JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Kinetic Characterization of O-Phospho-L-tyrosine Phosphohydrolase Activity of Two Fungal Phytases

ABUL H. J. ULLAH,* KANDAN SETHUMADHAVAN, AND EDWARD J. MULLANEY

Commodity Utilization Research Unit, Southern Regional Research Center, ARS, United States Department of Agriculture, 1100 Robert E. Lee Boulevard, New Orleans, Louisiana 70124

Fungal phytases belonging to "histidine acid phosphatase" or HAP class of phosphohydrolases that catalyze the hydrolysis of phytic acid could also hydrolyze O-phospho-L-tyrosine, which is also called phosphotyrosine. Two phytases from *Aspergillus niger* and *Aspergillus awamori* with pH optima 2.5 were tested for phosphotyrosine hydrolase activity; both enzymes cleaved the phosphomonoester bond of phosphotyrosine efficiently at acidic pH. The K_m for phosphotyrosine ranged from 465 to 590 μM as opposed to 135 to 160 μM for phytate. The V_{max}, however, is 2−4 times higher for phosphotyrosine than it is for phytate. The catalytic efficiency of phytase for phosphotyrosine is on the same order as it is for phytate (3.5×10^6 to 1.6×10^7 M^{-1} s^{-1}); the pH versus activity profile for phosphotyrosine is, however, different from what it is for phytate. The temperature optima shifted 5 °C higher to 70 °C when phosphotyrosine was used as the substrate. Taken together, the kinetic data show that fungal HAPs that are known as PhyB are capable of cleaving the phosphomonoester bond in phosphotyrosine. This is the first time that phosphotyrosine phosphatase (PTPase) activity has been reported for the subgroup of HAP known as phytase.

KEYWORDS: Phytase; PTPase; phosphohydrolase; phosphomonoesterase; histidine acid phosphatase

INTRODUCTION

Under limited phosphate *Aspergillus niger* (*ficuum*) NRRL 3135 produces three acid phosphatases (*1*–*3*). Of the three, two belong to subgroup "histidine acid phosphatase" or HAP; these acid phosphatases are capable of cleaving phosphates from phytic acid and, as such, are called phytases (*4*). The third acid phosphatase, pH 6.0-optimum phosphomonoesterase, lacks phytate degrading ability and belonged to "purple acid phosphatase" subclass of phosphomonoesterase (*5*). The phytase (*phyA*) gene from *A. niger* NRRL 3135 was cloned, overexpressed, and commercialized as a feed additive for poultry and swine industries (*6, 7*). The mechanism of hydrolysis of the monoester bond by phytase was elucidated, the enzyme's three-dimensional structure was deduced, and the sensitive Arg, His, and Asp residues were mapped (*8, 9*). The *A. niger* NRRL 3135 PhyB phytase, which cleaves phytate phosphates at pH 2.5 at an accelerated rate than PhyA phytase, is also a well-studied enzyme; its gene (*phyB*) was cloned and the three-dimensional structure had been elucidated (*10, 11*). Both PhyA and PhyB could hydrolyze synthetic substrate, *p*-nitrophenylphosphate, and a variety of phosphorylated sugars (*2*). The *phyB* was also cloned from *Aspergillus awamori* and overexpressed in mutant strains of the same species of fungi (*12*). To ascertain whether fungal PhyA and PhyB could hydrolyze phosphorylated amino acids, we tested phosphotyrosine, phosphoserine, and phosphothreonine as substrate. While PhyA could utilize all three phosphorylated amino acids with difficulty (data not presented), PhyB phytase could degrade phosphotyrosine facilely. The other two phosphorylated amino acids were hydrolyzed albeit slowly by PhyB phytase (A. Ullah, unpublished observation). A non-HAP phytase has been reported in *Selenomonas ruminantium* that share the active site motif and overall three-dimensional structure with protein tyrosine phosphatase (*13*). In this communication we present kinetic data demonstrating that PhyB phytases catalyze the dephosphorylation of phosphotyrosine. In near anaerobic condition in the rumen, a unique phosphatase with cysteine at the active site is implicated as being the phosphoprotein phosphatase or PTPase (*13*). This is however the first time that anyone is reporting PTPase activity in HAP type of acid phosphatase. All the work described here was done using two members of PhyB, namely, pH 2.5 optimum phytase produced by *A. niger* (*14*) and *A. awamori* (*12*).

MATERIALS AND METHODS

Source of Phytase. *A. niger* (*ficuum*) NRRL 3135 was grown in starch media supplemented with glucose, sodium nitrate, and other salts (*1*). The culture media containing PhyB activity was harvested by centrifugation at 12000 rpm in a Sorval GSA rotor in ice-cold conditions. The enzyme, PhyB, was purified using sequential ion-exchange chromatographies using MacroPrep S and Q columns (*2*). The purified phytase had a specific activity of 3570 nKat/mg protein.

The Finase enzyme produced by *A. awamori* was originally obtained from Alko Ltd. Biotechnology, SF-05200 Rajamaki, Finland, by Prof. E. R. Miller of Michigan State University. Prof. Xin-Gen Lei of Cornell University had subsequently received the same enzyme and made it

* To whom correspondence should be addressed. E-mail: abul.ullah@ars.usda.gov.

10.1021/jf800597f
Published on Web 07/16/2008

available to us. The phytase was then purified by chromatographic methods identical to the purification of *A. niger* PhyB as mentioned above. The specific activity of this highly homogeneous preparation was 4357 nKat/mg protein.

Phytase Assay. Phytase assays were carried out in 1.0 mL volume at 58 °C in assay buffer, 25 mM glycine, pH 2.5, similar to the *A. niger* phytase assay (*1*). The liberated inorganic *ortho*-phosphates were quantitated spectrophotometrically using a freshly prepared AMA reagent consisting of acetone, 10 mM ammonium molybdate, and 2.5 M sulfuric acid (2:1:1, v/v). Adding 2.0 mL AMA solution per assay tube terminated phytase assay. After 30 s, 0.1 mL of 1.0 M citric acid was added to each tube to fix the color generated by AMA reagent. Absorbance was read at 355 nm after blanking the spectrophotometer with appropriate control. Values were expressed as nkat/mL where kat stands for katal (mole substrate converted per second). One international unit (IU) is equivalent to 16.67 nkat.

Phosphotyrosine Phosphohydrolase Activity. The assay is identical to phytase assay except that the substrate 10 mM phosphotyrosine was used in incubations and the assay buffer was 25 mM glycine, pH 2.5 or 25 mM imidazole, pH 6.0; the final concentration of phosphotyrosine being 750 μM. The enzyme concentration was held at 65 pmol for PhyB and 112 pmol for Finase.

Phenylglyoxal Treatment. A 10 μL aliquot of the PhyB (650 pmol) was mixed in 240 μL of phenylglyoxal (250 mM) in bicarbonate (125 mM, pH 8.3) buffer and 45 μL of Finase (1120 pmole) was mixed with equal volume of phenylglyoxal (250 mM) in the same bicarbonate buffer. Reaction with the enzyme was carried out at 37 °C for 30 min. At the end of incubation, 2 μL of the sample was used for the phosphotyrosine phosphohydrolase activity with one min incubation time. The control experiments contained bicarbonate buffer without phenylglyoxal.

***myo*-inositol Hexasulfate Treatment (MIHS).** A 2 μL aliquot of PhyB (130 pmol) and 10 μL of Finase (249 pmol) was pipetted into assay tubes containing 25 mM glycine, pH 2.5 buffer raised to 58 °C. A 50 μL aliquot of 10 mM MIHS was added before the addition of 10 mM phosphotyrosine. The phosphotyrosine phosphohydrolase activity was measured after incubating for 1 min. Parallel control experiments were run without MIHS.

RESULTS

The PhyB phytase, first identified as a pH 2.5 optimum acid phosphatase, is capable of hydrolyzing the phosphomonoester bond of phosphotyrosine. Based on protein sequence, Finase, a recombinant phytase from *A. awamori*, is a homologue of *A. niger* PhyB.

Phosphotyrosine Phosphohydrolase Activity as a Function of pH. While performing the substrate selectivity study of *A. niger* PhyB, we discovered that this phosphomonoesterase (PhyB) could efficiently hydrolyze phosphotyrosine at pH 2.5, which is the pH optimum of this class of phytase (unpublished observation). Therefore, activity was measured as a function of pH for *A. niger* PhyB and Finase using phosphotyrosine as the substrate (*15*). The results are shown in **Figure 1**. Both the enzymes show bihump pH optima, one at pH 2.5 and the other at 6.0. The ratio of activity at pH 2.5 over pH 6.0 is much higher for Finase (3:1) than it is for *A. niger* phytase (0.9:1). Nonetheless, these two members of PhyB could hydrolyze phosphotyrosine quite facilely.

Phosphotyrosine Phosphohydrolase Activity as a Function of Temperature. The PhyB phytase from *A. niger* is a thermotolerant biocatalyst performing optimally at 63 °C (*2*). To determine the optimum temperature of phosphotyrosine phosphohydrolase activity catalytic measurements were done at various temperatures. The results are shown in **Figure 2**. The temperature optimum for *A. awamori* PhyB was found to be 70 °C, which is 7 °C higher than what it is for degrading synthetic substrate, p-nitrophenyl phosphate (*2*). However, at 75 °C the activity of both phytases dropped precipitously.

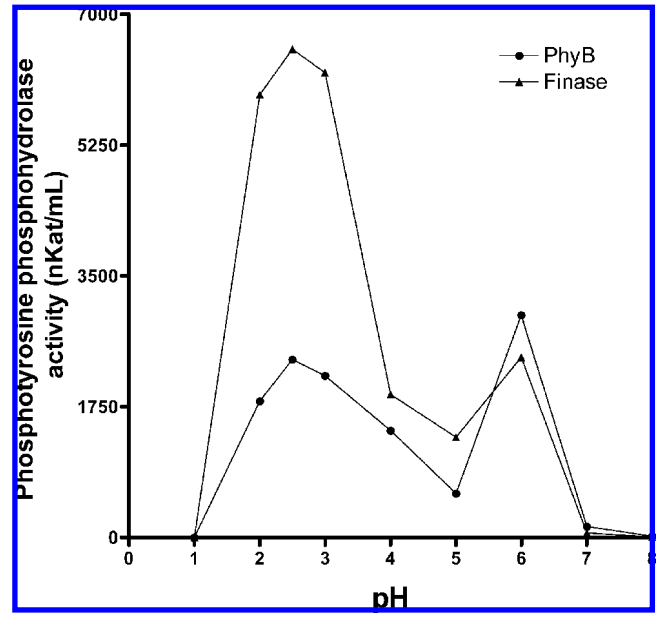

Figure 1. The pH optima of the fungal phytases (PhyB) using phosphotyrosine as substrate. The buffers used were glycine (pH 1.0−3.0), acetate (pH 3.5−5.5), and imidazole (pH 6.0−8.0).

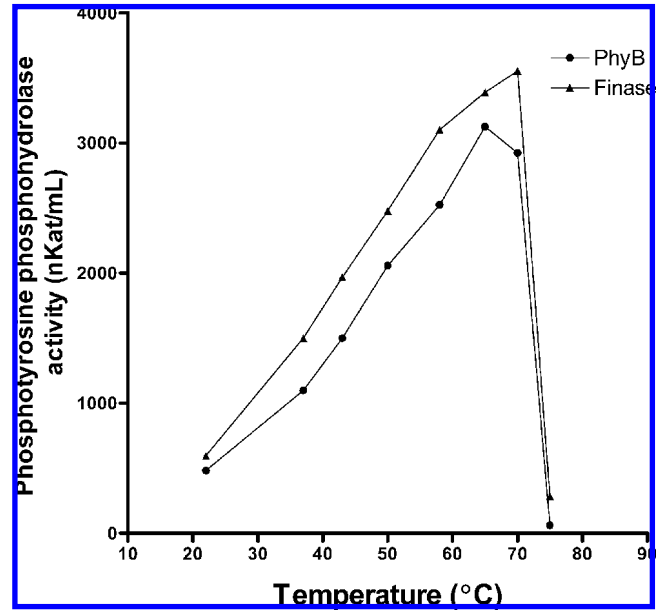

Figure 2. Temperature optima of the fungal phytases using phosphotyrosine as substrate. Phytase assays were carried out at 58 °C in assay buffer, 25 mM glycine, pH 2.5.

Phytate versus Phosphotyrosine Hydrolysis by PhyB. Figure 3 shows the results of phytate and phosphotyrosine hydrolysis catalyzed by fungal PhyB phytases. Phosphotyrosine was hydrolyzed more efficiently than phytic acid. **Figure 3A** shows the concentration dependent breakdown of both phytate and phosphotyrosine by *A. niger* PhyB phytase while panel B depicts the breakdown of phytate and phosphotyrosine by recombinant *A. awamori* PhyB phytase (Finase). The *A. niger* PhyB cleaved phosphate nearly 2.25 times faster from phosphotyrosine than it did from phytate. The recombinant *A. awamori* PhyB phytase demonstrated overall a higher rate of phosphohydrolase activity, but was similar to *A. niger* PhyB in its preferential hydrolysis of phosphotyrosine as compared to phytate. In *A. niger* PhyB when phosphotyrosine was used as substrate, the turnover number increased while the kinetic

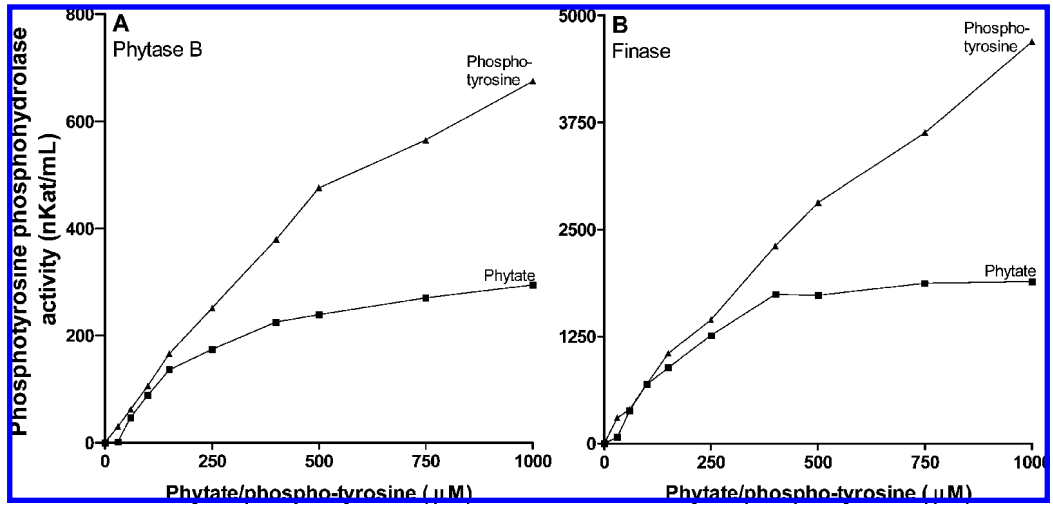

Figure 3. Phosphohydrolase activity of fungal phytases as function of substrate concentration. Both the phytase and the phosphotyrosine phosphatase assays were carried out at 58 °C in assay buffer, 25 mM glycine, pH 2.5.

Table 1. Kinetic Parameters and Catalytic Efficiency of PhyB Phytase and Finase for Phytate and Phosphotyrosine

enzymes	substrate	K_m (μM)	turnover (number s^{-1})	catalytic efficiency (M^{-1} s^{-1})
PhyB phytase	phytate	135	2178	1.61×10^7
PhyB phytase	phosphotyrosine	465	4894	1.05×10^7
Finase	phytate	160	710	4.43×10^6
Finase	phosphotyrosine	590	2118	3.59×10^6

efficiency decreased; similar result was observed when Finase was used to hydrolyze phosphotyrosine (**Table 1**). Phytate shows a higher affinity for binding to the active site as exemplified by a lower K_m. Like Finase, *A niger* PhyB phytase shows a higher K_m for phosphotyrosine (**Table 1**).

Phenylglyoxal Modification of PhyB. To examine if both phytate and phosphotyrosine utilize the same active site in PhyB subclass of phytase, both the proteins were treated with phenylglyoxal to modify arginine in the active site. The results are shown in **Figure 4**. Phenylglyoxal treated PhyB from both *A. niger* and *A. awamori* were totally inactivated. Phosphotyrosine phosphohydrolase activity of the treated PhyB phytase performed at both pH 3.0 and 6.0 were inactivated. This indicates that both phytate and phosphotyrosine utilize the same active site of phyB phytase.

***myo*-inositol Hexasulfate (MIHS) Inhibition of Phosphotyrosine Phosphohydrolase Activity.** MIHS, both a substrate analog and a known active site inhibitor of phytase (*16*) was used to investigate whether the mechanistic enzymology of PhyB subclass of phytase is same for phytate-driven and phosphotyrosine-driven phosphohydrolase reaction. The result of MIHS inhibition of phosphotyrosine phosphohydrolase activity of both *A. niger* and *A. awamori* PhyB phytase is shown in **Figure 5**. The enzymatic data clearly shows that at pH 3.0 MIHS is a potent inhibitor of both the PhyB phytases. However, at pH 6.0 MIHS had failed to inhibit the phosphotyrosine phosphohydrolase activity. Therefore, pH plays an important role in the three-dimensional structure of the active site.

DISCUSSION

PhyB phytase from *A. niger* and *A. awamori* hydrolyzed phosphotyrosine efficiently. The pH optimum for both phytate and *p*-nitrophenylphosphate (p-NPP) consisted of a single peak with optimum pH at 2.5 (*2*). However, when phosphotyrosine was used as the substrate, the PhyB phytases from both *A. niger* and *A. awamori* gave two distinct pH optima, one at 2.5 and the other at 6.0 (**Figure 1**). Based on the pH versus activity profile, it seems as if phosphotyrosine binds to the active site differently than phytate.

Earlier, it was noticed that PhyB had a higher temperature optimum for synthetic substrate, p-NPP than PhyA phytase. Previously, *A. ficuum* PhyB phytase was shown to be a homodimer based on gel filtration in native state (*2*); it was also shown to be more thermo-tolerant than PhyA phytase. Later, the hydrodynamic radius (R_H) of PhyB and Finase was found to be 5.5 nm (180-kDa), which supports the dimeric state of the native enzyme (A. Ullah, unpublished data). The dimeric structure may play a role in enhancing the thermo-stability in PhyB phytase. However, when phosphotyrosine was used as the substrate, the enzyme was found to be even more thermo-tolerant. The optimum temperature for phosphotyrosine hydrolysis was found to be 70 °C (**Figure 2**), which is 7 °C higher than phytate degradation.

Phosphotyrosine was hydrolyzed more efficiently than phytate by both the fungal PhyB phytases. The higher turnover number for phosphotyrosine driven catalysis over phytate reflects this enhanced catalysis. However, the affinity for phytate was much stronger than phosphotyrosine (**Table 1**). The combined kinetic parameters of K_m and turnover number when taken together explain why kinetic efficiency is not different in the case of these two PhyB enzymes for phytate and phosphotyrosine.

A sensitive Arg residue in the active site of phytase was implicated for catalyzing the phosphomonoester bond in phytate (*9*). To test whether the same Arg is involved in the catalysis of phosphotyrosine, both the PhyBs were treated with phenylglyoxal, a known modifier of Arg. The modified PhyBs from both the sources failed to hydrolyze phosphotyrosine at pH 3.0 and 6.0 indicating that the same active site for phytate hydrolysis is used to cleave phosphotyrosine.

MIHS, a phytate analog, was developed as an active site inhibitor for HAP subclass of acid phosphatase (*15*). At pH 3.0 the inhibitor bound to the substrate-binding domain of the active site of both the PhyBs and did not allow hydrolysis of phosphotyrosine; however, at pH 6.0 the catalysis of phosphotyrosine was not inhibited by MIHS (**Figure 5**). The precise

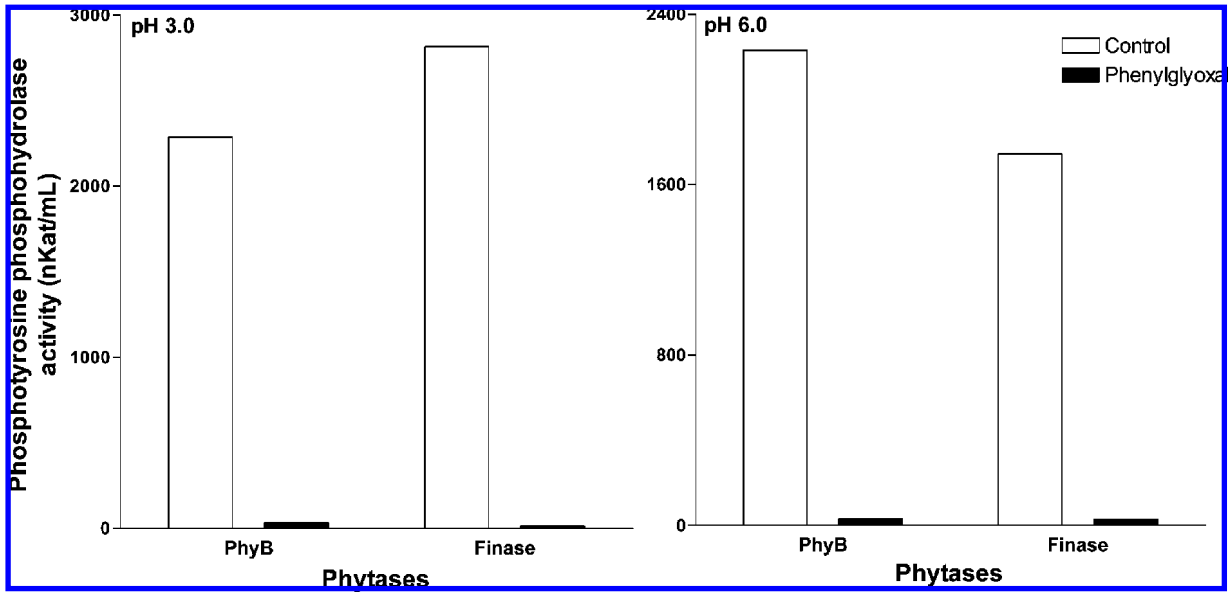

Figure 4. Inhibition of phosphotyrosine phosphohydrolase activity of fungal phytases by phenylglyoxal. After inactivation the enzyme activity was performed at 58 °C in assay buffer, 25 mM glycine, pH 2.5.

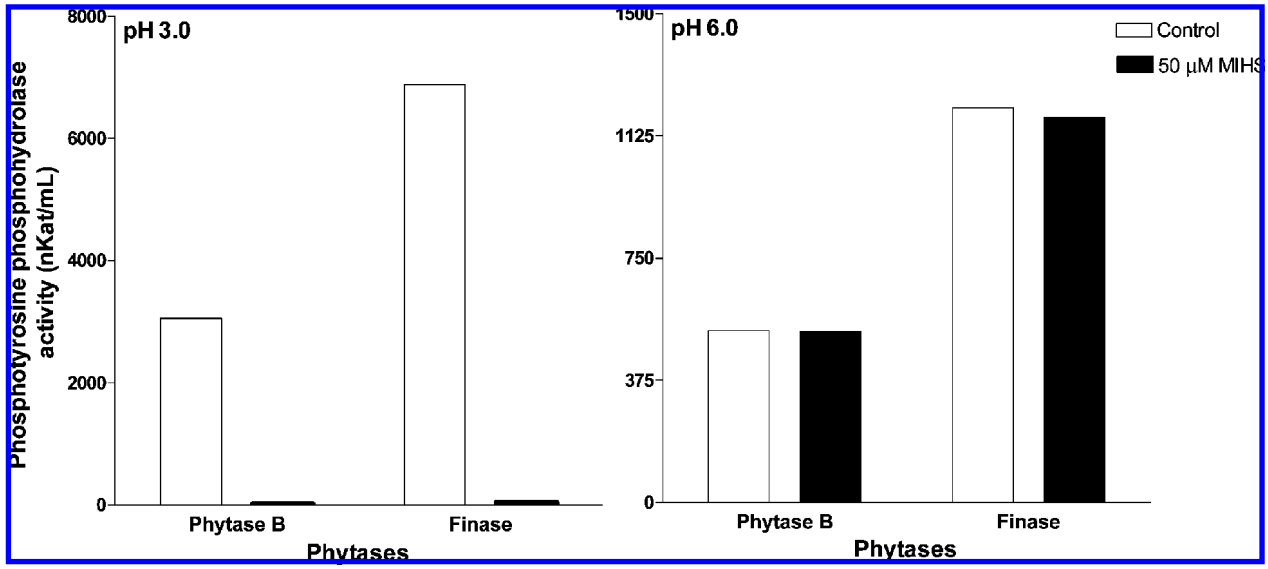

Figure 5. Inhibition of phosphotyrosine phosphohydrolase activity of the fungal phytases by MIHS. The MIHS treated enzyme was assayed for activity at 58 °C in assay buffer, 25 mM glycine, pH 2.5.

molecular explanation of this pH dependency for MIHS to block phosphohydrolase activity is lacking at this time; however, one possible explanation for no inhibition at pH 6.0 might be due to absence of negatively charged sulfate groups on MIHS at pH 6.0. Perhaps the X-ray deduced three-dimensional structure of PhyB in the presence of MIHS at pH 3.0 and 6.0 might unravel this differential effect of pH on catalysis in the presence of MIHS.

Protein-tyrosine-phosphatase or phosphotyrosine phosphatase (PTPase) are enzymes that catalyze the removal of a phosphate group attached to a tyrosine residue. These enzymes are very important in the control of cell growth, proliferation, differentiation and transformation (*17*). Multiple forms of PTPase have been characterized and could be classified into two categories: soluble PTPases and transmembrane receptor proteins that contain PTPase domain(s). One member of the PTP subclass, cysteine phosphatase, is known to be able to degrade phytic acid (*18*). The active site of cysteine phosphatase contains a conserved octa-peptide with sequence HCXXGXXR and shows no sequence homology with HAP subclass of acid phosphatase (E.C. 3.1.3.2 and E.C. 3.1.3.8) whose active site sequence is RHGXRXP. Furthermore, PTPase or tyrosine phosphatases have a signature sequence in their active site, with a cysteine residue that is essential for activity. The cysteine serves as the active site nucleophile (*19*). However, in fungal HAP, 4−5 disulfide bridges are formed by 8−10 Cys residues. Therefore, from the perspectives of active site and sequence homology, these two classes of phosphatases are markedly different. This is the first documentation that PhyB from *Aspergillus* is capable of efficiently hydrolyzing phosphotyrosine. It is noteworthy that a new class of phytase related to PTPase was isolated from several anaerobic bacteria from the rumen of cattle (*19*). The observed diversity of this new class of phytase in the rumen may account for the ability of ruminants to hydrolyze phytate. Despite no homology between the active site of phytase and PTPase both classes of enzymes degrade a variety of phosphorylated metabolites such as *myo*-inositol phos-

phates and phosphorylated amino acids. This indicates that during evolution the substrate selectivity of phosphatases was kept broad to allow for scavenging phosphates from an array of metabolites.

LITERATURE CITED

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

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

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

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

(5) Mullaney, E. J.; Ullah, A. H. J. The term phytase comprises several different classes of enzymes. *Biochem. Biophys. Res. Commun.* **2003**, *312*, 179–184.

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

(7) Yi, Z.; Kornegay, E. T. Evaluation of response criteria for assessing biological availability of phosphorus supplements in swine. In *Phytase in Animal Nutrition and Waste management*, 2nd revised edition; BASF: Mount Olive, NJ, 1999; pp 137−143.

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

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

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

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

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

(13) Chu, H-M.; Guo, R-T.; Lin, T-W.; Chou, C-C.; Shr, H-L.; Lai, H-L.; Tang, T-Y.; Cheng, K-J.; Selinger, B. L.; Wang, A.H.-J. Structures of Selenomonas ruminantum phytase in complex with persulfated phytate: DSP phytase fold and mechanism for sequential substrate hydrolysis. *Structure* **2004**, *12*, 2015–2024.

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

(15) Kremerskothen, J.; Barnekow, A. Determination of phosphotyrosine phosphatase (PTPase) activity in normal and transformed cells. *Molec. Cell. Biochem.* **1993**, *125*, 1–9.

(16) Ullah, A. H. J.; Sethumadhavan, K. Myo-inositol hexasulfate is a potent inhibitor of *Aspergillus ficuum* phytase. *Biochem. Biophys. Res. Commun.* **1998**, *251*, 260–263.

(17) Walton, K. M.; Dixon, J. E. Protein tyrosine phosphatases. *Annu. Rev. Biochem.* **1993**, *62*, 101–120.

(18) Chu, H. M.; Guo, R. T.; Lin, T. W.; Chou, C. C.; Shr, H. L.; Lai, H. L.; Tang, T. Y.; Cheng, K. J.; Selinger, B. L.; Wang, A. H. J. Structures of *Selenomonas ruminantium* phytase in complex with persulfated phytate: DSP phytase fold and mechanism for sequential substrate hydrolysis. *Structure* **2004**, *12*, 2015–2024.

(19) Nakashima, B. A.; McAllister, T. A.; Sharma, R.; Selinger, L. B. Diversity of phytases in the rumen. *Microb. Ecol.* **2007**, *53*, 82–88.

Received for review February 27, 2008. Revised manuscript received June 13, 2008. Accepted June 18, 2008.

JF800597F