Advances in Phytase Research

EDWARD J. MULLANEY, CATHERINE B. DALY, AND ABUL H. J. ULLAH

Southern Regional Research Center
Agricultural Research Service
United States Department of Agriculture
New Orleans, Louisiana 70124

ADVANCES IN APPLIED MICROBIOLOGY, VOLUME 47
0065-2164/00 $25.00

I. Introduction

A. Phytase Today

At the close of the twentieth century, annual sales of phytase as an animal feed additive were estimated to be $500 million and growing (Abelson, 1999). Evolution of the market for this feed additive can be attributed to a chain of events during the late twentieth century that both created the need for this enzyme and provided the means for its commercial development. Earlier reviews on phytase (Wodzinski and Ullah, 1996; Dvorakova, 1998) have chronicled the events since 1907, when Suzuki *et al.* (1907) first discovered phytase, up to its commercialization in 1994. Since then, both the use of, and the research on, phytase have expanded considerably. A search of the scientific literature for the period 1992–98 for studies involving phytase research (Fig. 1) demonstrates the recent increased interest in this enzyme. An examination of current phytase research and its relationship to a contemporary environmental concern provides insight into how a convergence of technologies fosters additional research and development on this key biocatalyst.

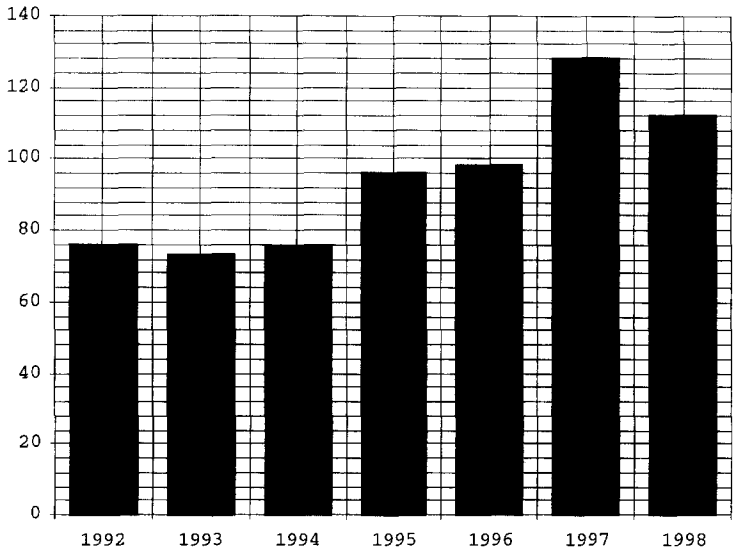

FIG. 1. Number of published studies pertaining to phytase each year for the period 1992–98. The total for each year was obtained by a search of PubMed, Food and Science Technology Abstracts (FSTA), Agricola, CAB abstracts, and Biological Abstracts for research involving phytase.

B. Phytic Acid in Plants

Phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate) is the primary storage form of phosphorus and the source of inositol in plant seeds (Reddy *et al.*, 1982). Studies on the structure of phytic acid (phytate) and the amounts of phytin phosphorus in various feedstuffs obtained from various plant meals have been published (Nelson *et al.*, 1968a; Reddy *et al.*, 1982; Wodzinski and Ullah, 1996). The storage function of phytase may be a part of a larger regulatory function that enables plants to control their phosphate and mineral concentrations (Raboy and Gerbasi, 1996). In addition to its role in phosphate storage, phytase may function as an antioxidant in the seeds (Graf *et al.*, 1987; Graf and Eaton, 1990). In plant seeds, most of the iron is complexed with phytate (Morris and Ellis, 1976), thereby alleviating the potentially lethal combination of free iron and unsaturated fatty acids in close proximity. Very low phytic acid maize mutants produce either stunted plants or seeds that are unable to germinate (Maugenest *et al.*, 1999).

C. Increased Use of Plant Meals in Animal Feed

The soybean industry has successfully expanded its markets in the latter half of the twentieth century. Feeds for monogastric animals increasingly use soybeans, grains, and other plant seeds that contain high levels of phytic acid. Considerable research efforts have resulted in a transition in one segment of agriculture into an "animal agriculture" that requires large quantities of cereals and meals (Berlan *et al.*, 1977). Between 1972 and 1992, the poultry industry was able to switch from fish meal as its primary protein source in its feed ration to lower-cost plant protein sources such as soybean meal (Rumsey, 1993). This was achieved by the efforts of poultry nutritionists who successfully removed inhibitors and antinutritional factors from plant meals, and then developed amino-acid and mineral supplements that enhanced the nutritional profile of these plant meals until they were essentially equal to fish meal. The phytin phosphorus in plant meals was unavailable to monogastric animals because they lack phytase. The lower cost achieved through substituting soybean and other plant meals in these animal feeds was a major consideration for this research.

Economic pressures continue to create a trend toward larger animal production units coupled with lower production costs (Mallin, 2000). Cost-effectiveness also dictated the means to deal with high levels of phytic acid in these meals. All the phytin phosphorus in these meals was unavailable to monogastric animals because they lack phytase.

This lack of adequate phosphorus was corrected by supplementing soybean and other meals with relatively inexpensive rock phosphate, which provided the animal with this necessary nutrient. The excess phytin phosphorus was disposed of in the animals' manure. However, this practice added even more phosphate to the animals' feed ration and resulted ultimately in phosphorus levels in the manure that far exceeded the land's capability.

D. Early Research Identifies a Fungal Phytase

The growth of the market for phosphate to supplement animal feed fostered a critical step in the commercial development of phytase. Wodzinski and Ullah (1996) detailed the role that International Mineral and Chemical (IMC), a supplier of rock phosphate to the feed industry, had in initiation of the research that first identified a phytase from *Aspergillus niger* (*ficuum*) NRRL 3135 (ATCC 66876) in 1968 (Nelson *et al.*, 1968b) with high enough activity to be considered a candidate for production as an animal feed supplement. In a remarkable display of foresight, IMC management sponsored research that surveyed hundreds of microorganisms for phytase activity. They apparently realized that a microbial phytase might one day be marketed as an effective means to hydrolyze phytic acid in plant meals and that they could supply this enzyme to animal feed producers. However, the necessary techniques to achieve overexpression of fungal phytase had not yet been developed and the project was terminated in 1968 before commercialization could be achieved.

Dr. Rudy Wodzinski, a member of that IMC research effort, remained convinced of the merits of developing phytase as an animal feed additive. His continued interest in this project served to attract other scientists to this research, and he remained until his death in 1997 a strong advocate for the future potential of this enzyme. One of the early problems for researchers was the lack of a commercial supply of phytase. When Dr. Wodzinski was contacted by the Linus Pauling Institute in 1986 about a request for a supply of phytase, he supplied Sigma Chemical Company with both the necessary growth requirements and the *A. niger* isolate to produce enough phytase to supply the research efforts of several scientists. Dr. Wodzinski also served as a consultant to the Agricultural Research Service of the U.S.D.A. in 1984 when it started its own phytase research program. His support of this ARS study resulted in the initial characterization of phytase (Ullah and Gibson, 1987) and partial cloning of the phytase gene (Mullaney *et al.*, 1991). In time, when advances in biotechnology made it possible, the phytase gene from *A. niger* NRRL 3135 was overexpressed and its product commercialized (Van Hartingsveldt *et al.*, 1993).

II. Phytases that are Histidine Acid Phosphatases (HAPs)

A. PhyA

Several types of acid phosphatases have been reported in biological systems. These include purple acid phosphatases with a dinuclear Fe–Fe or Fe–Zn center in their active site (Klabunde *et al.*, 1996), the low-molecular-weight acid phosphatases, and the high-molecular-weight acid phosphatases (Vincent *et al.*, 1992). *A. niger* NRRL 3135 phytase A (phyA) belongs to this last group and features a conserved active site motif, RHGXRXP (Fig. 2), unique to this class of enzyme, that hydrolyzes phosphomonoesters in a two-step mechanism (Ullah *et al.*, 1991; Van Etten *et al.*, 1991). Several other fungal (Table I), bacterial, and plant phytases are now known to belong to the histidine acid phosphatase (HAP) class of enzymes (Wodzinski and Ullah, 1996). All these phytases share this common active site structure. As one of the best characterized HAPs, the *A. niger* NRRL 3135 phytase molecule is being employed as a model to better understand this class of enzymes.

TABLE I

FUNGAL HAP PHYTASES

PhyA	Accession numbers	Number of Cys
A. niger (*ficuum*) NRRL 3135	JN0656	10
A. niger var. *awamori* ATCC 38854	P34753	10
A. niger T213 (*A. niger* CB)	–	10
A. niger SK-57	BAA74433	10
A. fumigatus ATCC 130703[a]	AAB96872	10
Emericella nidulans (*A. nidulans*) (Roche Nr. R1288)	O00093	10
A. terreus 9A1	AAB52507	11
A. terreus CBS	AAB58465	11
Myceliophthora thermophila[2] ATCC 48102	AAB52508	10
Talaromyces thermophilus ATCC 20186	AAB96873	11
Thermomyces lanuginosus CBS 586.94	–	8
Peniophora lycii CBS 686.95	–	10
Consensus phytase (Lehmann, 1998)	–	10

[a]Erroneously designated ATCC 34625 in Wyss *et al.*, 1998.

[b]*Thielavia heterothallica.*

```
                              #   1         1          * *        #
MGVSAVLLPLYLLSGVTSGLAVPASRNQSSCDTVDQGYQCFSETSHLWGQYAPFFSLANE   60

          2          *** *  * **   *          #                #
SVISPEVPAGCRVTFAQVLSRHGARYPTDSKGKKYSALIEEIQQNATTFDGKYAFLKTYN 120
                           N

                                                *
YSLGADDLTPFGEQELVNSGIKFYQRYESLTRNIVPFIRSSGSSRVIASGKKFIEGFQST 180

                         *    #       3  **          #
KLKDPRAQPGQSSPKIDVVISEASSSNNTLDPGTCTVFEDSELADTVEANFTATFVPSIR 240

                              4 *                4          *
QRLENDLSGVTLTDTEVTYLMDMCSFDTISTSTVDTKLSPFCDLFTHDEWINYDYLQSLK 300

*  *                                          #          #
KYYGHGAGNPLGPTQGVGYANELIARLTHSPVHDDTSSNHTLDSSPATFPLNSTLYADFS 360

**             #           #                                2
HDNGIISILFALGLYNGTKPLSTTTVENITQTDGFSSAWTVPFASRLYVEMMQCQAEQEP 420
 C

               5       5                    3
LVRVLVNDRVVPLHGCPVDALGRCTRDSFVRGLSFARSGGDWAECFA              467
```

FIG. 2. The *A. niger* NRRL 3135 phyA amino-acid sequence (NCBI Accession No. JN0656). The conserved residues having sidechains protruding into the reaction cavity have an asterisk (*) above them (Pasamontes *et al.*, 1997b) and the # above the 10 Asn residues denotes glycosylation. The N-terminal (**N**) (RHGXRXP) and C-terminal (**C**) (HD) motifs found in histidine acid phosphates are highlighted. The number above each of the 10 cysteine residues refers to the individual disulfide bridge to which it belongs (Kostrewa *et al.*, 1997). The two acidic and four basic amino acids, respectively, that compose the substrate specificity site (Kostrewa *et al.*, 1999)—Glu228, Asp262, Lys91, Lys94, Lys300, and Lys301—are underlined.

A. niger NRRL 3135 phyA is a monomeric protein with a molecular weight of 48.5 kDa for the unglycosylated enzyme (Ullah and Dischinger, 1993). However, the native enzyme, which is heavily glycosylated, shows a molecular weight of 85 kDa (Ullah, 1988). Fungal phytase, when purified from shake-flask-produced cells grown in the presence of starch and low phosphate conditions, shows microheterogeneity in molecular weight distribution as evidenced by the diffuse band in

SDS-PAGE gel electrophoresis giving a molecular weight of 85–100 kDa (Ullah and Gibson, 1987).

It is noteworthy that *A. niger* NRRL 3135 phyA shows inherent thermostability since its optimum temperature was determined to be 58°C (Ullah and Gibson, 1987). One of the reasons for higher thermostability in this enzyme could be the presence of 10 cysteine (Cys) residues, which allow the protein to have 5 disulfide bridges (Fig. 2). Both biochemical (Ullah and Mullaney, 1996) and X-ray crystallographic data (Kostrewa *et al.*, 1997) indicate that all 10 Cys residues are involved in forming 5 disulfide bridges. When the putative phyA gene from *Arabidopsis thaliana* was checked for Cys residues, it also revealed the presence of 10 Cys residues (Mullaney and Ullah, 1998b). The presence of five disulfide bridges is becoming a hallmark of phyA proteins, especially in microbes (Table I).

The active site residues in phyA from various sources are well described. The active site motif containing the conserved septapeptide had been discovered independently by two labs in 1991 (Ullah *et al.*, 1991; Van Etten *et al.*, 1991). The vestiges of phyA's active site, however, were first described in 1989 by Bob Fletterick's research team while searching for sequence similarities among diverse phosphate-metabolizing enzymes such as fructose 2,6-biphosphatase, phosphate glycerate mutase, and acid phosphatases (Bazan *et al.*, 1989).

The other hallmark of phyA protein is the C-terminal HD motif (His361 and Asp362, Fig. 2), which had been recognized first by the Van Etten group as early as 1991 (Van Etten *et al.*, 1991). The HD motif is well conserved in a wide variety of phytase sequences (Ehrlich *et al.*, 1993; Mitchell *et al.*, 1997; Pasamontes *et al.*, 1997b). The catalytic His (His82) and Asp (Asp362) residues come from very different parts of the protein and are located in close proximity to each other in the active site area of the phytase molecule, as shown in the X-ray-deduced three-dimensional structure of phyA (Kostrewa *et al.*, 1997). The equivalent His residue in *E. coli* acid phosphatase is thought to perform a nucleophilic attack on the scissile phosphoester bond (Ostanin *et al.*, 1992), and the equivalent Asp residue is thought to protonate the leaving group (Ostanin and Van Etten, 1993).

A. niger phytase is extensively glycosylated, as pointed out earlier by the banding pattern in SDS-PAGE (Ullah and Gibson, 1987). The secreted phytase was found to be stable for months in the crude culture filtrate. In addition, during chemical sequencing of the protein it was necessary to add urea to a concentration of 4.0 *M* so that trypsin and chymotrypsin could cleave the peptide bonds (Ullah and Dischinger, 1993). Thus, it was thought that glyco-conjugates that are present in phytase must be preventing the protease from degrading the peptide

bonds. In phytase, there are 10 (originally reported as 9) asparagine (Asn) residues that could be glycosylated, and all of them were found to be glycosylated (Ullah and Dischinger, 1993). The role of glycosylation in the functional expression of *A. niger* phyA in *Pichia pastoris* has been investigated by Han and Lei (1999). They found that glycosylation was vital to the enzyme's thermostability. Glycosylation patterns for several fungal phyAs expressed in different expression systems have been determined (Wyss *et al.*, 1999b). The extent of glycosylation varied, but no significant effect on specific activity, thermostability, or the refolding properties of individual phytases was reported.

The role of glycosylation in fungal phyA protein was studied by expressing the gene in *E. coli* where glycosylation would not be possible. When the gene was expressed, the protein did not fold properly to produce activity (Phillippy and Mullaney, 1997). Thus, glycosylation may be assisting in folding the enzyme. In fungi grown in phosphate-limiting conditions, the *phy*A gene product has to travel across the membrane to reach the phosphate source, that is, starch in the medium. It is plausible that glycosylation of Asn residues not only facilitates the enzyme's transport across the membrane, but it may also help the linear polypeptide to fold appropriately as it exits the cell. To the contrary, in *E. coli* where *A. niger phyA* gene was overexpressed, the enzyme was found in inclusion bodies where it did not fold properly. This may explain why fungal phytase has not been successfully cloned and expressed in bacteria.

In 1996, a process for the deglycosylation of proteins for crystallization using a recombinant glycosidase fusion protein was developed (Grueninger-Leitch *et al.*, 1996) that enabled Kostrewa *et al.* (1997) to first crystallize *A. niger* NRRL 3135 phyA and then determine its structure by X-ray crystallography. The X-ray crystal structure of phyA established how the 5 disulfide bridges are formed from the 10 Cys residues: Cys31–Cys40, Cys71–Cys414, Cys215–Cys465, Cys264–Cys282, and Cys436–Cys444 (Fig. 2). The structure can be subdivided into a large α/β and a smaller α-domain. A deep indentation that contains the catalytically essential amino acids Arg81 and His82 is formed at the interface of these two domains.

By comparing the crystal structure of *A. niger* NRRL 3135 phyA with the previously determined structure of another HAP, rat acid phosphatase (Schneider *et al.*, 1993), Kostrewa concluded that several conserved residues—Arg81, His82, Arg85, Arg165, His361, and Asp362—from the α/β domain are essential for the catalytic process of phosphoester hydrolysis. In a model of substrate binding for this study, all these conserved amino-acid residues are involved with the scissile 3-phosphate group of phytate when hydrolysis is initiated. Several

applications of this X-ray crystallization study to facilitate enzyme engineering of phytase are provided in Section V.

A phytase from *Peniophora lycii*, a basidiomycete, has recently been reported and is awaiting approval for marketing as Bio-Feed® Phytase by Novo Nordisk (Lassen *et al.*, 1997). Based on a comparison of its amino-acid sequence with *A. niger* NRRL 3135 phyA, it appears to be an HAP with 10 cysteine residues; 4 of the 5 disulfide bridges show strong conservation (Fig. 3). Only the two N-terminal cysteine residues, forming the first disulfide bridges in *A. niger* phyA, are different in *P. lycii* phytase. One unique feature of *P. lycii* phytase is that, unlike *A. niger* NRRL 3135 phyA, it is a 6-phytase. All the known *Aspergillus* phytases start the hydrolysis of phytic acid at the third phosphorus group, that is, 3-phytase. Preliminary studies suggest that this enzyme

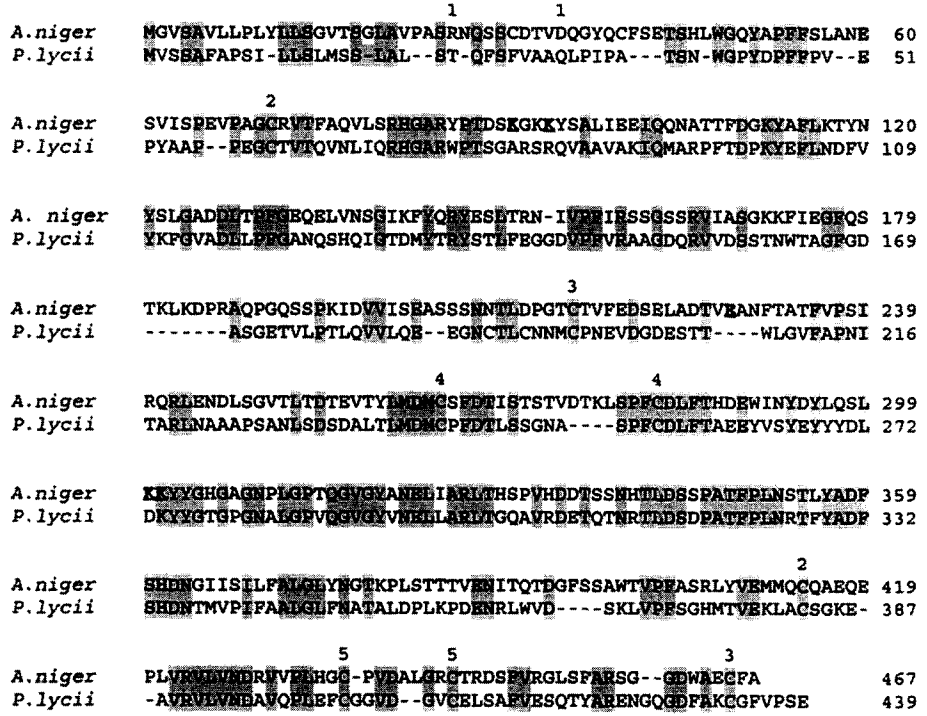

FIG. 3. A comparison of the amino-acid sequences of phyA of *A. niger* NRRL 3135 (NCBI Accession No. JN0656) and *P. lycii* phytase (Lassen *et al.*, 1997). Regions of conserved sequence are highlighted and the amino acids that compose the substrate specificity site (Kostrewa *et al.*, 1999) are underlined.

has a high initial rate of liberating phosphate from phytic acid coupled with high specific activity.

B. PhyB

Another extracellular enzyme from *A. niger* NRRL 3135 with phytase activity, which is also an HAP, is phytase B (phyB). This enzyme had been referred to in the literature initially as a pH-2.5-optimum acid phosphatase and was thought not to have phytase activity (Ullah and Cummins, 1987). However, additional studies have now shown that it does (Ullah and Phillippy, 1994), and one study has recently reported on its overexpression in a fungal expression system and the high phytase activity from the recombinant enzyme (Meittinen-Oinonen *et al.*, 1997). The deduced amino-acid sequence is currently available for three phyB genes: *phy*B from *A. niger* NRRL 3135 (Ehrlich *et al.*, 1993), *aph* from *A. niger* var. *Awamori* ATCC 38854 (Piddington *et al.*, 1993), and pH-2.5 acid phosphatase *A. niger* T213 (Kostrewa *et al.*, 1999).

Irving and Cosgrove (1972) were first to identify an *A. niger* NRRL 3135 phosphomonoesterase with phytase activity and a pH optimum for phytic acid of 2.0, which later was shown to be phyB. The enzyme was not purified, and, as such, molecular details and kinetic characterization were not available at the time. In 1987 the purification and characterization of a phosphomonoesterase with a pH optimum of 2.5 was reported (Ullah and Cummins, 1987). When this enzyme was tested for phytate breakdown at pH 5.0, the results were negative. Later, when the same enzyme from *A. niger* was assayed at pH 2.5 for phytate hydrolysis, it turned out that this enzyme is also an efficient phytase with a turnover number of 628 per second as opposed to 348 per second for phyA when phytate was used as a substrate (Ullah and Phillippy, 1994).

Like phyA, pH-2.5 acid phosphatase or phyB is a 3-phytase (Irving and Cosgrove, 1972). The phyB gene, which has been reported in other *Aspergillus* isolates, was also cloned from *Aspergillus awamori* by ALKO, a Finnish biotechnology company (Piddington *et al.*, 1993). Wyss and colleagues showed a fungal phyB to have low phytase activity (Wyss *et al.*, 1999a). A close examination indicates that the activity of the enzyme was tested at a substrate concentration of 5 m*M* phytate (Fig. 2, Wyss *et al.*, 1999a). We examined the effect of substrate concentration on enzyme activity with *A. niger* NRRL 3135 phyB phytase at pH 5.0 and 2.5. Maximum activity was observed at 1.5 m*M* phytate concentration. A concentration above this value was found to be inhibitory. This inhibition by excess substrate followed a concentration de-

pendency (Ullah, unpublished data). For *A. niger* NRRL 3135 phyA, at pH 2.5, a substrate concentration above 4 m*M* completely inactivated phytase activity (Fig. 3; Ullah, 1988). Wyss and his coworkers have interpreted their results to favor the view that phyB had low phytate-degrading ability. If the enzyme assays had been carried out at 1 m*M* phytate concentration, these authors would have drawn a very different conclusion. Thus, it appears that substrate concentration should be maintained at or about the 1 m*M* level to assess the enzymatic activity of extracellular phytase (Ullah, 1988).

The X-ray crystal structure of *A. niger* T213 phyB is now known (Kostrewa *et al.*, 1999). Unlike *A. niger* phyA, it is a tetramer formed by two dimers. Wyss *et al.* (1998) had reported *A. niger* T213 phyB (pH-2.5 acid phosphatase) to be an oligomer that is most likely composed of four identical protomers. The crystal structure reveals that the main contacts for each unit come from the N terminus, each interfacing with its neighboring molecule in that region. In this structure two dimers form a tetramer that allows each active site ready access to the substrates.

As in phyA, phyB also shows five disulfide bridges in its X-ray crystal structure. The three bridges analogous to Cys71–Cys414, Cys215–Cys465, and Cys436–Cys444 (Fig. 2) in phyA are conserved in phyB. The N terminus of phyB stretches out to allow for interfacing with its neighbor, while in phyA the N terminus is a disulfide bridge formed by Cys31–Cys40, which results in a compact loop configuration in this region.

Despite having almost identical catalytic centers, phyA and phyB have different pH profiles for hydrolysis of phytate. PhyA hydrolyzes phytate at both pH 2.5 and 5.0, but phyB displays optimum phytase activity at pH 2.5 and lacks activity at pH 5.0. Kostrewa *et al.* (1999) attribute this variation to differences in the charge distribution at the substrate specificity sites. In the *A. niger* T213 phyB site, there are only two acidic amino acids, Asp75 and Glu272 (Fig. 4). At the *A. niger* NRRL 3135 phyA substrate specificity site, there are two acidic and four basic amino acids: Glu228, Asp262, Lys91, Lys94, Lys300, and Lys301 (Fig. 2) [the last two Lys residues were erroneously given as 250 (227)[1] and 251 (228)[1] in the report of Kostrewa *et al.*, 1999]. The active site of phyB is thus more acidic than the active site of phyA. Therefore, at pH 2.5 the acidic amino acids (Asp75 and Glu272, Fig. 4) in the phyB substrate specificity site are uncharged and can accommodate the negatively charged phytate as a substrate. At this pH in phyA the four basic

[1]The amino-acid residue location in the abbreviated sequence in Fig. 4 (Kostrewa *et al.*, 1999).

```
                                                                    1
T213 phyB    FSYGAAIPQSTQEKQFSQEFRDGYSILKHYGGNGPYSERVSYGIARDPPTSCEVDQVIMV  60

                                                                2
T213 phyB    KRHGERYPSPSAGKDIEEALAKVYSINTTEYKGDLAFLNDWTYYVPNECYYNAETTSGPY 120
                N
T213 phyB    AGLLDAYNHGNDYKARYGHLWNGETVVPFFSSGYGRVIETARKFGEGFFGYNYSTNAALN 180

                             3         4
T213 phyB    IISESEVMGADSLTPTCDTDNDQTTCDNLTYQLPQFKVAAARLNSQNPGMNLTASDVYNL 240

                                                     4
T213 phyB    MVMASFELNARPFSNWINAFTQDEWVSFGYVEDLNYYYCAGPGDKNMAAVGAVYANASLT 300

T213 phyB    LLNQGPKEAGSLFFNFAHDTNITPILAALGVLIPNEDLPLDRVAFGNPYSIGNIVPMGGH 360
                                  C
                1                                 5         5
T213 phyB    LTIERLSCQATALSDEGTYVRLVLNEAVLPFNDCTSGPGYSCPLANYTSILNKNLPDYTT 420

                3                                 2
T213 phyB    TCNVSASYPQYLSFWWNYNTTTELNYRSSPIACQEGDAMD                      460
```

FIG. 4. The amino-acid sequence from the crystal structure study of the *A. niger* T213 phyB phytase gene (Kostrewa *et al.*, 1999). The N-terminal (N) (RHGXRXP) and C-terminal (C) (HD) motifs found in histidine acid phosphatase are underlined. The acidic amino acids of its substrate specificity site (Kostrewa *et al.*, 1999) are highlighted. The number above each of the 10 cysteine residues refers to the individual disulfide bridge to which it belongs (Kostrewa *et al.*, 1999).

amino acids (Lys91, Lys94, Lys300, and Lys301; Fig. 2) of the substrate specificity site are all positively charged and would attract the negatively charged phosphate groups of the phytate molecule. When the pH is raised to 5.0, the acidic amino acids become negatively charged, while the basic amino acids remain positively charged. The substrate binding site of phyB would thus repulse the negatively charged phytate molecule while the site in phyA would still attract the phosphate groups of phytate.

Applying this model for the substrate specificity site to *A. niger* NRRL 3135 phyB, it is interesting to note that only one of the two amino acids is acidic. In *A. niger* NRRL 3135 phyB, while Glu272 is conserved, Asp75 is serine, an aliphatic neutral amino acid with a hydroxyl side-chain (Fig. 5). This may indicate that only a single acidic residue at position 272 is necessary.

PhyB has been reported to have a broader substrate specificity than *A. niger* phyA (Wyss *et al.*, 1999a; Ullah and Cummins, 1988). The more

```
3135 phyB    MPRTSLLTLACALATGASAFSYGAAIPQSTQEKQFSQEFRDGYSILKHYGGNGPYSERVS  60
T213 phyB                          FSYGAAIPQSTQEKQFSQEFRDGYSILKHYGGNGPYSERVS  41

                       1
3135 phyB    YGIARDPPTGCEVDQVIMVKRHGERYPSPSAGKSIEEALAKVYSINTTEYKGDLAFLNDW 120
T213 phyB    YGIARDPPTSCEVDQVIMVKRHGERYPSPSAGKDIEEALAKVYSINTTEYKGDLAFLNDW 101
                                    N         *
             2
3135 phyB    TYYVPNECYYNAETTSGPYAGLLDAYNHGNDYKARYGHLWNGETVVPFFSSGYGRVIETA 180
T213 phyB    TYYVPNECYYNAETTSGPYAGLLDAYNHGNDYKARYGHLWNGETVVPFFSSGYGRVIETA 161

                                                  3         4
3135 phyB    RKFGEGFFGYNYSTNAALNIISESEVMGADSLTPTCDTDNDQTTCDNLTYQLPQFKVAAA 240
T213 phyB    RKFGEGFFGYNYSTNAALNIISESEVMGADSLTPTCDTDNDQTTCDNLTYQLPQFKVAAA 221

                                                                       4
3135 phyB    RLNSQNPGMNLTASDVYNLIVMASFELNARPFSNWINAFTQDEWVSFGYVEDLNYYYCAG 300
T213 phyB    RLNSQNPGMNLTASDVYNLMVMASFELNARPFSNWINAFTQDEWVSFGYVEDLNYYYCAG 281
                                                                    *

3135 phyB    PGDKNMAAVGAVYANASLTLLNQGPKEAGPLFFNFAHDTNITPILAALGVLIPNEDLPLD 360
T213 phyB    PGDKNMAAVGAVYANASLTLLNQGPKEAGSLFFNFAHDTNITPILAALGVLIPNEDLPLD 341
                                                 C
                                          1                          5
3135 phyB    RVAFGNPYSIGNIVPMGGHLTIERLSCQATALSDRGTYVRLVLNEAVLPFNDCTSGPGYS 420
T213 phyB    RVAFGNPYSIGNIVPMGGHLTIERLSCQATALSDEGTYVRLVLNEAVLPFNDCTSGPGYS 401

             5                       3                              2
3135 phyB    CPLANYTSILNKNLPDYTTTCNVSASYPQYLSFWWNYNTTTELNYRSSPIACQEGDAMD  479
T213 phyB    CPLANYTSILNKNLPDYTTTCNVSASYPQYLSFWWNYNTTTELNYRSSPIACQEGDAMD  460
```

FIG. 5. A comparison of the amino-acid sequences of phyB of *A. niger* NRRL 3135 (NCBI Accession No. P34754) and the partial phyB sequence of *A. niger* T213 (Kostrewa *et al.*, 1999). The N-terminal (**N**) (RHGXRXP) and C-terminal (**C**) (HD) motifs found in histidine acid phosphatase are underlined. The acidic amino acids of its substrate specificity site (Kostrewa *et al.*, 1999) are denoted by an *. The number above each of the 10 cysteine residues refers to the individual disulfide bridge to which it belongs (Kostrewa *et al.*, 1999). All of the nonconserved amino-acid sequence in T213 is highlighted.

neutral electrostatic field of the phyB substrate specificity site (Kostrewa *et al.*, 1999) provides a reason for this. A wider variety of phosphomonoesters can be utilized effectively by phyB at its optimum pH as a substrate. The highly positive electrostatic field of phyA's substrate-specific site is optimized for binding negatively charged phytate. Consequently, other less charged substrates bind less effectively at that site.

PhyB (pH-2.5 acid phosphatase) from *A. niger* T213 is more thermostable than recombinant phyA from either *A. niger* T213 or *A. fumigatus* ATCC 13070 (erroneously designated ATCC 34625) (Wyss *et al.*,

1998). However, exposure of *A. niger* T213 phyB to 90°C results in an irreversible conformational change and complete inactivation of the enzyme. PhyA from *A. niger* and *A. fumigatus* were denatured at temperatures between 50 and 70°C. *A. fumigatus* phyA, after cooling to 30°C, refolds into a native-like conformation and regains most of its activity. But the less heat-tolerant *A. niger* T213 phyA is capable of properly refolding only after heating to 50°C. The tetrameric structure of *A. niger* T213 phyB provides this higher thermostability. But it also explains why its individual protomers are unable to properly reassociate into an active tetramer once the molecule is denatured.

C. *E. COLI* HAP PHYTASE

Members of the HAP group also occur in prokaryotes. For example, *E. coli* HAP phytase is also known. Just as *A. niger* phyB was first reported as a pH-2.5 acid phosphatase, this periplasmic enzyme encoded by the *E. coli app*A gene was first identified in the literature as an acid phosphatase with a pH optimum of 2.5 (Dassa and Boquet, 1985). Subsequently, it was denoted a phytase P2, because of its high phytase activity (Greiner *et al.*, 1993). This study also elucidated the hydrolytic pathway this enzyme employs for phytin and accordingly established it as a 6-phytase. Recently, a recombinant form of *E. coli* phytase was purified and crystallized to provide a three-dimensional structure of a 6-phytase (Jia *et al.*, 1998). This X-ray-deduced model can be used with the crystal structure of *A. niger* phyA, a 3-phytase (Kostrewa *et al.*, 1997) to define the structural basis for their different catalytic pathways.

The complete nucleotide sequence of the *E. coli app*A gene has been determined (Dassa *et al.*, 1990). Rodriguez *et al.* (1999) utilized this sequence to design primers in order to clone the phytase gene from an *E. coli* isolate selected for its high phytase activity. The nucleotide sequence for this second *E. coli* phytase gene, *app*A2, was 95% homologous to *app*A. This translated into seven different amino acids in its deduced sequence. The significance of these sequence changes was established when both the *E. coli app*A and *app*A2 genes were expressed in *Pichia pastoris* and their recombinant proteins, r-appA and r-appA2, were markedly different in their pH profile and other catalytic characteristics (Rodriquez *et al.*, 1999).

Both r-appA and r-appA2 have identical sequence in the regions of the N-terminal motif (RHGVRAP, positions 38–44) and the C-terminal motif (HD, positions 325–326) that is characteristic of HAP. It was by a

site-directed mutagenesis study with this gene, *E. coli app*A, that Ostanin and Van Etten (1993) demonstrated the critical nature of the C-terminal motif, HD, to the catalytic function of the HAP class of enzymes.

D. Yeast HAP Phytase

Phytases have been reported previously in yeast (Nayini and Markakis, 1984). Several yeast acid phosphatase genes have now been cloned and characterized. For a review, see Wodzinski and Ullah (1996). In this review, three yeast acid phosphates were surveyed as HAP representatives and shown to have both the RHGXRXP and HD motifs in their amino-acid sequence. These three enzymes are encoded by the following genes: *Schizosaccharomyces pombe pho*1, *Saccharomyces cerevisiae pho*3, and *Schizosaccharomyces pombe pho*4. Subsequently the *S. cerevisiae pho*3 gene was cloned and transformed into an *Aspergillus oryzae* expression system (Moore *et al.*, 1995). An assay of the resulting recombinant *S. cerevisiae* HAP indicated high phytase activity. In addition, two other *S. cerevisiae* HAP genes were included in that screening, and they were shown to encode recombinant *S. cerevisiae* HAP with phytase activity. This suggests that similar yeast HAPs also can effectively hydrolyze phytic acid. Additional details of this transformation study are given in Section V.E.

E. Plants

Genes containing the HAP active site motif have recently been reported in diverse species of plants. In maize, a phytase cDNA, phy S11, was cloned and sequenced (Maugenest *et al.*, 1997). This cDNA was then utilized to screen a maize genomic library. Two different genes, *PHYT I* and *PHYT II*, were identified (Maugenest *et al.*, 1999). The study indicated that both *PHYT I* and *PHYT II* are expressed in germination of this monocotyledon, but only *PHYT I* is expressed in adult roots. A high level of homology is evident in the transcribed sequences of these two genes. However, other than partial homology to the region of amino acids around the HAP consensus motif, RHGXRXP, little sequence homology is found with *A. niger* phytase.

A putative *Arabidopsis thaliana* phytase, recently discovered by searching the Genbank database, shares more features with *A. niger* phyA phytase (Mullaney and Ullah, 1998b). The enzyme encoded by the *A. thaliana* gene has not only the septapeptide active sequence

RHGXRXP, but also the dipeptidic HD region necessary for phosphatase activity in fungal phytase. In addition, *A. thaliana* and *A. niger* HAP both have 10 Cys residues, necessary for proper structure folding by forming 5 crucial disulfide bridges in *A. niger* phyA. Moreover, the primary structure of both the plant and fungal phyA are comprised of 464 to 448 amino-acid residues, respectively.

III. Phytases with an Undefined Active Site

A. *Bacillus* Phytase (Phytase C)

A *Bacillus subtilis* phytase that hydrolyzed only phytate was reported by Powar and Jagannathan (1982). It required calcium for activity and had optimum activity at pH 7.5. A thermostable phytase from *Bacillus* sp. DS11, later identified as *B. amyloliquefaciens* (Ha *et al.*, 1999), was then purified and characterized (Kim *et al.*, 1998a) and its gene (*phy*) cloned and overexpressed in *E. coli* (Kim *et al.*, 1998b). In that same year a different research group, Kerovuo and colleagues (1998), reported on the characterization, gene cloning, and sequencing of a phytase (*phy*C) from *Bacillus subtilis* VTT E-68013. Sequence from the two cloned phytases indicates that neither of these contain the active site motif found in HAPs. Analysis of their putative amino-acid sequence shows they are highly homologous (93% sequence identity). However, while they both require calcium for activity and have a similar pH optimum, *B. subtilis* VTT E-68013 pH 7–7.5 and *B. amyloliquefaciens* pH 7–8.0, have different optimum temperatures: 55°C for *B. subtilis* and 70°C for *B. amyloliquefaciens*. Preliminary X-ray crystallographic analysis of the *B. amyloliquefaciens* thermostable phytase (TS-Phy) has been initiated (Ha *et al.*, 1999). Additional research on the contributions that their divergent sequence contributes to different temperature optima will advance our understanding of this novel class of phytase.

B. *Klebsiella* Phytase

Phytases have been isolated from both *Klebsiella terrigena* and *K. aerogenes*. A cytoplasmic phytase has been isolated from *K. terrigena* (Greiner *et al.*, 1997). It is reported to be a monomeric 3-phytase, with a molecular weight of 40 kDa. Like *A. niger* NRRL 3135 phyA, the *K. terrigena* phytase has an optimum temperature of 58°C and is not a metalloenzyme. This study also indicated that it is rather specific for phytate and has an optimum pH of 5.0. Tambe *et al.* (1994) found two inducible molecular forms of phytase from *K. aerogenes* (*Aerobacter*

aerogenes). The molecular weight of the large form is 700 kDa, and of the other form 10–13 kDa, making it the smallest known fraction to exhibit phytase activity. This indicates that, despite its size, an intact active site is present in the 13-kDa form. The possibility that the 700-kDa fraction was formed by noncovalent bonding of the 13-kDa enzyme with other larger proteins was discussed. The two also differed in their optimum pH value: 5.2 for the low-molecular-weight form and 4.5 for the larger.

C. Yeast

Two yeast species, *Schwanniomyces castellii* (*Schwanniomyces occidentalis*) (Segueilha *et al.*, 1992) and *Arxula adeninivorans* (Sano *et al.*, 1999), have secreted phytase that has been characterized. *S. castelli* phytase is tetrameric in structure with a molecular weight of 490 kDa. When deglycosylated, this tetramer is composed of one large unit (125 kDa) and three identical subunits (70 kDa). Its phytase has been reported to be thermostable up to 74°C. Conditions to optimize *S. castelli* CBS 2863 phytase yield have been determined (Lambrechts *et al.*, 1993). Both *S. castelli* CBS 2863 and *A. adeninivorans* secreted phytases have high optimum temperatures: 77 and 75°C, respectively.

D. Plants and Microbes

A phytase has been purified from soybeans (Morgan *et al.*, 1998). Based on limited amino-acid sequence, it does not show homology with any known HAP phytase. Amino-acid sequence analysis reveals its only match is with a region in the N terminus of a putative purple acid phosphatase in *Arabidopsis* (NCBI Accession #AAC04486). Phytase from scallion (*Allium fistulosum* L.) leaves has also been purified and determined to have a maximum activity at pH 5.5 and an optimum temperature of 51°C (Phillippy, 1998). The phytase from another monocotyledon, oats (*Avena sativa*), has also been isolated. It is a monomeric enzyme with a molecular weight of about 67 kDa and has pH and temperature optima of 5.0 and 35°C, respectively (Greiner and Alminger, 1999).

McElhinney and Mitchell (1993) demonstrated phytase activity in ectomycorrhizal fungi. Isolates of *Paxillus involutus*, *Suillus grevillei*, and two unidentified basidiomycetes from Sitka spruce were shown to have phytase activity. The enzyme activity appears to be membrane-

bound and not extracellular. It was postulated that this might be an adaptation for growth in soils where available phosphorus is low and there is intensive competition for limiting nutrients.

Paramecium phytase was found to degrade phytate to $Ins(1,2)P_2$ via the dephosphorylation sequence 6/5/4/1 (Van Der Kaay and Van Haastert, 1995). In investigations of phytate synthesis, the *Paramecium* phytase allows the kinetics of incorporation and release of radiolabeled phosphate to be precisely followed.

IV. Increased Phosphorus Levels in Our Environment Creates Need for Phytase

After the Second World War, industrial nitrogen fixation technology that was originally developed for munitions production was adapted to supply agriculture with nitrogen for fertilizer. This ushered in the age of fertilizer and transformed agriculture. With this development, nitrogen could be combined with phosphorus and potassium to precisely match the nutrient requirement of crops. This has contributed to the specialization found in agriculture, and in the 1990s the upper Midwestern states became the major users of phosphorus fertilizer, primarily for feed-grain production; this grain was then exported (Lanyon, 1999).

This abundance of feed grain has fostered the emergence of industrial animal production units in various regions of this country (Mallin, 2000). During the last decade, numerous large poultry and swine production units have been constructed in coastal regions of the Southeastern United States. Traditional reliance on crops to utilize and crop lands to bind the high level of phosphorus in the manure from these operations has been inadequate. When the capacity of the soil to bind phosphate is exceeded, it enters groundwater or adjacent surface water bodies. This is especially problematic in the sandy soils that are typical in coastal plains.

An increased concern has recently emerged about the environmental consequences of this nutrient runoff from agricultural operations. This concern has been generated in part by an increasing number of blooms of toxin-producing microbes around the world. A recent example of the phenomenon was large-scale fish kills in the Chesapeake Bay attributed to a toxin-producing dinoflagellate, *Pfiesteria piscicida*, which in 1997 attracted national news coverage (Mlot, 1998).

Very limited research has been carried out on the effects of phytic acid or inositol phosphates on aquatic microbes. Chu (1946) studied

the ability of marine diatoms to utilize various phosphorus compounds. Phytin was shown to support the growth of *Phaeocystis pouchetii*, *Skeletonema costatum*, and *Nitzschia closterium*. The oceanic dinoflagellate *Pyrocystis noctiluca* is also known to utilize phytic acid (Rivkin and Swift, 1980). The involvement of inositol phosphates has been cited in cyst formation in another dinoflagellate, *Crypthecodium cohnii* (Tsim *et al.*, 1998). Research on *Pfiesteria piscicida* has established that organic phosphorus does stimulate both its toxic form and the nontoxic zoospores. Phosphorus is also believed to play a role in the transformation of various stages of *P. piscicida* into a toxic zoospore stage (Burkholder and Glasgow, 1997).

The widespread concern caused by the 1997 *Pfiesteria* fish kill in the Chesapeake Bay resulted in a Blue Ribbon Citizens' *Pfiesteria* Action Commission. This commission investigated the phenomenon and issued a report to the Governor of Maryland. Among its findings was that the large poultry-producing region of the state was applying more phosphorus in manure than the crops could utilize (Mallin, 2000). A complex of factors resulting from developments in the fertilizer and feed industries have resulted in the phosphorus supplies on many farms now exceeding their crops' nutrient requirements (Lanyon, 1999).

The commission considered several means for reducing the amount of phosphorus manure applied to the land. They concluded that the most encouraging proposal in this area was the use of phytase in animal feed. The efficacy of phytase in reducing phosphorus levels in livestock manure has been established (Yano *et al.*, 1999; Ward, 1993). Acting on this part of the commission's report, Governor Glendening proposed that as of 1 January 2000 phytase was to be added to all poultry feed produced in Maryland. To help defray the added cost of preparing the feed mills to use this enzyme, it was also proposed that the state support a cost-sharing program with feed producers.

V. Engineering Phytase

A. Heat Tolerance

The cost of using phytase as an animal feed additive can be reduced if the heat tolerance of the enzyme is increased. This is because, during processing the plant meal, most of the meal mash is heated briefly to a high temperature (65–95°C). The *A. niger* phytase that is currently marketed would be denatured at these temperatures. This means an additional processing step is required after pelletization to add the phytase, thus adding to the cost. A phytase that combines the desirable

traits in the *A. niger* NRRL 3135 phytase that is now commercially available with high heat tolerance would therefore be a superior enzyme for most current animal feed applications.

The search for higher thermostability has led to the cloning of phytase genes from thermophilic fungi. The phytase genes from *Myceliophthora thermophila* (Mitchell *et al.*, 1997) and *Talaromyces thermophilis* (Pasamontes *et al.*, 1997b) have been cloned. Analysis of their predicted amino-acid sequence revealed that both have a high identity to known phytases and are HAPs. Their optimum temperatures were not reported, but the optimum pH for enzyme activity for phytic acid for *M. thermophila* is 5.5 (Wyss *et al.*, 1999b). Another thermophilic fungus, *Thermomyces lanuginosus*, has been cloned and the expressed enzyme characterized (Berka *et al.*, 1998). *T. lanuginosus phy*A encodes a mature protein of 442 amino acids that has 47% homology with the *A. niger* phytase (phyA). The *T. lanuginosus* phytase gene was transformed and expressed in *Fusarium venenatum*. The optimum temperature for phytase activity for the recombinant phytase was 65°C. While the pH profiles for *A. niger* and *T. lanuginosus* phytase (phyA) are similar, *T. lanuginosus* has a slightly higher optimum pH, pH 6, and its enzyme is active at neutral pH, where *A. niger* phytase lacks activity.

Several other phytases have been investigated as possible enzymes with increased heat tolerance. They include phytase from *Aspergillus terreus* No. 9A-1, optimum temperature 70°C (Yamada *et al.*, 1968); *Schwanniomyces castelii* (Segueilha *et al.*, 1992); *Arxula adeninivorans* (Sano *et al.*, 1999); *Bacillus* sp. DS11 (Kim *et al.*, 1998a); and *A. fumigatus* ATCC 13073 (Pasamontes *et al.*, 1997a).

The *A. fumigatus* ATCC 13073 phytase gene has been cloned and overexpressed in *A. niger* NW205 (Pasamontes *et al.*, 1997a). The recombinant phytase was reported to withstand temperatures up to 100°C for more than 20 minutes with only minimal loss (10%) of its enzymatic activity. In a subsequent study (Wyss *et al.*, 1998), this recombinant phytase was compared with the phytase encoded by the *A. niger* T213 phytase gene, which was cloned and also overexpressed in *A. niger* NW205. A comparison of the two recombinant phytases revealed that, while *A. niger* T213 phytase does not have the capacity to refold properly after heat denaturation, the recombinant *A. fumigatus* phytase refolds into a native-like and fully active configuration.

Studies to support the potential use of *A. fumigatus* ATCC 13073 phytase as a feed additive that can withstand the brief period of high temperatures required in pelletization of animal feed have been performed. When the recombinant *A. fumigatus* phytase was added to feed

mash that was then pelleted at 85°C, approximately 50% of the enzymatic activity was recovered (Wyss *et al.*, 1998). Nunes and Guggenbuhl (1998) evaluated the efficacy of recombinant *A. fumigatus* phytase in a feeding trial with pigs. Recombinant *A. fumigatus* phytase was shown to significantly decrease the phosphorus content in feces, and it also increased the growth rate for the pigs over pigs fed with a control diet.

Analysis of the *A. fumigatus* ATCC 13073 phytase sequence reveals no obvious reason for its higher heat tolerance. The only unique feature of its sequence is a higher pI (7.28) than the other known fungal phytases (Wyss *et al.*, 1999b). This pI value is also higher than most of the extracellular proteins produced by the expression strains used (*A. niger* NW205). This would make it especially well suited for rapid purification if it is produced on an industrial scale.

Using a new biotechnology technique, the first "engineered" phytase has already been assembled. A phytase with an increased optimum temperature has been reported by using a consensus technique. This technique compares the amino-acid sequence of several known phyAs and then selects the most conserved choice for each residue. This sequence is then back-translated into a DNA sequence, and then this DNA is transformed into an expression system (Lehmann, 1998). A high degree of conservation of amino-acid sequence and features such as preservation of cysteines for disulfide bridges are observed in a comparison of the consensus and the *A. niger* NRRL 3135 phyA (Fig. 6).

The use of compounds to enhance the thermostability of phytase has also been investigated (Phillippy, 2000). Phytate is reported to enhance the activity of *A. adeninivorans* phytase (Sano *et al.*, 1999), and calcium contributes to the heat tolerance of *Bacillus* sp. DS11 (Kim *et al.*, 1998a). The effects of different buffers on the heat tolerance of *A. fumigatus* phytase expressed in *Pichia pastoris* indicates they can facilitate refolding of the enzyme into the native-like, active configuration after heat denaturation (Rodriguez *et al.*, 2000). Immobilization of *E. coli* phytase is reported to enhance its thermostability (Greiner and Konietzny, 1996). Glycosylation of recombinant *A. niger* phyA expressed in *S. cerevisiae* (Han *et al.*, 1999) and in *Pichia pastoris* (Han and Lei, 1999) contributes to the thermostability of the enzyme produced.

B. Temperature and pH Optima

The optimum temperature for *A. niger* NRRL 3135 phyA activity is 58°C (Ullah and Gibson, 1987). This is approximately 20° higher than the

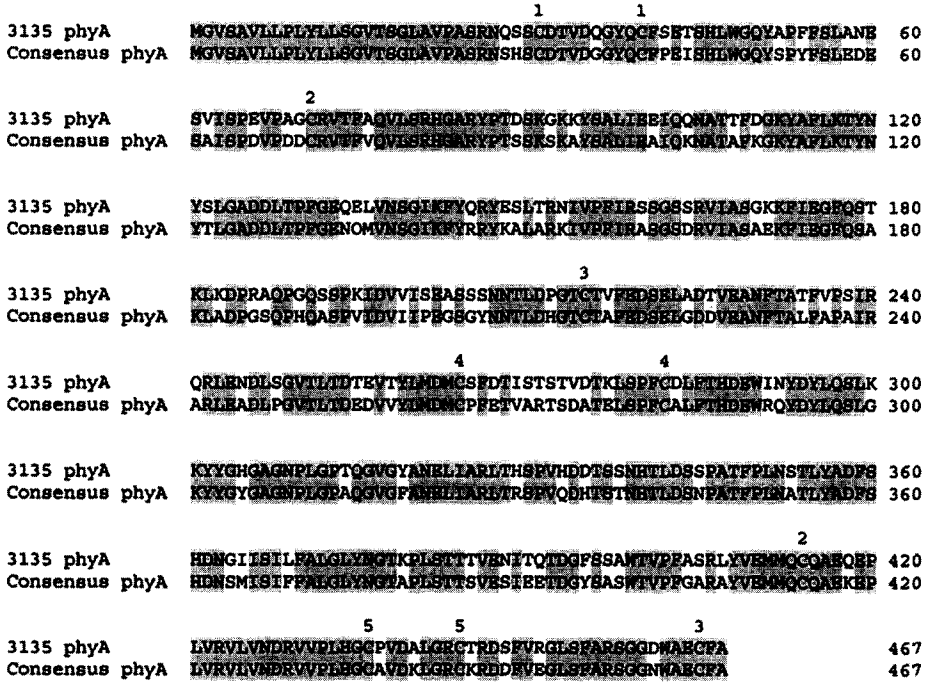

FIG. 6. A comparison of the amino-acid sequences of phyA of *A. niger* NRRL 3135 (NCBI Accession No. JN0656) and the consensus phytase (Lehmann, 1998). Regions of conserved amino-acid sequence are highlighted, and the number above each of the 10 cysteine residues refers to the individual disulfide bridge to which it belongs (Kostrewa *et al.*, 1999).

body temperatures of poultry and swine, at which maximum activity is desirable. When used in aquaculture, with lower body temperatures of the animals, there is even a more pronounced activity reduction. Having a pH optimum similar to the pH level found in the digestive tract of the animal is essential for maximum effectiveness of the enzyme. Having a broad pH optimum for phytase activity as reported for *A. fumigatus*, pH 2.5–7.5 (Pasamontes *et al.*, 1997a), would expand the enzyme's potential usefulness as a feed additive. Crystal structure studies of differences in charge distribution of the substrate specificity site (Kostrewa *et al.*, 1999) offers the first insight into the molecular basis for the pH optima of both phyA and phyB (see §II.B). When this pH optimum model is applied to *P. lycii* phytase, the local electrostatic

charge of the respective amino acids in the substrate specificity site (Fig. 3) at pH 5.0 would be negative. This would explain the lower pH optimum, pH 4.0, reported for *P. lycii* phytase (Lassen *et al.*, 1997). Further research into these mechanisms offers the potential to eventually engineer a feed additive superior to the native fungal phytase that is in commercial use today.

C. Substrate Specificity

To be effective in an animal diet high in phytic acid, high phosphohydrolytic activity associated with phytase is essential. The phytases that have been characterized to date do not all have the same affinity for phytate as a substrate. Wyss *et al.* (1999a) have categorized *E. coli* and several fungal phytases into two groups based on activity levels for phytic acid and the ability of the enzyme to hydrolyze other substrates. The first group has high activity (102 to 811 U/mg) but a narrow substrate specificity and includes *A. niger*, *A. terreus*, and *E. coli* phytase. The second, with a low activity (23 to 41 U/mg), but a broad substrate range, includes phytases from *A. fumigatus*, *A. nidulans*, and *M. thermophila*. The activity of the second group is similar to the activity level of *A. niger* NRRL 3135 pH-6.0-optimum acid phosphatase (Ullah and Cummins, 1988; Ullah and Dischinger, 1993), which is a metalloenzyme (Mullaney and Ullah, 1998a). A molecular basis for this division is not known at this time. Kostrewa *et al.* (1999) do offer some insight into the narrow substrate specificity of phyA for phytate. It is the positive charge of its substrate specificity site that, while optimized for binding phytate, is less attractive to other substrates not as negatively charged.

D. Enzyme Stability

The stability of plant and microbial phytases have been reviewed by Phillippy (2000). *A. niger* phytase is known to be significantly more resistant to proteolytic digestion than either wheat (Phillippy, 1999) or *A. fumigatus* phytase (Wyss *et al.*, 1999b). Recombinant phytases from *A. fumigatus*, *A. nidulans*, *A. terrus* 9A1, and *M. thermophila* when expressed in *A. niger* undergo proteolytic degradation. To address this problem, Wyss *et al.* (1999b) have successfully engineered the higher level of proteinase resistance found in *A. niger* phytase into *A. fumigatus* phytase. They compared the N-terminal amino-acid sequence of *A. fumigatus* phytase to the three-dimensional structure model of *A.*

niger phytase, and deduced that the proteolytic cleavage site was within one of the exposed loop structures. A site between amino acids 152 and 153 of *A. fumigatus* phytase (Wyss *et al.*, 1999b) was then identified as the probable cleavage site. Site-directed mutagenesis at that site with S152N and R151L/S152N yielded mutant proteins with reduced susceptibility to proteolysis. The utilization of this information from the available three-dimensional structure model marks a significant achievement toward the goal of actually being able to engineer a phytase molecule with improved enzymatic characteristics for use as an animal feed additive.

E. Synergistic Effect

When *A. niger* (*ficuum*) NRRL 3135 is grown under limiting phosphate conditions, it produces four different extracellular acid phosphatases. These four enzymes are phyA (Ullah and Gibson, 1987), phyB (Ullah and Cummins, 1987; Ullah and Phillippy, 1994), Apase6 (Ullah and Cummins, 1988; Mullaney *et al.*, 1995), and phoA (Ehrlich *et al.*, 1994). Whereas phyA and phyB, both histidine acid phosphatases, can effectively hydrolyze phytic acid, Apase6, which is a purple acid phosphatase, cannot. The active site motif and hydrolytic mechanism of phoA is not known, but this enzyme cannot effectively utilize phytic acid as a substrate. The genes for these enzymes are not clustered, but rather are dispersed throughout its genome (Table II). Their simultaneous expression by *A. niger* suggests that in nature, when this fungus experiences certain nutrient conditions, it needs all these enzymes to scavenge enough phosphorus from the available sources. A single acid phosphatase/phytase did not evolve with superior hydrolytic activity for all the phosphate sources this mold encounters in its surroundings. It also may be advantageous to combine different phytases or acid phosphatases in animal feed rations to achieve a more efficient utilization of the phytin phosphorus by the animal.

A study by Moore *et al.* (1995) suggested that an increase in phytin phosphorus availability may be achieved by combining two acid phosphatases. Three *Saccharomyces cerevisiae* histidine acid phosphatase genes (*pho*3, *pho*5, and *pho*11) were cloned by polymerase chain reaction and overexpressed separately in an *Aspergillus oryzae* isolate. Phytase activity was then measured in the control *A. oryzae* and in individual isolates from the three transformations. All three transformations yielded isolates that displayed up to a four- to sixfold increase

TABLE II

CHROMOSOME LOCATION OF *A. NIGER* ACID PHOSPHATASE GENES

Gene	Chromosome
*phy*A	III
*phy*B	VIII
*aph*A	IV
*pho*A	I

Chromosome location of the four *A. niger* phosphate-repressible acid phosphatase genes determined by CHEF electrophoresis and Southern blot analysis (Mullaney, unpublished data).

in phytase activity. Phytase activity of the native yeast acid phosphatases were not, however, reported in that study.

In the same study, the *A. niger aph*A gene (MacRae *et al.*, 1988; Mullaney *et al.*, 1995), which encodes Apase6, a purple acid phosphatase that has low phytase activity, was also cloned and overexpressed in *A. oryzae*. One isolate from this transformation, with an estimated number of 20 copies of the *aph*A gene, was reported to have a fivefold higher level of phytase activity than the control *A. oryzae*. This suggests that the increase in phytase activity was due to a synergistic effect between the recombinant purple acid phosphatase and the host *A. oryzae* phytase.

The efficacy of the pho5 and the aphA recombinant acid phosphatases was established in a feeding trial. In a chicken feed trial, the basal diet contained 0.25% unavailable P (phytic acid). Both enzymes were effective and raised the plasma phosphorus levels in the test animals to a level equivalent to that obtained in chickens fed a diet of supplemental inorganic phosphorus. Compared to a commercially available phytase, the recombinant acid phosphatases increased P utilization by 40% compared to 48% for phytase.

Park *et al.* (1999) showed that more enzymatic activity might be induced by combining *A. niger* NRRL 3135 phyA and *B. amyloliquefaciens* phyC. Their study indicated that different combination ratios of phyA and phyC, because of their different pH profiles, would be more effective at hydrolyzing phytate over the entire gastrointestinal tract than either single phytase alone.

Another synergistic effect from combining two acid phosphatases was recently reported by Wyss *et al.* (1999a). Their study showed that *A. fumigatus* and several other fungal phyAs only release five of the six phosphate groups in phytic acid. However, when a combination of *A. fumigatus* phytase (phyA) and *A. niger* pH-2.5 acid phosphatase (phyB) was utilized, all six of the phosphate groups were released. This suggests that this combination of enzymes would be beneficial in increasing phosphorus utilization in animal feed rations with high phytic acid levels.

Evidence supporting this synergistic effect has also been described by Vanderbeke *et al.* (1994). By blending phyA and phyB, a higher synergetic phytate hydrolyzing efficiency after thermal treatment was observed. The higher thermal stability of phyB (pH-2.5 acid phosphatase) and its importance in achieving this effect was noted. The molecular basis for phyB's greater heat stability is discussed in Section II.B. However, in a recent hog feeding trial and an *in vitro* study, no evidence of any synergistic interaction between phyA and phyB was reported (Nasi *et al.*, 1999). In this study, genes encoding the *A. niger* var. *Awamori* phytase (phyA) and pH-optimum-2.5 acid phosphatase (phyB) (Piddington *et al.*, 1993) were transformed separately into *Trichoderma reesei*. The transgenic phytases were then utilized in both *in vitro* and *in vivo* tests that showed no significant synergistic effect in hydrolyzing phytin phosphorus from barley, maize, and soybean meal, when the diet was fed without thermal processing.

Research was also conducted on the benefits of producing phytase in a fungal expression system that simultaneously produces another hydrolytic enzyme. The gene for *A. niger* pH-2.5 acid phosphatase (phyB) (Piddington *et al.*, 1993) was transformed and expressed in a high-cellulase-production strain of *Trichoderma reesei* (Meittinen-Oinonen *et al.*, 1997). The recombinant phytase was secreted into the culture medium broth and retained its activity. This enzyme mixture would increase the nutritional value of animal feeds containing significant amounts of both phytic acid and cellulose.

An enzymic "cocktail" composed of phytase, an acid phosphatase, an *Aspergillus saitoi* acid protease, citric acid, and an *A. niger* pectinase was studied under simulated intestinal conditions to measure enzymatic dephosphorylation of corn (Zyla *et al.*, 1995). The results indicated that the "cocktail" improved digestibility of phytate phosphorus, protein, and carbohydrates. The use of citric acid alone to lower gastric pH levels and thus enhance phytase efficacy has been tested in hog

feeding experiments (Goihl, 1998). However, no significant effects were reported.

VI. Enzyme Production

A. Filamentous Fungi

Today, large-scale fermentation operations employ overexpression and other biotechnological techniques to produce nearly all the recombinant phytase used by the animal feed industry. One company, Gist-Brocades, has cloned multiple copies of the *A. niger* NRRL 3135 *phy*A gene into their PluGBug® system that yields high levels of phytase in their *A. niger* host. This product is now being marketed as Natuphos™ (van Dijck, 1999). Another enzyme producer, Novo Nordisk, is replacing their current phytase product, Phytase Novo™, with another phytase cloned from *Peniophora lycii*, a basidiomycete (Novo Nordisk A/S, 1999). This *P. lycii* phytase will be overexpressed in an *A. niger* expression system and sold under the product name Bio-Feed Phytase (Ronozyme™ P). Alltech Inc. produces another phytase, Allzyme Phytase 115. This is a nonrecombinant phytase from a proprietary isolate.

B. Expression in Yeast

The potential to employ a yeast expression system for commercial phytase production has been examined in several studies. Han *et al.* (1999) described the use of a relatively low-cost medium containing yeast extract–peptone–dextrose (YEPD) to produce *A. niger* NRRL 3135 phyA in a *Saccharomyces cerevisiae* system. This research utilized the pYES2 expression vectors (Invitrogen) to construct a plasmid, pYPP1, that when transformed and expressed in *S. cerevisiae* yielded up to 2797 units per liter of extracellular phytase activity in the medium supernatant within 15 hours.

Hansenula polymorpha and *Pichia pastoris*, facultative methylotrophic yeasts, have been investigated as potential high-yield production systems for phytase. *A. niger* NRRL 3135 phyA has been expressed in *P. pastoris* and with the production of high levels of active phytase (25–65 U/ml of medium) (Han and Lei, 1999). This recombinant phyA shared the same characteristics with phyA overexpressed by *A. niger* with slightly improved thermostability profile. Phytase titers in the fermentation supernatant up to 13.5 g/liter for a "consensus" phytase (Lehmann, 1998) have been achieved by an expression system using a

recombinant strain of *H. polymorpha* (Mayer *et al.*, 1999). This system featured the use of an economical carbon source, glucose or glucose syrups, as a means to make this a low-cost process. However, lower yields (6.1 g/liter) were obtained when the *A. fumigatus phy*A gene was expressed in the system.

C. Expression in Plants

The gene for fungal phytase has been successfully overexpressed in several transgenic plants (Day, 1996; Verwoerd *et al.*, 1995; Li *et al.*, 1997). The ability to express recombinant phytase in plants offers the possibility for the development of plant varieties that would contain sufficient amounts of phytase in their grain or seed so that phytase supplements would not be required. In addition, the potential use of crop plants to serve as bioreactors to produce phytase commercially is being investigated. In 1995, Verwoerd and coworkers in Holland did express a functional phytase in *Nicotiana tabacum* through a constitutive expression of phytase cDNA and showed that the enzyme was secreted out of the cells. They achieved secretion to the extracellular fluid by the use of a signal sequence from the tobacco pathogen-related protein S (Verwoerd *et al.*, 1995). The expressed phytase was found to be biologically active and accumulated in leaves up to 14.4% of total soluble protein during plant maturation. Researchers at the University of Wisconsin Biotechnology Center have independently expressed the *A. niger* NRRL 3135 *phy*A gene in tobacco leaves. The full 441-aa protein was made in leaf tissue, which was purified to homogeneity and extensively characterized (Ullah *et al.*, 1999). Except for a decrease in molecular mass due to reduced glycosylation, the expressed recombinant phytase was virtually the same as native fungal phytase. The catalytic properties of the cloned phytase were encouraging enough to open the possibility of overexpressing the fungal phyA gene in other crop plants. This could pave the way for producing phytase commercially in field crops.

University of Wisconsin researchers have also developed alfalfa plants to commercially produce phytase. They performed cloning and expression of the fungal phytase gene, so that most of its product was contained in the juice collected after the alfalfa was processed (Gutknecht, 1997). The equipment investment for this biofarming process is minimal and potentially turns a byproduct into a source of additional income for the farmer. Other enzymes have also been expressed in these

plant "bioreactors." But the results achieved with phytase enhances the feasibility of future development of this technology to produce this enzyme commercially.

Another application of biotechnology in plants is to reduce the need for phytase by lowering phytate levels in the plant's cereal or meal. Maize cultivars with reduced levels of phytic acid have already been produced (Raboy and Gerbasi, 1996). Transgenic soybean isolates that overexpress fungal phytases and thus eliminate or reduce the need to supplement meal with phytase are also being pursued. Li *et al.* (1997) have expressed the *A. niger* NRRL 3135 phytase gene in soybean (*Glycine max*). The recombinant phytase had a lower molecular weight than the native fungal enzyme, but its temperature and pH optimum were almost identical to that of the native enzyme.

This strategy of having transgenic plant overexpressed phytase still requires a heat-tolerant phytase to survive the elevated temperatures often required in feed production. Recent work at the Swiss Federal Institute of Technology in Zurich details the transfer of a heat-tolerant *A. fumigatus* phytase gene by transformation and its expression in rice (*Oryza sativa* L.). This research is targeted at improving the nutritional profile of rice by reducing the amount of phytate. Phytate binds up to 95% of the iron in rice and keeps it from being absorbed. Therefore, individuals in parts of the world with a high-rice diet are prone to iron deficiency (Gura, 1999).

D. Transgenic Animals

In the future, transgenic poultry, hogs, and so on may produce phytase in their own digestive tract. Several attempts have already been made to transform and express a fungal phytase in an animal (privileged information, personal communication). To date none of these attempts have been successful. Similar results were obtained when the *phyA* gene was expressed in *E. coli* (Phillippy and Mullaney, 1997). The problem could very well be associated with glycosylation or its lack in animal and bacterial cells, respectively. Native fungal phyA protein contains 10 asparagine (Asn) residues with glycosylation signals that are all *N*-glycosylated. In *E. coli* these Asn residues are not glycosylated, and perhaps the recombinant protein does not fold appropriately to produce active site geometry, which is essential for activity. Similarly, in animal systems where *O*-glycosylation is preferred, the 10 *N*-glycosylation sites will be left unglycosylated. This may explain the lack of activity of phyA protein expressed in animals. Perhaps engi-

neering *O*-glycosylation sites at these locations may allow the mammalian cells to glycosylate either the threonine or serine residues and thereby allow for appropriate folding of recombinant phytase. This, however, needs to be tested.

As more is understood about the structure–function relationships of the microbial phytases, another possible avenue is to modify an animal's HAP, that is, rat acid phosphatase (Kostrewa *et al.*, 1997; Schneider *et al.*, 1993), or multiple inositol polyphosphate phosphatase (Craxton *et al.*, 1997) to enhance its ability to hydrolyze phytin. If successful, this would reduce the difficulty of obtaining expression in animal tissue.

VII. Expanding Uses of Phytase

A. Potential in Aquaculture

Numerous studies have been conducted on the use of soybean meal or other plant meals in aquaculture, including feeding studies on rainbow trout (Watanabe and Pongmaneerat, 1993; Mwachireya *et al.*, 1999), the greenback flounder (Bransden and Carter, 1999), and the African catfish (van Weerd *et al.*, 1999). By substituting lower-cost plant protein for a more expensive protein source, such as menhaden fish meal, a significant cost reduction could be achieved. Feed costs constitute up to 70% of total fish production costs (Rumsey, 1993). The consumer price index over the period of 1982–92 showed that, while the price index for seafood increased by more than 50%, the cost of alternative proteins increased only an average of 30% (Chamberlain, 1993).

As in poultry and hogs, fish lack an adequate digestive enzyme to effectively utilize the phytin phosphorus in this feed. Moreover, as aquatic animals, the problems associated with high phosphorus levels in the water from their waste is an immediate problem. Therefore, phytase has been evaluated as a means to both increase the use of low-cost plant meals in the aquaculture industry, and also to maintain acceptable phosphorus levels in the water. Several fish feeding studies have documented the potential value of phytase in diets containing high levels of plant feedstuffs (Robinson *et al.*, 1996; Oliva-Teles *et al.*, 1998: Mwachireya *et al.*, 1999).

Chamberlain (1993) has projected that global seafood consumption will increase 35% by 2025. The aquaculture industry will supply an increasing amount of the world's need for seafood. Whereas the major market for phytase today is as a food additive in poultry and hog feed, there is great potential to expand the market into feedstuffs used in

aquaculture. The higher temperature required for pelletization of feed in aquaculture and the lower body temperature of fish may require the development of commercial phytases tailored for aquaculture feeds.

B. Phytase as a Soil Amendment

In certain locations, phytic acid and its derivatives may represent up to 50% of the total organic phosphorus in the soil (Dalal, 1978). This abundance of phytic acid in the soil and the possibility that the addition of phytase might stimulate plant growth in these soils has been investigated. Findenegg and Nelemans (1993) studied the effect of phytase (phyA) on the availability of phosphorus from phytic acid in the soil for maize plants. Growth stimulation was reported as the result of an increased rate of phytin hydrolysis when phytase was added to the soil. However, the amount of phytase necessary for a significant effect meant that this was not a practical technique at this time. This study also suggested that the expression of phytase in the roots of transgenic plants might increase the availability of phosphorus to plant roots (Day, 1996). To better understand the role root phytase plays in the phosphorus nutrition of plants, the phytase and acid-phosphatase activity of extracts from several temperate pasture grass and legumes were isolated and studied (Hayes *et al.*, 1999).

C. Production of *myo*-Inositol Phosphates

Greiner and Konietzny (1996) investigated the use of *E. coli* phytase to generate specific breakdown products from phytic acid. A packed-bed bioreactor containing covalently attached *E. coli* phytase was constructed in this study to economically produce special isomers of the lower *myo*-inositol phosphate esters. The bioreactor chiefly yielded $I(1,2,3,4,5)P_5$, $I(2,3,4,5)P_4$, $I(2,4,5)P_3$, and $I(2,5)P_2$ isomer forms. Because only one major isomer of each *myo*-inositol phosphate species was formed, further purification could be easily achieved by ion-exchange chromatography.

The K_m for phytate increased from 130 μM for free enzyme to 240 μM when *E. coli* phytase was immobilized. However, the catalytic turnover number was lowered from 6209 per second for free phytase to 1182 per second. Thus, on crosslinking, *E. coli* phytase's catalytic activity was slowed down drastically. However, the immobilized bacterial enzyme performed much better than the immobilized *A. niger* NRRL 3135 phyA (Ullah and Cummins, 1988; Dischinger and Ullah, 1992). One reason

that the fungal phytase performed so poorly on immobilization could be due to extensive crosslinking of the enzyme through its glyco-conjugates. The active site geometry of *Aspergillus niger* phytase was altered significantly to cause such loss of activity. To the contrary, crosslinking of protein due to glycosylation would not be a problem when *E. coli* phytase is used in a bioreactor.

D. Semisynthesis of Peroxidase

A semisynthetic peroxidase was designed by taking advantage of the structural similarity of the active site of vanadium-dependent haloperoxidases and fungal phytases and acid phosphatases (van de Velde *et al.*, 2000). The Delft group incorporated vanadate ion into the active site of *A. niger* (*ficuum*) NRRL 3135 phytase. This resulted in transformation of native phosphohydrolase activity of phytase into semisynthetic peroxidase. The "new" enzyme was able to catalyze enantioselective oxidation of prochiral sulfides, with H_2O_2 affording the *S*-sulfoxide. Under the reaction conditions, this semisynthetic vanadium peroxidase was found to be stable for over 3 days with only a slight loss in turnover number. The other exceptional feature of this "new" enzyme, being polar water-miscible, was that cosolvents, such as methanol, dioxane, and dimethoxyethane, could be used up to a concentration of 30% (v/v) with only a slight loss in activity. It is remarkable that of all acid phosphatases and phytases tested by the Delft group, only those enzymes belonging to the "Histidine Acid Phosphatase" class with the active site sequence RHGXRXP could function as a peroxidase when vanadate ion was incorporated into the active site.

The idea of transforming certain acid phosphatases into enzymes acting as vanadium chloroperoxidase stems from the observation that vanadium chloroperoxidase shares structural similarities with some membrane-bound acid phosphates. Furthermore, the apoenzyme of vanadium chloroperoxidase could exhibit phosphatase-like activity (Hemrika *et al.*, 1997; Neuwald, 1997).

VIII. Occupational Health Concerns

There have been several occasions when workers exposed to microbial enzymes have suffered allergic responses (Slavin and Lewis, 1971). Being aware of this, commercial producers of phytase now routinely

include a safety warning on their product about the need for workers to exercise caution when handling the enzyme. Extended exposure and breathing of dust are the main concerns. Bio-Feed® Phytase from Novo Nordisk is available in a coated granulated form that is advertised as a nondusty product that offers several advantages over the powdered enzyme. Doekes *et al.* (1999) present evidence that phytase is an occupational allergen that can cause specific IgE immune responses among exposed workers. Because the commercially available phytase in this study was not purified to homogeneity, this allergic response could not be positively linked to phytase. However, it was concluded that measures to prevent airborne occupational exposure should be implemented at sites where phytase is handled. Recombinant phytase expressed in *A. niger* still contains a glyco-conjugate with *N*-acteyl-glucosamine and a high mannose chain. Prior research has demonstrated that these oligosaccharides are immunodominant in *Aspergillus*, and antibodies to these epitopes are readily detected in the serum of individuals with aspergillosis (Hearn and Shimizu, 1996). *N*-glycosylation of asparagine residues may play a role in the antigenicity of phytase. However, this needs to be studied in detail before one can conclusively ascertain what role, if any, this glyco-conjugate is playing in stimulating the IgE immune response.

IX. Future Prospects

A. Phosphorus for Future Generations

Today, agricultural operations are supplying rock phosphate at minimal cost from mining operations throughout the world. The current major use of this phosphate is in fertilizers. Phosphorus, like nitrogen, is essential for plants, just as it is for all other forms of life (Abelson, 1999). It is a basic component in nucleic acids, ATP, and numerous other biological compounds. Nitrogen, however, has a cycle that constantly replenishes the earth's supply. To the contrary, phosphorus has no analogous cycle. The geologic phosphorus deposits that are being mined today are millions of years old. They contain the phosphorus that was removed from our biosphere when it precipitated from prehistoric oceans (Lanyon, 1999). This fact is beginning to generate concern that future generations may face a shortage as the demands on the world's phosphate reserves accelerates. Phytase is now being recognized for its beneficial environmental role in reducing the phosphorus levels in manure. But as the need to conserve the world's phosphate reserves increases in significance, the role of phytase may broaden.

B. Phytase's Role in Our Future

Some insight in gauging the future role of phytase may be obtained by examining the development of another group of industrial enzymes, subtilisin proteases. It is estimated that, of the total amount of enzymes produced in the world, 60% is destined for the laundry detergent market (Horikoshi, 1999). This huge market for subtilisin proteases and other enzymes developed in the last quarter of the twentieth century because of the phosphorus loading of the Great Lakes and other waterways (Alexander, 1977). Biotechnology responded to the need to replace detergent phosphate with alkaliphilic enzymes that would perform effectively as laundry proteases. Considerable research effort was channeled into the development of the superior laundry proteases that are available today (Wolff *et al.*, 1996). The parallel between phytase and subtilisins is that they both were developed in response to a need to reduce excess phosphate in our environment.

Phytase seems destined to become increasingly important, along with other innovations, as a measure to maximize efficient utilization of the earth's phosphorus supply. While it is not possible to predict how greatly the market for this enzyme will expand, trends in agriculture today suggest continued growth in demand. The development of a consensus phytase (Lehmann, 1998) points to still more research to develop a second-generation phytase with superior attributes as an animal feed additive. Continued research on lowering the production cost and expanding its utilization to other applications also suggests an increased importance of phytase in the immediate future.

X. Summary

Since its discovery in 1907, a complex of technological developments has created a potential $500 million market for phytase as an animal feed additive. During the last 30 years, research has led to increased use of soybean meal and other plant material as protein sources in animal feed. One problem that had to be overcome was the presence of antinutritional factors, including phytate, in plant meal. Phytate phosphorus is not digested by monogastric animals (e.g., hogs and poultry), and in order to supply enough of this nutrient, additional phosphate was required in the feed ration. Rock phosphate soon proved to be a cost-effective means of supplying this additional phosphorus, and the excess phytin phosphorus could be disposed of easily with the animals' manure. However, this additional phosphorus creates a massive environmental problem when the land's ability to bind it is exceeded. Over

the last decade, numerous feed studies have established the efficacy of a fungal phytase, *A. niger* NRRL 3135, to hydrolyze phytin phosphorus in an animal's digestive tract, which benefits the animal while reducing total phosphorus levels in manure. The gene for phytase has now been cloned and overexpressed to provide a commercial source of phytase. This monomeric enzyme, a type of histidine acid phophatase (HAP), has been characterized and extensively studied. HAPs are also found in other fungi, plants, and animals. Several microbial and plant HAPs are known to have significant phytase activity. A second *A. niger* phytase (phyB), a tetramer, is known and, like phyA, has had its X-ray crystal structure determined. The model provided by this crystal structure research has provided an enhanced understanding of how these molecules function. In addition to the HAP phytase, several other phytases that lack the unique HAP active site motif RHGXRXP have been studied. The best known group of the non-HAPs is phytase C (phyC) from the genus *Bacillus*. While a preliminary X-ray crystallographic analysis has been initiated, no enzymatic mechanism has been proposed.

Perhaps the pivotal event in the last century that created the need for phytase was the development of modern fertilizers after the Second World War. This fostered a transformation in agriculture and a tremendous increase in feed-grain production. These large quantities of cereals and meal in turn led to the transition of one segment of agriculture into "animal agriculture," with their its animal production capability. The huge volumes of manure spawned by these production units in time exceeded both the capacity of their crops and crop lands to utilize or bind the increased amount of phosphorus. Nutrient runoff from this land has now been linked to a number of blooms of toxin-producing microbes. Fish kills associated with these blooms have attracted public and governmental concern, as well as greater interest in phytase as a means to reduce this phosphorus pollution.

Phytase research efforts now are focused on the engineering of an improved enzyme. Improved heat tolerance to allow the enzyme to survive the brief period of elevated temperature during the pelletization process is seen as an essential step to lower its cost in animal feed. Information from the X-ray crystal structure of phytase is also relevant to improving the pH optimum, substrate specificity, and enzyme stability. Several studies on new strategies that involve synergistic interactions between phytase and other hydrolytic enzymes have shown positive results.

Further reduction in the production cost of phytase is also being pursued. Several studies have already investigated the use of various yeast expression systems as an alternative to the current production method for phytase using overexpression in filamentous fungi. Expression in plants is underway as a means to commercially produce phytase, as in biofarming in which plants such as alfalfa are used as "bioreactors," and also by developing plant cultivars that would produce enough transgenic phytase so that additional supplementation of their grain or meals is not necessary. Ultimately, transgenic poultry and hogs may produce their own digestive phytase.

Another active area of current phytase research is expanding its usage. One area that offers tremendous opportunity is increasing the use of phytase in aquaculture. Research is currently centered on utilizing phytase to allow producers in this industry to switch to lower-cost plant protein in their feed formulations. Development of a phytase for this application could significantly lower production costs. Other areas for expanded use range from the use of phytase as a soil amendment, to its use in a bioreactor to generate specific *myo*-inositol phosphate species. The transformation of phytase into a peroxidase may lead to another novel use for this enzyme. As attempts are made to widen the use of phytase, it is also important that extended exposure and breathing its dust be avoided as prudent safety measures to avoid possible allergic responses.

In expanding the use of phytase, another important consideration has been achieved. Conservation of the world's deposits of rock phosphate is recognized as important for future generations. Phosphorus is a basic component of life like nitrogen, but, unlike nitrogen, phosphorus does not have a cycle to constantly replenish its supply. It is very likely that the use of phytase will expand as the need to conserve the world's phosphate reserves increases.

References

Abelson, P. H. (1999). A potential phosphate crisis. *Science* **283**, 2015.

Alexander Jr., G. R. (1977). The rationale for a ban on detergent phosphate in the Great Lakes Basin. *Ciba Found. Symp.* **57**, 269–284.

Bazan, J. F., Fletterick, R. J., and Pilkis, S. J. (1989). Evolution of a bifunctional enzyme: 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *Proc. Natl. Acad. Sci. U.S.A.* **86**, 9642–9646.

Berka, R. M., Rey, M. W., Brown, K. M., Byun, T., and Klotz, A. V. (1998). Molecular characterization and expression of a phytase gene from the thermophilic fungus *Thermomyces lanuginosus*. *Appl. Environ. Microbiol.* **64**, 4423–4427.

Berlan, J.-P., Bertrand, J.-P., and Lebas, L. (1977). The growth of the American "soybean complex." *Eur. R. Agric. Eco.* **4**, 395–416.

Bransden, M. P., and Carter, C. G. (1999). Effect of processing soybean meal on the apparent digestibility of practical diets for the greenback flounder *Rhombosolea tapirina* (Gunther). *Aquacul. Res.* **30**, 719–723.

Burkholder, J. M., and Glasgow Jr., H. B. (1997). *Pfiesteria piscicida* and other *Pfiesteria*-like dinoflagellates: Behavior, impacts, and environmental controls. *Limnol. Oceanogr.* **42**, 1052–1075.

Chamberlain, G. W. (1993). Aquaculture trends and feed projections. *World Aquacul.* **24**, 19–29.

Chu, S. P. (1946). The utilization of organic phosphorus by phytoplankton. *J. Mar. Biol. Assoc. U.K.* **26**, 285–295.

Craxton, A., Caffrey, J. J., Burkhart, W., Safrany, S. T., and Shears, S. B. (1997). Molecular cloning and expression of a rat hepatic multiple inositol polyphosphate phosphatase. *Biochem. J.* **328**, 75–81.

Dalal, R. C. (1978). Soil organic phosphorus. *Adv. Agronom.* **29**, 83–117.

Dassa, E., and Boquet, P. L. (1985). Identification of the gene *app*A for the acid phosphatase (pH optimum 2.5) of *Escherichia coli*. *Mol. Gen. Genet.* **200**, 68–73.

Dassa, J., Marck, C., and Boquet, P. L. (1990). The complete nucleotide sequence of the *Escherichia coli* gene *app*A reveals significant homology between pH 2.5 acid phophatase and glucose-1-phosphatase. *J. Bacteriol.* **172**, 5497–5500.

Day, P. R. (1996). Genetic modification of plants: Significant issues and hurdles to success. *Am. J. Clin. Nutr.* **63**, 651S–656S.

Dischinger Jr., H. C., and Ullah, A. H. J. (1992). Immobilization of *Aspergillus ficuum* phytase by carbohydrate moieties onto crosslinked agarose. *Ann. N.Y. Acad. Sci.* **672**, 583–587.

Doekes, G., Kamminga, N., Helwegen, L., and Heederik, D. (1999). Occupational IgE sensitisation to phytase, a phosphatase derived from *Aspergillus niger*. *Occup. Environ. Med.* **56**, 454–459.

Dvorakova, J. (1998). Phytase: Sources, preparation and exploitation. *Folia Microbiol.* **43**, 323–338.

Ehrlich, K. C., Montalbano, B. G., Mullaney, E. J., Dischinger Jr., H. C., and Ullah, A. H. J. (1993). Identification and cloning of a second phytase gene (*phy*B) from *Aspergillus niger* (*ficuum*). *Biochem. Biophys. Res. Commun.* **195**, 53–57.

Ehrlich, K. C., Montalbano, B. G., Mullaney, E. J., Dischinger Jr., H. C., and Ullah, A. H. J. (1994). An acid phosphatase from *Aspergillus ficuum* has homology to *Penicillium chrysogenum pho*A. *Biochem. Biophys. Res. Commun.* **204**, 63–68.

Findenegg, G. R., and Nelemans, J. A. (1993). The effect of phytase on the availability of P from *myo*-inositol hexaphosphate (phytate) for maize roots. *Plant Soil* **154**, 189–196.

Goihl, J. (1998). Phytase, citric acid interaction in weaning pig diets investigated. *Feedstuffs* **70**, 10.

Graf, E., and Eaton, J. W. (1990). Antioxidant functions of phytic acid. *Free Radic. Biol. Med.* **8**, 61–69.

Graf, E., Empson, K. L., and Eaton, J. W. (1987). Phytic acid: A natural antioxidant. *J. Biol. Chem.* **1987**, 11647–11650.

Greiner, R., and Alminger, M. L. (1999). Purification and characterization of a phytate-degrading enzyme from germinated oat (*Avena sativa*). *J. Sci. Food Agric.* **79**, 1453–1460.

Greiner, R., and Konietzny, U. (1996). Construction of a bioreactor to produce special breakdown products of phytate. *J. Biotechnol.* **48**, 153–159.

Greiner, R., Konietzny, U., and Jany, K. D. (1993). Purification and Characterization of Two Phytases from *Escherichia coli*. *Arch. Biochem. Biophys.* **303**, 107–113.

Greiner, R., Haller, E., Konietzny, U., and Jany, K. D. (1997). Purification and characterization of a phytase from *Klebsiella terrigena*. *Arch. Biochem. Biophys.* **341**, 201–206.

Grueninger-Leitch, F., D'Arcy, A., D'Arcy, B., and Chene, C. (1996). Deglycosylation of proteins for crystallization using recombinant fusion protein glycosidases. *Protein Sci.* **5**, 2617–2622.

Gura, T. (1999). Biotechnology: New genes boost rice nutrients. *Science* **285**, 994–995.

Gutknecht, K. (1997). Green genes: Alfalfa biofarming is about to take root. *Wisconsin Agriculturist*, Mid-March, pp. 8–10.

Ha, N. C., Kim, Y. O., Oh, T. K., and Oh, B. H. (1999). Preliminary X-ray crystallographic analysis of a novel phytase from a *Bacillus amyloliquefaciens* strain. *Acta Crystallogr.* **D55**, 691–693.

Han, Y., and Lei, X. G. (1999). Role of glycosylation in the functional expression of an *Aspergillus niger* phytase (phyA) in *Pichia pastoris*. *Arch. Biochem. Biophys.* **364**, 83–90.

Han, Y., Wilson, D. B., and Lei, X. G. (1999). Expression of an *Aspergillus niger* phytase gene (*phyA*) in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **65**, 1915–1918.

Hayes, J. E., Richardson, A. E., and Simpson, R. J. (1999). Phytase and acid phosphatase activities in extracts from roots of temperate pasture grass and legume seedlings. *Aust. J. Plant Physiol.* **26**, 801–809.

Hearn, V. M., and Shimizu, M. (1996). Effects of tunicamycin on glycoprotein antigens of *Aspergillus fumigatus*. *Microbios* **85**, 239–250.

Hemrika, W., Renirie, R., Dekker, H. L., Barnett, P., and Weaver, R. (1997). From phosphatases to vanadium peroxidases: A similar architecture of the active site. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 2145–2149.

Horikoshi, K. (1999). Alkaliphiles: Some applications of their products for biotechnology. *Microbiol. Mol. Biol. Rev.* **63**, 735–750.

Irving, G. C. J., and Cosgrove, D. J. (1972). Inositol phosphate phosphatases of microbiological origin: The inositol pentaphosphate products of *Aspergillus ficuum* phytases. *J. Bacteriol.* **112**, 434–438.

Jia, Z., Golovan, S., Ye, Q., and Forsberg, C. W. (1998). Purification, crystallization and preliminary X-ray analysis of the *Escherichia coli* phytase. *Acta Crystallogr.* **D54**, 647–649.

Kerovuo, J., Lauraeus, M., Nurminen, P., Kalkkinen, N., and Apajalahti, J. (1998). Isolation, characterization, molecular gene cloning, and sequencing of a novel phytase from *Bacillus subtilis*. *Appl. Environ. Microbiol.* **64**, 2079–2085.

Kim, Y. O., Kim, H. K., Bae, K. S., Yu, J. H., and Oh, T. K. (1998a). Purification and properties of a thermostable phytase from *Bacillus* sp. DS11. *Enzyme Microb. Technol.* **22**, 2–7.

Kim, Y. O., Lee, J. K., Kim, H. K., Yu, J. H., and Oh, T. K. (1998b). Cloning of the thermostable phytase gene (*phy*) from *Bacillus* sp. DS11 and its overexpression in *Escherichia coli*. *FEMS Microbiol. Lett.* **162**, 185–191.

Klabunde, T., Strater, N., Frohlich, R., Witzel, H., and Krebs, B. (1996). Mechanism of Fe(III)–Zn(II) purple acid phosphatase based on crystal structures. *J. Mol. Biol.* **259**, 737–748.

Kostrewa, D., Grueninger-Leitch, F., D'Arcy, A., Broger, C., Mitchell, D., and van Loon, A. P. G. M. (1997). Crystal structure of phytase from *Aspergillus ficuum* at 2.5 Å resolution. *Natl. Struct. Biol.* **4**, 185–190.

Kostrewa, D., Wyss, M., D'Arcy, A., and van Loon, A. P. G. M. (1999). Crystal structure of *Aspergillus niger* pH 2.5 acid phosphatase at 2.4 Å resolution. *J. Mol. Biol.* **288**, 965–974.

Lambrechts, C., Boze, H., Segueilha, L., Moulin, G., and Galzy, P. (1993). Influence of culture conditions on the biosynthesis of *Schwanniomyces castellii* phytase. *Biotechnol. Lett.* **15**, 399–404.

Lanyon, L. E. (1999). Nutrient management: Regional issues affecting the bay. *In* "Agriculture and Phosphorus Management" (A. N. Sharpley, ed.), pp. 145–158. Lewis Publishers, Boca Raton, FL.

Lassen, S. F., Bech, L., Fuglsang, C. C., Breinholt, J., Ohmann, A., and Ostergaard, P. R. (1997). "Phytase Polypeptides." International Patent Application, No. WO98/28408.

Lehmann, M. (1998). "Consensus Phytase." International Patent Application, No. EP0897985A2.

Li, J., Hegeman, C. E., Hanlon, R. W., Lacy, G. H., Denbow, D. M., and Grabau, E. A. (1997). Secretion of active recombinant phytase from soybean cell-suspension cultures. *Plant Physiol.* **114**, 1–9.

MacRae, W. D., Buxton, F. P., Sibley, S., Garven, S., Gwynne, D. I., Davies, R. W., and Arst Jr., H. N. (1988). A phosphate-repressible acid phosphatase gene from *Aspergillus niger*: Its cloning, sequencing and transcriptional analysis. *Gene* **71**, 339–348.

Mallin, M. A. (2000). Impacts of industrial animal production on rivers and estuaries *Am. Sci.* **88**, 26–37.

Maugenest, S., Martinez, I., and Lescure. A. M. (1997). Cloning and characterization of a cDNA encoding a maize seedling phytase. *Biochem. J.* **322**, 511–517.

Maugenest, S., Martinez, I., Godin, B., Perez, P., and Lescure, A.-M. (1999). Structure of two maize phytase genes and their spatio-temporal expression during seedling development. *Plant Mol. Biol.* **39**, 503–514

Mayer, A. F., Hellmuth, K., Schlieker, H., Lopez-Ulibarri, R., Oertel, S., Dahlems, U., Strasser, A. W. M., and van Loon, A. P. G. M. (1999). An expression system matures: A highly efficient and cost-effective process for phytase production by recombinant strains of *Hansenula polymorpha*. *Biotechnol. Bioeng.* **63**, 373–381.

McElhinney, C., and Mitchell, D. T. (1993). Phosphatase activity of four ectomycorrhizal fungi found in a Sitka spruce–Japanese larch plantation in Ireland. *Mycol. Res.* **97**, 725–732.

Meittinen-Oinonen, A., Torkkeli, T., Paloheimo, M., and Nevalainen, H. (1997). Overexpression of the *Aspergillus niger* pH 2.5 acid phosphatase gene in a heterologous host, *Trichoderma reesei*. *J. Biotechnol.* **58**, 13–20.

Mitchell, D. B., Vogel, K., Weimann, B., Pasamontes, L., and van Loon, A. P. G. M. (1997). The phytase subfamily of histidine acid phosphatases: Isolation of genes for two novel phytases from the fungi *Aspergillus terreus* and *Myceliophthora thermophila*. *Microbiology* **143**, 245–252.

Mlot, C. (1998). *Pfiesteria piscicida* puts focus on harmful aquatic microbes. *ASM News* **63**, 590–592.

Moore, E., Helly, V. R., Conneely, O. M., Ward, P. P., Power, R. F., and Headon, D. R. (1995). Molecular cloning, expression and evaluation of phosphohydrolases for phytate-degrading activity. *J. Ind. Microbiol.* **14**, 396–402.

Morgan, A. J., Hessing, M., and Sleijster-Selis, H. E. (1998). "Phytase from Germinated Soybeans." International Patent Application, No. WO98/20139.

Morris, E. R., and Ellis, R. (1976). Isolation of monoferric phytate from wheat bran and its biological value as an iron source to the rat. *J. Nutr.* **106**, 753–760.

Mullaney, E. J., and Ullah, A. H. J. (1998a). Conservation of the active site motif in *Aspergillus niger* (*ficuum*) pH 6.0 optimum acid phosphatase and kidney bean purple acid phosphatase. *Biochem. Biophys. Res. Commun.* **243**. 471–473.

Mullaney, E. J., and Ullah, A. H. J. (1998b). Identification of a histidine acid phosphatase (*phy*A)-like gene in *Arabidopsis thaliana*. *Biochem. Biophys. Res. Commun.* **251**. 252–255.

Mullaney, E. J., Gibson, D. M., and Ullah, A. H. J. (1991). Positive identification of a lambda gt11 clone containing a region of fungal phytase gene by immunoprobe and sequence verification. *Appl. Microbiol. Biotechnol.* **35**, 611–614.

Mullaney, E. J., Daly, C. B., Ehrlich, K. C., and Ullah, A. H. J. (1995). The *Aspergillus niger* (*ficuum*) *aph*A gene encodes a pH 6.0-optimum acid phosphatase. *Gene* **162**, 117–121.

Mwachireya, S. A., Beames, R. M., Higgs, D. A., and Dosanjh, B. S. (1999). Digestibility of canola protein products derived from the physical, enzymatic and chemical processing of commercial canola meal in rainbow trout *Oncorhynchus mykiss* (Walbaum) held in fresh water. *Aquacul. Nutr.* **5**, 73–82.

Nasi, M., Piironen, J., and Partanen, K. (1999). Efficacy of *Trichoderma reesei* phytase and acid phosphatase activity ratios in phytate phosphorus degradation in vitro and in pigs fed maize-soybean meal or barley-soybean meal diets. *Anim. Feed Sci. Technol.* **77**. 125–137.

Nayini, N. R., and Markakis, P. (1984). The phytase of yeast. *Lebensm. u. Technol.* **17**, 24–26.

Nelson, T. S., Ferrara, L. W., and Storer, N. L. (1968a). Phytate phosphorus content of feed ingredients derived from plants. *Poult. Sci.* **47**, 1372–1374.

Nelson, T. S., Shieh, T. R., Wodzinski, R. J., and Ware, J. H. (1968b). The availability of phytate phosphorus in soybean meal before and after treatment with a mold phytase. *Poult. Sci.* **47**, 1842–1848.

Neuwald, A. F. (1997). An unexpected structural relationship between integral membrane phosphatases and soluble haloperoxidases. *Protein Sci.* **6**, 1764–1767.

Novo Nordisk A/S (1999). New phytase could make a world of difference. *BioTimes* **14**(3), 6–7.

Nunes, C. S., and Guggenbuhl, P. (1998). Effects of *Aspergillus fumigatus* phytase on phosphorus digestibility, phosphorus excretion, bone strength and performance in pigs. *Reprod. Nutr. Dev.* **38**, 429–440.

Oliva-Teles, A., Pereira, J. P., Gouveia, A., and Gomes, E. (1998). Utilisation of diets supplemented with microbial phytase by seabass (*Dicentrarchus labrax*) juveniles. *Aquat. Living Resour.* **11**, 255–259.

Ostanin, K., and Van Etten, R. L. (1993). Asp^{304} of *Escherichia coli* acid phosphatase is involved in leaving group protonation. *J. Biol. Chem.* **268**, 20778–20784.

Ostanin, K., Harms, E. H., Stevis, P. E., Kuciel, R., Zhou, M.-M., and Van Etten, R. L. (1992). Overexpression, site-directed mutagenesis, and mechanism of *Escherichia coli* acid phosphatase. *J. Biol. Chem.* **267**, 22830–22836.

Park, S. C., Choi, Y. W., and Oh, T. K. (1999). Comparative enzymatic hydrolysis of phytate in various animal feedstuff with two different phytases. *J. Vet. Med. Sci.* **61**, 1257–1259.

Pasamontes, L., Haiker, M., Wyss, M., Tessier, M., and van Loon, A. P. G. M. (1997a). Gene cloning, purification, and characterization of a heat-stable phytase from the fungus *Aspergillus fumigatus*. *Appl. Environ. Microbiol.* **63**, 1696–1700.

Pasamontes, L., Haiker, M., Henriquez-Huecas, M., Mitchell, D. B., and van Loon A. P. G. M. (1997b). Cloning of the phytases from *Emericella nidulans* and the thermophilic fungus *Talaromyces thermophilus*. *Biochim. Biophys. Acta* **1353**, 217–223.

Phillippy, B. Q. (1998). Purification and catalytic properties of a phytase from scallion (*Allium fistulosum* L.) leaves. *J. Agric. Food Chem.* **46**, 3491–3496.

Phillippy, B. Q. (1999). Susceptibility of wheat and *Aspergillus niger* phytases to inactivation by gastrointestinal enzymes. *J. Agric. Food Chem.* **47**, 1385–1388.

Phillippy, B. Q. (2000). Stability of plant and microbial phytases. *In* "Food Phytates" (N. R. Reddy and S. K. Sathe, eds.). Technomic, Lancaster, PA. In press.

Phillippy, B. Q., and Mullaney, E. J. (1997). Expression of an *Aspergillus niger* phytase (*phy*A) in *Escherichia coli*. *J. Agric. Food Chem.* **45**, 3337–3342.

Piddington, C. S., Houston, C. S., Paloheimo, M., Cantrell, M., Meittinen-Oinonen, A., Nevalainen, H., and Rambosek, J. (1993). The cloning and sequencing of the genes encoding phytase (*phy*) and pH 2.5-optimum acid phosphatase (*aph*) from *Aspergillus niger* var. *awamori*. *Gene* **133**, 55–62.

Powar, V. K., and Jagannathan, V. (1982). Purification and properties of phytate-specific phosphatase from *Bacillus subtilis*. *J. Bacteriol* **151**, 1102–1108.

Raboy, V., and Gerbasi, P. (1996). Genetics of *myo*-inositol phosphate synthesis and accumulation. *In* "Subcellular Biochemistry," Vol. 26: "*myo*-Inositol Phosphates, Phosphoinositides, and Signal Transduction" (B. B. Biswas and S. Biswas, eds.), pp. 257–285. Plenum, New York.

Reddy, N. R., Sathe, S. K., and Salunkhe, D. K. (1982). Phytates in legumes and cereals. *Adv. Food Res.* **28**, 1–92.

Rivkin, R. B., and Swift, E. (1980). Characterization of alkaline phosphatase and organic phosphorus utilization in the oceanic dinoflagellate *Pyrocystis noctiluca*. *Mar. Biol.* **61**, 1–8.

Robinson, E. H., Jackson, S., and Li, M. H. (1996). Supplemental phytase in catfish diets. *Aquacul. Mag.* **22**, 80–82.

Rodriguez, E., Han, Y., and Lei, X. G. (1999). Cloning, sequencing, and expression of an *Escherichia coli* acid phosphatase/phytase gene (*app*A2) isolated from pig colon. *Biochem. Biophys. Res. Commun.* **257**, 117–123.

Rodriguez, E., Mullaney, E., and Lei, X. G. (2000). Expression of *Aspergillus fumigatus* phytase gene in *Pichia pastoris* and characterization of the recombinant enzyme. *Biochem. Biophys. Res. Commun.* **268**, 373–378.

Rumsey, G. L. (1993). Fish meal and alternate sources of protein in fish feeds: Update 1993. *Fisheries* **18**, 14–19.

Sano, K., Fukuhara, H., and Nakamura, Y. (1999). Phytase of the yeast *Arxula adeninivorans*. *Biotechnol. Lett.* **21**, 33–38.

Schneider, G., Lindqvist, Y., and Vihko, P. (1993). Three-dimensional structure of rat acid phosphatase. *EMBO J.* **12**, 2609–2615.

Segueilha, L., Lambrechts, C., Boze, H., Moulin, G., and Galzy, P. (1992). Purification and properties of the phytase from *Schwanniomyces castellii*. *J. Ferment. Bioeng.* **74**, 7–11.

Slavin, R. G., and Lewis, C. R. (1971). Sensitivity to enzyme additives in laundry detergent workers. *J. Allergy Clin. Immunol.* **48**, 262–266.

Suzuki, U., Yoshimura, K., and Takaishi, M. (1907). Ueber ein Enzym "Phytase" das "Anhydro-oxy-methylen diphosphorsaure" Spaltet. *Tokyo Imper. Univ. Coll. Agric. Bull.* **7**, 503–512.

Tambe, S. M., Kaklij, G. S., Kelkar, S. M., and Parekh, L. J. (1994). Two distinct molecular forms of phytase from *Klebsiella aerogenes*: Evidence for unusually small active enzyme peptide. *J. Ferment. Bioeng.* **77**, 23–27.

Tsim, S.-T., Wong, J. T., and Wong, Y. H. (1998). Regulation of calcium influx and phospholipase C activity by indoleamines in dinoflagellate *Crypthecodinium cohnii*. *J. Pineal Res.* **24**, 152–161.

Ullah, A. H. J. (1988). Production, rapid purification and catalytic characterization of extracellular phytase from *Aspergillus ficuum*. *Prep. Biochem.* **18**, 443–458.

Ullah, A. H. J., and Cummins, B. J. (1987). Purification, N-terminal amino-acid sequence and characterization of the pH 2.5 optimum acid phosphatase (E.C. 3.1.3.2) from *Aspergillus ficuum*. *Prep. Biochem.* **17**, 397–422.

Ullah, A. H. J., and Cummins, B. J. (1988). *Aspergillus ficuum* extracellular pH 6.0 optimum acid phosphatase: Purification, N-terminal amino-acid sequence, and biochemical characterization. *Prep. Biochem.* **18**, 37–65.

Ullah, A. H. J., and Dischinger Jr., H. C. (1993). *Aspergillus ficuum* phytase: Complete primary structure elucidation by chemical sequencing. *Biochem. Biophys. Res. Commun.* **192**, 747–753.

Ullah, A. H. J., and Gibson, D. M. (1987). Extracellular phytase (E.C. 3.1.3.8) from *Aspergillus ficuum* NRRL 3135: Purification and characterization. *Prep. Biochem.* **17**, 63–91.

Ullah, A. H. J., and Mullaney, E. J. (1996). Disulfide bonds are necessary for structure and activity in *Aspergillus ficuum* phytase. *Biochem. Biophys. Res. Commun.* **227**, 311–317.

Ullah, A. H. J., and Phillippy, B. Q. (1994). Substrate selectivity in *Aspergillus ficuum* phytase and acid phosphatases using *myo*-inositol phosphates. *J. Agric. Food Chem.* **42**, 423–425.

Ullah, A. H. J., Cummins, B. J., and Dischinger Jr., H. C. (1991). Cyclohexanedione modification of arginine at the active site of *Aspergillus ficuum* phytase. *Biochem. Biophys. Res. Commun.* **178**, 45–53.

Ullah, A. H. J., Sethumadhavan, K., Mullaney, E. J., Ziegelhoffer, T., and Austin-Phillips, S. (1999). Characterization of recombinant fungal phytase (phyA) expressed in tobacco leaves. *Biochem. Biophys. Res. Commun.* **264**, 201–206.

Vanderbeke, E. E. M., De Schrijver, M., and Vermeire, A. M. M. (1994). "Phytate Hydrolysis and Enzyme Composition for Hydrolyzing Phytate." European Patent Application 0619369A1.

Van Der Kaay, J., and Van Haastert, P. J. M. (1995). Stereospecificity of inositol hexakisphosphate dephosphorylation by *Paramecium* phytase. *Biochem. J.* **312**, 907–910.

van de Velde, F., Konemann, L., van Rantwijk, F., and Sheldon, R. A. (2000). The rational design of semisynthetic peroxidases. *Biotechnol. Bioeng.* **67**, 87–96.

van Dijck, P. W. M. (1999). Chymosin and phytase, made by genetic engineering. *J. Biotechnol.* **67**, 77–80.

Van Etten, R. L., Davidson, R., Stevis, P. E., MacArthur, H., and Moore, D. L. (1991). Covalent structure, disulfide bonding, and identification of reactive surface and active site residues of human prostatic acid phosphatase. *J. Biol. Chem.* **266**, 2313–2319.

Van Hartingsveldt, W., van Zeijl, C. M., Harteveld, G. M., Gouka, R. J., Suykerbuyk, M. E., Luiten, R. G., van Paridon, P. A., Selten, G. C., Veenstra, A. E., van Gorcom R. F. M., and van den Hondel, C. A. M. J. J. (1993). Cloning, characterization and overexpression of the phytase-encoding gene (*phyA*) of *Aspergillus niger*. *Gene* **127**, 87–94.

van Weerd, J. H., Khalaf, K. H. A., Aartsen, F. J., and Tijssen, P. A. T. (1999). Balance trials with African catfish *Clarias gariepinus* fed phytase-treated soybean meal-based diets. *Aquacul. Nutr.* **5**, 135–142.

Verwoerd, T. C., van Paridon, P. A., van Ooyen, A. J. J., van Lent, J. W. M., Hoekema, A., and Pen J. (1995). Stable accumulation of *Aspergillus niger* phytase in transgenic tobacco leaves. *Plant Physiol.* **109**, 1199–1205.

Vincent, J. B., Crowder, M. W., and Averill, B. A. (1992). Hydrolysis of phosphate monoesters: A biological problem with multiple chemical solutions. *TIBS* **17**, 105–110.

Ward, N. E. (1993). Phytase in nutrition and waste management. *Poult. Dig.* **52**, 10–15

Watanabe, T., and Pongmaneerat, J. (1993). Potential of soybean meal as a protein source in extruded pellets for rainbow trout. *Nippon Suisan Gakkaishi* **59**, 1415–1423.

Wodzinski, R. J., and Ullah, A. H. J. (1996). Phytase. *Adv. Appl. Microbiol.* **42**, 263–302.

Wolff, A. M., Showell, M. S., Venegas, M. G., Barnett, B. L., and Wertz, W. C. (1996). Laundry performance of subtilisin proteases. *Adv. Exp. Med. Biol.* **379**, 113–120,

Wyss, M., Pasamontes, L., Remy, R., Kohler, J., Kusznir, E., Gadient, M., Muller, F., and van Loon, A. P. G. M. (1998). Comparison of the thermostability properties of three acid phosphatases from molds: *Aspergillus fumigatus* phytase, *A. niger* phytase, and *A. niger* pH 2.5 acid phosphatase. *Appl. Environ. Microbiol.* **64**, 4446–4451.

Wyss, M. R., Brugger, R., Kronenberger, A., Remy, R., Fimbel, R., Oesterhelt, G., Lehmann, M., and van Loon, A. P. G. M. (1999a). Biochemical characterization of fungal phytases (*myo*-inositol hexakisphosphate phosphohydrolases): Catalytic properties. *Appl. Environ. Microbiol.* **65**, 367–373.

Wyss, M., Pasamontes, L., Friedlein, A., Remy, R., Tessier., M., Kronenberger, A., Middendorf, A., Lehmann, M., Schnoebelen, L., Rothlisberger, U., Kusznir, E., Wahl, G., Muller, F., Lahm, H.-W., Vogel, K., and van Loon, A. P. G. M. (1999b). Biophysical characterization of fungal phytases (*myo*-inositol hexakisphosphate phosphohydrolases): Molecular size, glycosylation pattern, and engineering of proteolytic resistance. *Appl. Environ. Microbiol.* **65**, 359–366.

Yamada, K., Minoda, Y., and Yamamoto, S. (1968). Phytase from *Aspergillus terreus*, Part I: Production, purification and some general properties of the enzyme. *Agric. Biol. Chem.* **32**, 1275–1282.

Yano, F., Nakajima, T., and Matsuda, M. (1999). Reduction of nitrogen and phosphorus from livestock waste: A major priority for intensive animal production. *Asian-Aust. J. Anim. Sci.* **12**, 651–656.

Zyla, K., Ledoux, D. R., and Veum, T. L. (1995). Complete enzymic dephosphorylation of corn-soybean meal feed under simulated intestinal conditions of the turkey. *J. Agric. Food Chem.* **43**, 288–294.