mg. Other mutations also produced significant effects on specific activity. Based on the crystal structure of the *A. niger* PhyA (Kostrewa et al. 1997) and computer models of phytase-substrate complexes, they suggested that Glu-27 of the wild-type *A. fumigatus* PhyA might form a hydrogen bond with the 6-phosphate group of *myo*-inositol hexakisphosphate that would be weakened or removed by the mutations, inducing an accelerated product release.

Similar structure-based mutations have also been conducted with the *A. niger* and *A. terreus* phytases (Tomschy et al. 2000b; Jermutus et al. 2001). After comparing the amino acid sequences of several fungal phytases, Lemann et al. (2000a) constructed a consensus phytase that contained the most frequently occurring amino acid at each residue. This phytase had an unfolding temperature 15–22°C higher than any of the other phytases used in its design. Because this increase in thermostability, however, concurred with a low specific activity for phytic acid, they replaced all divergent amino acids in the active site with those found in the *A. niger* PhyA (Lehmann et al. 2000b). This approach successfully increased the specific activity of the consensus phytase by almost 50%, but it was still approximately 35% lower than the specific activity of the *A. niger* PhyA. The pH profile of this new phytase was nearly identical to that of *A. niger* PhyA. The new consensus phytase had an 8°C lower unfolding temperature than the original consensus phytase; however, its unfolding temperature was still >7°C higher than any of the other fungal phytases used in its design (Lehmann et al. 2000a, b). Apparently, this technique has substantial benefits compared to random mutagenesis or single amino acid substitutions in the development of a more thermostable enzyme, but it will most likely benefit from fine-tuning with single amino acid substitutions to the consensus backbone.

Which expression system is efficient to produce phytase?

As the cost effectiveness of phytase production is a major limiting factor for its application, different heterologous expression systems and hosts have been evaluated. These are plants, bacteria, and fungi including yeast. As expected, each system bears some unique advantages, along with certain limitations.

Plants

The *A. niger phyA* gene has been successfully expressed in tobacco seeds (Pen et al. 1993) or leaves (Verwoerd et al. 1995) and soybean cells (Li et al. 1997). In the transformed tobacco leaves, phytase accumulated extracellularly during plant maturation to approximately 14% of the total soluble plant protein (Verwoerd et al. 1995). Ullah et al. (1999) purified the expressed *A. niger* PhyA in tobacco leaves and found that the recombinant protein was 17% less glycosylated than the wild-type. While this change did not affect its temperature profile, the pH optima of the less glycosylated recombinant protein became more acidic, with peaks at 4.0 and 2.0 as opposed to the wild-type enzyme with peaks at 5.5 and 2.5. Expression of the PhyA of *A. niger* in soybeans (Li et al. 1997), a typical component of swine and poultry diets, seems to be a convenient approach to eliminate costs associated with phytase production, purification, and supplementation. However, the thermo-tolerance constraints of the *A. niger* PhyA protein would not allow the enzyme to sustain the heat encountered in the production of soybean meal. Because feeding raw soybeans to production animals is impractical due to the presence of protease inhibitors, future researchers interested in developing phytase-overexpressing soybeans should consider transforming thermo-tolerant enzymes. High levels of functional phytase have also been produced in wheat (Brinch-Pedersen et al. 2000). Overexpressed phytase in soybean or canola seed has been shown to be as effective as microbial phytase in improving phytate-P utilization by broilers and pigs (Denbow et al. 1998; Zhang et al. 2000). Apparently, plants can be used as phytase producers and carriers for animal feeding. Whether this approach will be feasible in production depends on public acceptance to growing genetically modified organisms in

the field, and the effectiveness and stability of the heterologous phytases in plants used as animal feeds. It is also worth mentioning that the recently developed low-phytic-acid corn has been tested in animal feeding trials (Yan et al. 2000). However, there is concern over the reduced yield of the mutant corn and possible detrimental impacts of the removal of phytate, the energy and P storage for seed germination (Reddy et al. 1982), on plant survival under adverse conditions.

Fungi

Phytase genes from *A. niger*, *A. terreus*, *A. fumigatus*, *E. nidulans*, and *M. thermophila* have all been expressed and secreted as active enzymes by *A. niger* (van Hartingsveldt et al. 1993; Wyss et al. 1999a). Berka et al. (1998) examined the use of another fungi, *Fusarium venenatum*, as an expression system for the *phyA* gene from thermophilic fungus *Thermomyces lanuginosus*. While fungal systems produce and secrete active phytases, other proteins, including proteases, are often produced at relatively high levels. Thus, there may be a need for further purification or inhibition of proteolysis that adds to the production cost.

Yeast

Because *S. cerevisiae* and its by-products have long been known to be safe for human and animal consumption, we have examined it as a potential phytase expression host (Han et al. 1999). A hyper-glycosylated *A. niger* PhyA (120 kDa) was expressed and secreted into the medium by this yeast. The 40-kDa increase in molecular mass compared with the native enzyme or the enzyme expressed in *A. niger* (80 kDa) was caused by elevated *N*-glycosylation. Importantly, this hyper-glycosylation improved the thermo-tolerance of the enzyme. While other PhyA enzymes have also been expressed in this system (Wyss et al. 1999a), protein yield is a concern for industrial application.

To enhance the yield, we have used a methylotrophic yeast, *P. pastoris*, to express both fungal and bacterial phytases (Han and Lei 1999; Rodriguez et al. 1999a, 2000a, b). This organism shares many beneficial characteristics with *S. cerevisiae*, such as ease in cultivation and genetic manipulation, as well as being generally regarded as safe. It also represents one of the most efficient hosts for heterologous protein expression (see a recent review by Gellissen 2000). When the *A. niger phyA* gene was transformed into *P. pastoris* (Han and Lei 1999), a 95-kDa protein was secreted, indicating a glycosylation level greater than in *A. niger* (van Hartingsveldt et al. 1993) but less than in *S. cerevisiae* (Han et al. 1999). As tunicamycin (20 mg/ml) significantly inhibited both intra- and extra-cellular phytase activity expression, *N*-glycosyaltion seemed to be vital to the biosynthesis of the PhyA in *P. pastoris* (Han and Lei 1999). Both the *A. niger* and *A. fumigatus* PhyA proteins were secreted by the *P. pastoris* transformants, and the extracellular phytase activity accumulated linearly over 3–4 days (Han and Lei 1999; Rodriguez et al. 2000a). When the *E. coli* phytase gene (*appA*) was transformed into *P. pastoris* (Rodriguez et al. 1999a, 2000b), the expressed phytase protein had an increase in molecular mass by 5–10 kDa over the non-glycosylated native form (Greiner et al. 1993), giving an 8–17% increase in glycosylation. This glycosylation also helped stabilize the enzyme. Because of the promising feeding effects of AppA produced by this system (Stahl et al. 2000), we are trying to optimize large-scale fermentation with cheap carbon sources. Another methylotropic yeast, *Hansenula polymorpha*, has also been examined as an expression host of several fungal phytases (Wyss et al. 1999a). The reported yield is up to 13.5 g of protein/l of medium in a fermentation system utilizing low-cost glucose or glucose syrups (Mayer et al. 1999).

Bacteria

The obstacle in using bacterial systems to produce fungal phytases is their inability to sufficiently glycosylate the expressed proteins to the extent necessary for activity. Thus, inactive *A. niger* PhyA protein was expressed intracellularly in *E. coli* (Phillippy and Mullaney 1997) and extracellularly in *Streptomyces lividans* (Han and Lei, unpublished). In contrast, the *E. coli* pET expression system was able to express phytases of *E. coli* and *Bacillus* sp. origins (Kim et al. 1998b; Golovan et al. 2000). The expressed *Bacillus* sp. phytase accounted for 20% of the total soluble proteins in *E. coli* (Kim et al. 1998b), while the *E. coli* phytase transformants produced up to 600 U of phytase/ml (~0.3 g/l) of minimal media after 30 h growth (Golovan et al. 2000). However, the intracellular expression of phytases in *E. coli*, without further processing or purification prior to application, may affect their accessibility to feed phytate-P in digesta.

Kerovuo and Tynkkyen (2000) evaluated *Lactobacillus plantarum* as an extracellular expression system for the *phy*C gene from *B. subtilis*, but the expression or secretion level was too low to be considered for industrial use. Because *S. lividans* is able to express and secrete bio-active heterologous proteins from both prokaryotic and eukaryotic sources (Gilbert et al. 1995; Binnie et al. 1997), with a limited ability to glycosylate proteins (MacLeod et al. 1992; Ong et al. 1994), we examined the possibility of using this bacterium to express the *A. niger* phyA and the *E. coli appA* (Stahl and Lei, unpublished). While both proteins were expressed and secreted by *S. lividans*, only AppA was an active phytase. Neither protein was glycosylated and almost all of the protein was found extracellularly. The AppA expressed by *S. lividans* had enzymatic properties similar to those of AppA expressed by *E. coli* (Golovan et al. 2000), but different from the glycosylated AppA expressed by *P. pastoris* (Rodriguez et al. 1999a). This suggests that

either glycosylation of this protein or the expression host may affect the properties of the expressed enzyme. While this system is capable of producing and secreting an active AppA with minimal media, the expression yield is much lower than that with *P. pastoris*. We have also expressed the *E. coli appA* gene as an active phytase in *Lactococcus lactis* (Stahl and Lei, unpublished).

Concluding remarks

Public awareness of the environmental impact of animal agriculture has led to legislation that limits the quantity of P in animal excreta in some parts of the world, and will likely extend to other parts of the world in the near future. Under these conditions, phytase will be widely used in animal diets to improve phytate-P bioavailability and reduce P excretion. Annual world market of phytase as an animal feed additive is estimated to be $500 million (Abelson 1999). Significant progress has been made in phytase research during the last 15 years. Transgenic mice expressing bacterial phytase have been recently developed as an experimental model to study the efficacy of endogenous phytase expression (Golovan et al. 2001). Nevertheless, our scientific knowledge of phytase has yet to yield a solution to meet its enormous nutritional and environmental demand. Further research into discovering new phytases, engineering better phytases based on three-dimensional structures, and developing more cost-effective expression systems should be continued. As no single phytase or expression system is likely to be able to meet the diverse needs for this enzyme, a series of phytases specifically designed for different species at different physiological stages and feeding conditions should be developed. Multiple approaches, such as using low-phytic acid corn, along with microbial or plant phytase supplementation, may be the right direction to pursue. With the collaborative efforts of phytase scientists from different fields, effective solutions to the biotechnological development of phytases for mineral nutrition and environmental protection will be available in the near future.

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