J. Microbiol. Biotechnol. (2001), **11**(6), 1066–1070

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Expression of the *Aspergillus niger* var. *awamori* Phytase Gene in *Pichia pastoris*, and Comparison of Biological Properties

CHOI, JAE-MUN[1], DOO-SANG KIM[3], MOON-SICK YANG[2], HYUNG-RAK KIM[3], AND JAE-HO KIM[1]*

[1]*Graduate School of Biotechnology, Korea University, Anam-dong, Sungbuk-ku, Seoul 136-701, Korea*
[2]*Institute for Molecular Biology and Genetics, Chonbuk National University, Chonju 561-756, Korea*
[3]*Department of Food Science & Biotechnology, Pukyung National University, Pusan 608-737, Korea*

Received: September 19, 2001
Accepted: November 12, 2001

Abstract The *PhyA* gene, encoding *myo*-inositol hexakisphosphate phosphohydrolase in *Aspergillus niger* var. *awamori* (wild-type), was cloned and sequenced. The cDNA was overexpressed by a multicopy gene expression system in *Pichia pastoris* KM71. Recombinant, wild-type, and commercial phytase from *Aspergillus ficuum* NRRL 3135 (Natuphos) were purified. The *PhyA* gene of *Aspergillus niger* var *awamori* showed perfect homology to the phytase of *Aspergillus ficcum* and 97% homology to *A. niger* var *awamori* (L02421). Wild-type phytase was highly glycosylated and more thermostable than the other two, while deglycosylated forms of three phytases showed identical molecular weight, 50 kDa. After heating at 80°C, wild-type, commercial, and recombinant phytases retained 57%, 32%, and 8% of their original activities, respectively. In conclusion, glycosylation plays a key role in the thermostability of phytase and its enzymatic characterization.

Key words: Phytase, *Aspergillus niger* var. *awamori*, *Pichia pastoris*, multicopy gene expression system

Phytic acid (*myo*-inositol hexakis dihydrogen phosphate) is the major storage form of phosphate in food and feed of plant origin. Phytic acid is considered to be an antinutritional factor since it chelates minerals such as magnesium, zinc, and calcium. It may also react with proteins, thereby decreasing the bioavailability of proteins and nutritionally important minerals. When phytic acid-rich meals are fed to monogastric animals, they poorly digest phytic acid due to a lack of active phytases in their gastrointestinal tract. Thus, nearly all of the dietary phytate phosphorus ingested by these species is excreted into the environment, resulting in phosphorus pollution in the areas of intensive animal production [8]. Usage of phytase as a feed additive has been examined several times over the past two decades, resulting in improved phosphorus availability from poultry and swine feeds. However, the high cost of the enzyme production compared to the cheap cost of inorganic phosphate has prevented its universal usages. Recently, there has been a renewed interest in phytase due to the low-cost production of this enzyme by recombinant DNA technology and an increased concern for the environment. The phytase produced by *Aspergillus ficuum* NRRL 3135 has been isolated and well characterized by Ullah and Gibson [20]. A bacterial strain producing high level of an extracellular phytase was isolated and defined for culture condition [2]. In addition, the cloning and expression of the *PhyA* gene have been reported for *A. ficuum*, *A. awamori*, *A. terreus*, and *A. fumigatus*. In particula, *A. fumigatus* phytase expressed in *A. niger* showed higher thermostability required for these enzyme to withstand inactivation during the feed-pelleting process [14]. However, while it displayed a higher level of heat tolerance than *A. niger* phytase, its level of specific phytase activity was significantly lower than that of *A. niger* phytase [21, 22]. *Pichia pastoris* was proven to be an economical host for the production of functional recombinant enzyme, which was cheaply inducible with methanol [16, 9].

The purpose of this experiment is to determine the sequence of the *PhyA* of *Aspergillus* sp. 5990 previously isolated [6], and to produce recombinant phytase in *Pichia pastoris* by a multicopy gene expression system applicable for industrialized mass production for characterization of its enzymatic properties.

Materials and Methods

cDNA Amplification and Sequencing, and Cloning of Phytase Gene

Aspergillus niger var. *awamori* [6] was cultured in a phosphate limiting condition, as previously described [18],

*Corresponding author
Phone: 82-2-3290-3443; Fax: 82-2-924-3094;
E-mail: jhkim@tiger.korea.ac.kr

and its genomic DNA was isolated according to the method described [14] with a minor modification. The phytase-encoding gene was amplified with PCR using *PhyA* gene specific primer pairs that were derived from regions that are conserved between the *A. niger* phytase and other histidine acid phosphatases. The following primers were used: forward AU (5'-TCA TAG GCA TCA TGG GCG TCT CTG CTG-3') and reverse AD2 (5'-TCA GCT AAG CAG AAC ACT CCG CCC AAT C-3'). The amplicon was inserted into the *Sma*I site of pBluescript II KS (Stratagene, CA, U.S.A.) vector. mRNA was isolated by hybridization with a magnetic-labeled oligo-dT probe, using an mRNA DIRECT kit (Dynabeads, Oslo, Norway), following the manufacturer's instruction. A cDNA fragment of the coding region of the *Aspergillus niger* var. *awamori* phytase gene was obtained by RT-PCR. The second round of PCR amplification was performed with a specific primer set which was confirmed by the earlier sequence analysis: primer A_UM*Eco*RI (5'-CGG AAT TCC TGG CAG TCC CCG CCT-3') and primer A_DM*Eco*RI (5'-CGG AAT TCA GGT AAT TCA GCT AAG CAA AAC ACTC-3'). Amplified RT-PCR product (1.4 kb) was inserted into the *Eco*RI site of pPIC9K (Invitrogen, CA, U.S.A.) expression vector. The resulting plasmid was designated as PKAMC (10.7 kb). The DNA sequence was determined by the dideoxy chain termination technique with an ALFexpress AutoRead sequencing kit (Pharmacia, Uppsala, Sweden).

Yeast Transformation, Expression, and Purification

PKAMC was linearized by restriction enzyme *Pme*I and transformed into *P. pastoris* KM71 by electroporation using Gene Pulser II (Bio-Rad, CA, U.S.A.). After the transformed cells were plated onto RDB (1 M sorbitol, 2% dextrose, 1.34% YNB, 4×10^{-5} % biotin, 0.005% amino acid), all His^+ transformants were transferred on YPD plates (1% yeast extract, 2% peptone, and 2% dextrose) containing G418 sulfate (0.25 mg/ml–2 mg/ml) to screen a multicopy gene insertion event [19]. A single colony, PKAMC/KM71, was inoculated to BMGY (1% yeast extract, 2% peptone, 100 mM of 1M potassium phosphate, 1.34% YNB, 4×10^{-5} % amino acid, 1% glycerol) medium and incubated at 30°C for 15 h until the culture reached to OD_{600}=5.0. The cells were harvested by centrifugation, resuspended in 100 ml of BMMY (1% yeast extract, 2% peptone, 100 mM of 1 M potassium phosphate, 1.34% YNB, 4×10^{-5}% amino acid, 0.5% methanol) medium, and incubated at 30°C for 5 days. For the induction, 0.5 ml of methanol was added to the medium every 24 h. For the purification, the supernatants were loaded onto a Q-Sepharose column (Pharmacia, Uppsala, Sweden) which was equilibrated with 20 mM Tris-HCl, pH 8.0, containing 1 mM $CaCl_2$. The phytase fractions were eluted with a 0.5 M sodium chloride gradient and subjected to a SP-Sepharose column (Pharmacia, Uppsala, Sweden). Then, the fractions were pooled and applied to a Mono-S HR 5/5 FPLC column (Pharmacia, Uppsala, Sweden).

Enzyme Activity and Properties

A phytase activity in the culture supernatant and total soluble fractions of the transformed cells were determined as previously described [11]. A sample was diluted with 0.2 M sodium citrate, pH 5.5, pre-warmed in a water bath (37°C) for 5 min. The sample was mixed with an equal volume of 1% (v/v) sodium phytate in the same buffer and incubated at 37°C for 15 min. The reaction was stopped by adding 1 ml of 15% (w/v) trichloroacetic acid, and the released phosphorus was measured. The optimal pH of phytases was determined (37°C) using 0.2 M glycine-HCl, 0.2 M sodium, or 0.2 M Tris-HCl. The thermostability of the expressed phytase and the commercial phytase were compared by measuring the remaining activity at 37°C after 0.2 U of phytase had been heated for 20 min at each temperature.

Deglycosylation of Phytases

Samples of purified proteins were subjected to 8% SDS-PAGE using a Mini-Protein II Cell (Bio-Rad, CA, U.S.A.). Protein was stained with Coomassie Brilliant Blue R-250 using a BenchMark protein ladder as the standard (BRL, MD, U.S.A.). Endo H_f (Boehringer Mannheim, Mannheim, Germany) was used to deglycosylate three phytases by a previously described method [7]. The reaction was carried out by incubating phytase samples with 3 units of Endo H_f in 20 mM Tris-HCl, pH 7.0, 10 mM EDTA, and 1 mM PMSF for 6 h at 37°C. After the reaction, the mixture was subjected to SDS-PAGE.

RESULTS AND DISCUSSION

Expression and Purification of Phytase

In the previous work, *Aspergillus* sp. 5990 was isolated from the soil near a root of a leguminous plant which was shown to have phytase activity [6]. Extracellular phytase secreted in a liquid culture by *Aspergillus* sp. 5990 showed a 5-fold higher activity when compared with that of *Aspergillus ficuum* NRRL 3135. The phytase from *Aspergillus* sp. 5990 showed the higher optimum temperature for its activity than the commercial enzyme from *Aspergillus ficuum* NRRL 3135. *PhyA* genes from genomic DNA and cDNA of *Aspergillus* sp. 5990 were cloned and sequenced for further study. The *PhyA* gene of *Aspergillus* sp. 5990 encodes an enzyme of 452 amino acids with identical homology to the phytase of *Aspergillus ficcum*, and 97%, 66%, and 62% homology to the phytases of *Aspergillus niger* var *awamori* (Entrez Accession Number; L02421), *Aspergillus fumigatus*, and *Aspergillus terrus*, respectively. The analysis of the protein sequence revealed a theoretical

pI value of 4.84. However, *Aspergillus* sp. 5990 was proven to be a *Aspergillus niger* var *awamori* through morphological and ribosomal RNA analyses. Therefore, this result suggests that a host-dependent post-translational modification plays a critical role in the naturally occurring diverse enzyme characteristics.

The RT-PCR product of the coding region of the *phyA* gene was inserted into the *Eco*RI site of the pPIC9K expression vector in a frame with the initiation codon of the a-factor signal sequence. The resulting clone, PKAMC, was expressed under the control of an AOX1 (alcohol oxidase1) promoter [3] and induced by methanol. The recombinant enzyme was purified from the culture of multicopy transformed *P. pastoris*. Phytases from the wild-type and the crude commercial one from Natuphos (BASF, Ulsan, Korea) were purified together for the comparison analysis.

Purified phytases from wild-type, Natuphos (BASF, Ulsan, Korea), and recombinants were deglycosylated by Endo H_f and subjected to SDS-PAGE. The molecular weight of recombinant, wild-type, and Natuphos (BASF, Ulsan, Korea) phytases A were 80, 85, and 75 kDa respectively, but all of them showed the same molecular weight, 50 kDa, after deglycosylation (Fig. 1). It was consistent with the theoretical M_r of 49,616, as was reported previously by another laboratory [4]. These results indicated that the recombinant was less glycosylated compared to the wild-types, while all of three phytases A have ten potential N-glycosylation sites. It suggests that glycosylation of phytases A is highly variable depending on the host, which is consistant with the findings of Wyss *et al.* [22].

Glycosylation has several biological significances and serves a vital role in the foldings and assemblies of viable proteins [1]. Some proteins required N-linked oligosaccharides

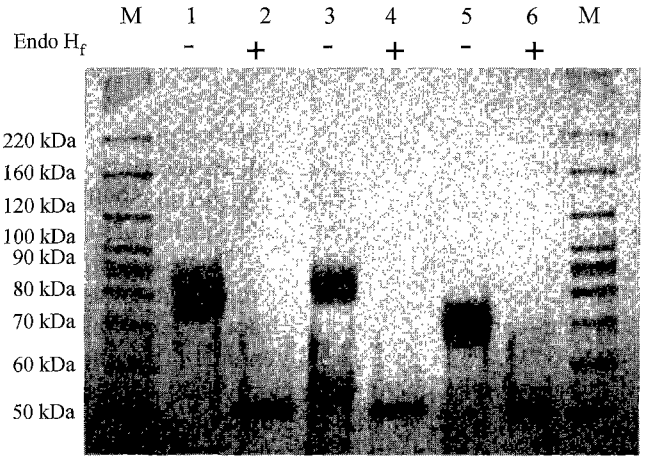

Fig. 1. SDS-polyacrylamide gel electrophoresis of the purified three phytases before and after deglycosylation by Endo H_f.
Lane M, protein standard marker from BRL. Lanes 1 and 2, recombinant PhyA expressed in *P. pastoris* with and without Endo H_f. Lanes 3 and 4, *Aspergillus niger* var. *awamori* PhyA with and without Endo H_f. Lanes 5 and 6, commercial PhyA; Natuphos from BASF with and without Endo H_f.

Table 1. Specific activities and kinetic constants for phytic acid.

Phytases	Specific activity (U/mg)	K_m (μM)	K_{cat} (S^{-1})
Wild-type	986	370	2,200
Recombinant	1,371	640	3,500

to maintain proper function (Rudd *et al.* [17]) or viral attachment [13] or to be correctly targeted [15]. It was reported that the different pattern of glycosylation could shift the pI of protein [21] and, thereby, it could change the behavior of protein purification. Therefore, the different behavior of wild-type phytase during the purification procedures is most likely due to the different patterns of glycosylation.

Determination of Kinetic Parameters

The kinetic properties of three purified phytases were compared. The K_m values and the substrate turnover number of phytases from wild-type were estimated to be 0.37 mM and 2,200 sec^{-1} at pH 5.5, while that of recombinant phytase from *P. pastoris* KM71 were 0.64 mM and 3,500 sec^{-1} at pH 5.5 (Table 1). It was reported that the apparent K_m values of the phytase A from *Aspergillus ficcum* were determined to be 0.25 mM [12], which was slightly lower than the presently isolated or recombinant PhyA. However, the substrate affinity of the presently purified recombinant PhyA was much higher than that of the enzyme from *A. fumigatus* gene expressed in *P. pastoris* [16].

Determination of Physical Properties of Purified Enzymes

The pH optima of wild-type and Natuphos phytases were 5.5 and 5.0, respectively (Fig. 2). Two distinct pH optima were observed with phytases from wild-type and Natuphos, while the highest activity was retained at pH 5.5–5.0. Two distinct pH optima of phytase A of *Aspergillus* were the common feature, which agrees with the results. However, the optimal pH of recombinant phytase from *P. pastoris* was quite different from those of the two other purified phytases, which maintained 100%–40% of the relative activity through the pH ranging from pH 1.0 to 5.5. Given the narrow pH optimum peak at pH 2.5 and low level of expression, contamination of phytase B could be completely excluded. Also, the size difference of phytase B excluded any chance of contamination of phytase B during purification. This result was contradictory to the results from other laboratories [4, 20], where two distinct pH optima, 2.5 and 5.5, were reported with yeast-expressed recombinant phytase A. These findings suggest that altered post-translational modification in the different host could cause a dramatic change in enzymatic characteristics. Recently, it was reported that even a single amino acid substitution could shift the pH optima of glycosidase [5]. If

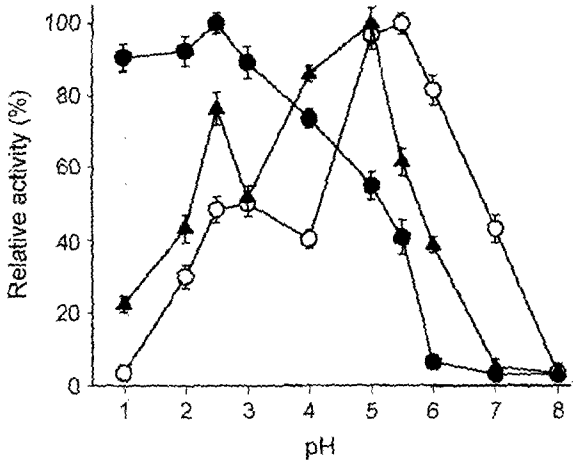

Fig. 2. Optimum pH of phytase activity.
The purified recombinant PhyA expressed in *P. pastoris* (●), *Aspergillus niger* var. *awamori* PhyA (○), and commercial PhyA (▲), with sodium phytate as the substrate at various pHs: 0.2 M glycine-HCl (pHs 1.0, 2.0, and 2.5), 0.2 M sodium citrate (pHs 3.0, 4.0, 5.0, and 5.5), and 0.2 M Tris-HCl (pHs 6.0, 7.0, and 8.0). Results are expressed as means of three experiments.

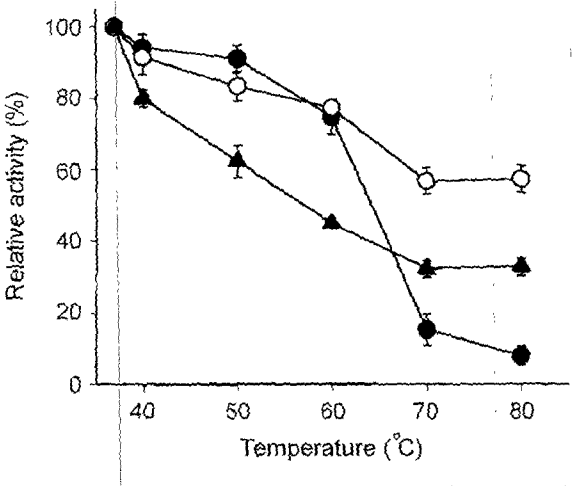

Fig. 4. Residual enzymatic activity of the purified recombinant PhyA from *P. pastoris* (●), *Aspergillus niger* var. *awamori* PhyA (○), and the purified commercial PhyA (▲) after exposure for 20 min to the indicated temperatures, in 0.2 M sodium citrate (pH 5.5) buffer.
Results are expressed as means of three experiments.

this was the case, the altered pH optimum of recombinant phytase might be due to mutation during RT-PCR or an integration process into the host genome.

Thermal stability of phytase is important in animal feed applications, where the enzyme is normally incorporated into the plant feed stuffs prior to pelletization. Recombinant phytase from *A. fumigatus*, which produced a thermostable phytase, was tested to overcome this problem [14]. But its lower specific activity toward phytic acid and susceptibility to the proteolytic degradation limit the practical use of this enzyme. Therefore, *A. niger* phytase is the only enzyme now commercially available [10]. The recombinant and Natuphos phytase showed optimum temperatures around 50°C (Fig. 3). Wild-type phytase showed a higher and broader range of active temperatures around 50°C–60°C than the recombinant and Natuphos phytase. At 60°C, *Aspergillus niger* var. *awamori* PhyA retained over 69% of its activity, while commercial phytase retained only 48% of its activity. The thermal stability of three purified phytases was compared by measuring the remaining activity at 37°C after being heated for 20 min at each temperature (40°C to 80°C). It was remarkable that PhyA from *Aspergillus niger* var. *awamori* retains 57% of its original activity at 80°C, while that of the commercial one retains only 32% of activity in the same condition (Fig. 4). The thermal stability of recombinant was dramatically reduced, whereas only 8% of activity was detected at 80°C. These results suggest that the thermal stability of phytases was affected by the pattern of glycosylation, which is contradictory to the findings of Wyss and coworkers [22].

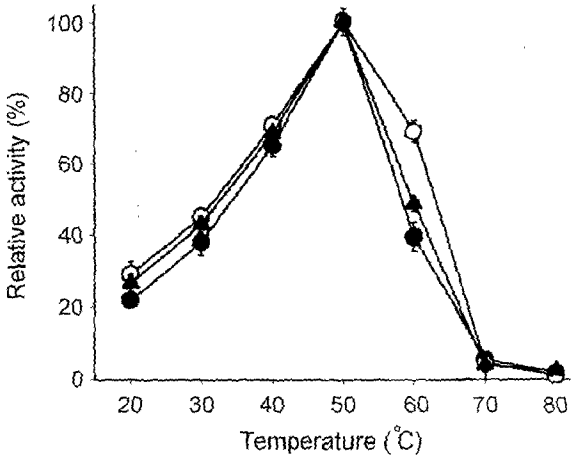

Fig. 3. Temperature dependence of phytase activity.
The purified recombinant PhyA expressed in *P. pastoris* (●), *Aspergillus niger* var. *awamori* (○), and commercial PhyA (▲) with sodium phytate as the substrate at pH 5.5. Results are expressed as means of three experiments.

Acknowledgment

This research work was financially supported by the Agricultural R & D Promotion Project in Ministry of Agriculture & Forestry, Korea.

REFERENCES

1. Cassader, M., G. Ruiu, R. Gambino, F. Guzzon, A. Pagano, F. Veglia, R. Pagni, G. Pagano, T. Marquardt, and A. Helenius. 1994. Influence of misfolding and aggregation of

newly synthesised proteins in the endoplasmic reticulum. *J. Cell Biol.* **117:** 505–513.
2. Choi, Y. M., D. O. Noh, S. H. Cho, H. K. Lee, H. J. Suh, and S. H. Chung. 1999. Isolation of a phytase-producing *Bacillus* sp. KHU-10 and its phytase production. *J. Microbiol. Biotechnol.* **9:** 223–226.
3. Ellis, S. B., P. F. Brust, P. J. Koutz, A. F. Waters, M. M. Harpold, and T. R. Gingeras. 1985. Isolation of alcohol oxidase and two other methanol regulatable genes from the yeast, *Pichia pastoris*. *Mol. Cell. Biol.* **5:** 1111–1121.
4. Han, Y. X. and G. Lei. 1999. Role of glycosylation in the functional expression of an *Aspergillus niger* phytase (*phy*) in *Pichia pastoris*. *Archives of Biochemistry and Biophysics* **364:** 83–90.
5. Joshi, D. M., G. Sidhu, I. Pot, G. D. Brayer, S. G. Withers, and L. P. McIntosh. 2000. Hydrogen bonding and catalysis: A novel explanation for how a single amino acid substitution can change the pH optimum of a glycosidase. *J. Mol. Biol.* **299:** 255–279.
6. Kim, D. S., J. S. Godber, and H. R. Kim. 1999. Culture conditions for a new phytase-producing fungus. *Biotechnol. Lett.* **21:** 1077–1081.
7. Kim, H. P., M. R. Jin, I. Y. Kim, B. Y. Ahn, S. M. Kang, E. J. Choi, J. Kim, I. H. Kim, and K. S. Ahn. 1999. Analysis of the major histocompatibility complex class I antigen presentation machinery in human lung cancer. *J. Microbiol. Biotechnol.* **9:** 346–351.
8. Kim, M. H., W. T. Chung, M. K. Lee, J. Y. Lee, S. J. Ohh, J. H. Lee, D. H. Park, D. J. Kim, and H. Y. Lee. 2000. Kinetics of removing nitrogenous and phosphorus compounds from swine waste by growth of microalga, *Spirulina platensis*. *J. Microbiol. Biotechnol.* **10(4):** 455–461.
9. Lim, H. K., K. Y. Kim, K. J. Lee, D. H. Park, S. I. Chung, and K. H. Jung. 2000. Genetic stability of the integrated structural gene of Guamerin in recombinant *Pichia pastoris*. *J. Microbiol. Biotechnol.* **10(4):** 470–475.
10. Mullaney, E. J., C. B. Daly, K. Sethumadhavan, E. Rodriquez, X. G. Lei, and A. H. J. Ullah. 2000. Phytase activity in *Aspergillus fumigatus* isolates. *Biochem. Biophys. Res. Commun.* **275:** 759–763.
11. Mullaney, E. J., D. M. Gibson, and A. H. J. Ullah. 1991. Positive identification of a lambda gt11 clone containing a region of fungal phytase gene by immunoprobe and sequence verification. *Appl. Microbiol. Biotechnol.* **55:** 611–614.
12. Nagashima, T., T. Tange, and H. Anazawa. 1999. Dephosphorylation of phytate by using the *Aspergillus niger* with a high affinity for phytate. *Appl. Environ. Microbiol.* **65:** 4682–4684.
13. Park, J. O., J. M. Yang, and I. S. Chung. 1999. Biochemical analysis of *Anagrapha falcifera* NPV attachment to *Spodoptera frugiperda* 21 cells. *J. Microbiol. Biotechnol.* **9(3):** 361–364.
14. Pasamontes, L., M. Haiker, M. Wyss, M. Tessier, and A. P. G. M. van Loon. 1997. Gene cloning, purification, and characterization of heat-stable phytase from the fungus *Aspergillus fumigatus*. *Appl. Environ. Microbiol.* **63:** 1696–1700.
15. Pfeffer, S. R. and J. E. Rothman. 1987. Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. *Annu. Rev. Biochem.* **56:** 829–852.
16. Rodriguez, E., E. J. Mullaney, and X. G. Lei. 2000. Expression of the *Aspergillus fumigatus* phytase gene in *Pichia pastoris* and characterization of the recombinant enzyme. *Biochem. Biophys. Res. Commun.* **268:** 373–378.
17. Rudd, P. M., H. C. Joao, E. Coghill, P. Fiten, M. R. Saunders, G. Opdenakker, and R. A. Dwek. 1994. Glycoforms modify the dynamic stability and functional activity of an enzyme. *Biochemistry* **33:** 17–22.
18. Sankpal, N. V., A. P. Joshi, and B. D. Kulkarni. 2000. Nitrogen-dependant regulation of gluconic and/or citric acid production by *Aspergillus niger*. *J. Microbiol. Biotechnol.* **10:** 51–55.
19. Scorer, C. A., J. J. Clare, M. A. Romanos, and K. Sreekrishna. 1993. Rapid selection using G418 of high copy number transformants of *Pichia pastoris* for high-level foreign gene expression. *Bio/Technology* **12:** 181–184.
20. Ullah, A. H. J. and D. M. Gibson. 1987. Extracellular phytase (EC. 3.1.3.8) from *Aspergillus ficuum* NRRL 3135: Purification and characterization. *Prep. Biochem.* **17:** 63–91.
21. Wyss, M., R. Bruger, A. Kronenberger, R. Remy, R. Fimbel, G. Oesterhelt, M. Lehmann, and A. P. G. M. van Loon. 1999. Biochemical characterization of fungal phytases (myo-inositol hexakisphosphate phosphohydrolase): Catalytic properties. *Appl. Environ. Microbiol.* **65:** 367–373.
22. Wyss, M., L. Pasamontes, A. Friedlein, R. Remy, M. Tessier, A. Kronenberger, A. Middenddorf, M. Lehmann, L. Schnoebelen, U. Rothlisberger, E. Kusznir, G. Wahl, F. Muller, H. W. Lahm, K. Vogel, and A. P. G. M. van Loon. 1999. Biophysical characterization of fungal phytases (myo-inositol hexakisphosphate phosphohydrolase): Molecular size, glycosylation pattern, and engineering of proteolytic resistance. *Appl. Environ. Microbiol.* **65:** 359–366.