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Cloning, Expression, and Enzyme Characterization of an Acid Heat-Stable Phytase from *Aspergillus fumigatus* WY-2

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Abstract. A novel thermostable phytase gene was cloned from *Aspergillus fumigatus* WY-2. It was 1459 bp in size and encoded a polypeptide of 465 amino acids. The gene was expressed in *Pichia pastoris* GS115 as an extracellular enzyme. The expressed enzyme was purified to homogeneity and biochemically characterized. The purified enzyme had a specific activity of 51 U/mg with an approximate molecular mass of 88 kDa. The optimum pH and temperature for activity were pH 5.5 and 55°C, respectively. After incubation at 90°C for 15 min, it still remained at 43.7% of the initial activity. The enzyme showed higher affinity for sodium phytate than other phosphate conjugates, and the K_m and K_{cat} for sodium phytate were 114 μM and 102 s^{-1}, respectively. Incubated with pepsin at 37°C for 2 h at the ratio (pepsin/phytase, wt/wt) of 0.1, it still retained 90.1% residual activity. These exceptional properties give the newly cloned enzyme good potential in animal feed applications.

Phytate (*myo*-inositol hexakisphosphate) is the predominant phosphorous storage form in cereals, oilseeds, and legumes, which are the major ingredients of animal feed [13]. Monogastric animals, such as poultry and pig, lack the ability to digest phytate phosphorus because of the low phytase activity in their gastrointestinal tracts [6]. As a consequence, the large amount of undigested phytate is excreted in animal manure, causing environmental problems in areas of intensive livestock production [19]. In addition, phytate is also regarded as an antinutrient factor because it can chelate multivalent cations and some proteins, thereby rendering these biologically unavailable to the animal [2, 19].

Phytases (*myo*-inositol hexakiphosphate phosphohydrolase, EC 3.1.3.8 and EC 3.1.3.26) catalyze the hydrolysis of phytate to various lower phosphate *myo*-inositol derivatives and inorganic phosphate [7]. Supplement of phytases to the feed can improve the phosphorus and mineral bioavailability in monogastric animals and reduce the phosphorus excretion in their manure [8]. Because poultry and pig feed commonly is pelleted, a commercially attractive phytase should be able to withstand high temperatures (60–90°C) that may be reached temporarily during the pelleting process. The phytase from *Aspergillus fumigatus* ATCC34625 was described to have very high thermostability by Pasamontes et al. [11], and its superior property has been confirmed by Martin et al., Peng et al., and Rodriguez et al. [9, 12, 14]. However, the specific activity of the enzyme is relatively low [20], limiting its feasibility in industrial applications, and makes it necessary to finding a phytase with high thermostability and specific activity for commercial use. Recently, we isolated a producing-phytase strain, named WY-2, from the soil of the Dalian University of Technology (Dalian, China). Through microbial taxonomy methods, it was identified as *A. fumigatus* (CICC accession no. 40699). In this paper, we cloned and sequenced the phytase gene from *A. fumigatus* WY-2 and expressed it in *Pichia pastoris* GS115.

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Materials and Methods

Strains, Plasmids, and Growth Conditions. *A. fumigatus* strain WY-2 was used as a phytase gene donor strain and cultured in Hylon starch medium [16] at 30°C. *Pichia pastoris* GS115 (*his4*) (Invitrogen, San Diego, USA) was used as a gene expression host and cultured in YPD medium at 30°C. Plasmid pMD18-T (TaKaRa, Dalian, China) was used for TA cloning and nucleotide sequencing, and plasmid pPIC9K (Invitrogen, San Diego, USA) was used for expression.

DNA Amplification, Cloning, and Sequencing. Genomic DNA of *A. fumigatus* WY-2 was isolated from cultures as previously described [3]. The primers derived from the published gene sequence of *A. fumigatus* ATCC34625 phytase (GenBank accession no. U59804) were used to isolate the phytase gene of our strain. The upstream primer was 5′-ATGGTGACTCTGACTTTCCTGCTT-3′ and the downstream primer was 5′-TCAACTAAAGCACTCTCCCCAGTTGCC-3′. A DNA fragment of approximately 1.5 kb was generated and cloned into the vector pMD18-T. The clone that contained the polymerase chain reaction (PCR) product was verified by restriction enzyme digestion, agarose gel electrophoresis, and sequencing. The nucleotides and deduced amino acid sequence homology searches were performed on the National Center for Biotechnology Information (NCBI) databases by BLAST search.

Plasmid Construction and Transformation. DNA amplification of the mature phytase sequence was performed by PCR using primers: the forward primer 5′-GCGAATTCTCCAAGTCGTGCGATAC-3′ and the reverse primer 5′-ACAGCGGCCGCCTAAAGCACTCTCC-3′. The forward and reverse primers contained *Eco*RI and *Not*I restriction site, respectively. The amplified PCR product was inserted into an expressing vector pPIC9K at *Eco*RI and *Not*I sites. The resulting plasmid was linearized by restriction enzyme Bpu1102I and transformed into *P. pastoris* GS115 by electroporation. The transformed cells were plated on MD medium (2% glucose, 1.34% YNB, 0.0004% biotin, and 1.5% agar, pH 5.5) at 30°C for 2 days.

Expression of the Recombinant Phytase in *P. pastoris* GS115 and Purification. The recombinant yeast was cultured in the optimized medium A (4% glucose, 0.2% yeast extract, 2% peptone, 2% NH_2SO_4, 0.05% KCl, 0.05% $MgSO_4{\cdot}7H_2O$, 0.003% $MnSO_4{\cdot}7H_2O$, and 0.003% $FeSO_4{\cdot}7H_2O$, pH 5.5) at 30°C and 200 rpm for 36 h. The cells were pelleted and suspended in medium B (A without glucose and yeast extract, pH 3.0) with 2.5% methanol to induce the phytase gene expression. The culture broth was harvested after 60 h of induction, and fractionated with ammonium sulfate. The precipitate obtained in 30%–80% ammonium sulphate saturation was collected by centrifugation, redissolved in a minimum volume of buffer A (20 mM Tris–HCl, pH 8.0), and dialyzed against the same buffer. The mixture was then centrifuged to remove any insoluble matter and concentrated by the Ultrafree-15 centrifugal filter device (Millipore, 10 kDa). The protein solution was directly loaded onto a 5-mL Q DEAE-Sepharose column (Pharmacia), pre-equilibrated with buffer A, and then the phytase was released with a linear gradient of 0–1 M NaCl in buffer A at a flow rate of 1.0 mL/min. The eluted fraction exhibiting the highest phytase activity was pooled and checked for purity by sodium dodecyl sulfate (SDS)–polyacrylamide gel (10%) electrophoresis [5]. Protein concentration was determined by the method of Bradford [1] using bovine serum albumin as a standard.

Phytase Assay. Phytase activity assay was carried out in 1.0-mL volume at 37°C for 15 min in 200 mM sodium acetate buffer (pH 5.5) containing 2 mM sodium phytate. The released inorganic orthophosphates were quantified spectrophotometrically by the molybdate-blue reaction [10]. One phytase activity unit is defined as the amount of enzyme that catalyzes the release of 1 μmol of inorganic phosphorus from sodium phytate per min at 37°C and pH 5.5.

Characterizations of the Recombinant Phytase. The profiles of pH versus activity and temperature versus activity were determined under the standard assay method. A pH range of 2.0–8.0 and a temperature range of 30–75°C were used. Thermal stability of the recombinant phytase was estimated by incubation of the purified enzyme or the culture supernatant (containing 0.5–1 U of phytase) in 200 mM sodium acetate buffer (pH 5.5) for 15 min at various temperatures ranging from 30 to 90°C, followed by 30-min incubation at 0°C before measuring. The kinetic parameters were determined from the Lineweaver-Burk plot with different concentrations of sodium phytate (0.05–2.0 mM). The substrate specificity was examined by measuring the phytase activity with different phosphate compounds (sodium phytate (Na-IHP), ρ-nitrophenyl phosphate (pNPP), ATP, ADP, AMP, fructose 1, 6-biphosphate (F-1, 6-PP), fructose 6-phosphate (F-6-P), ribose 5-phosphate (R-5-P), and glucose 6-phosphate (G-6-P)) as substrate. The effect of metal ions on the phytase activity was determined by pre-incubating the enzyme with the buffers containing 1 mM metal ion (Zn^{2+}, Cu^{2+}, K^{+}, Co^{2+}, Ca^{2+}, Mn^{2+}, Mg^{2+}, Al^{3+}, and Fe^{3+}) for 15 min at 30°C before performing the enzyme assay. The proteolytic effects of pepsin and trypsin on phytase activity were investigated as follows: pepsin (800 units/mg protein) and trypsin (1500 N, α-benzoyl-L-arginine ethyl ester units/mg protein) was dissolved into 10 mM HCl, pH 2.0 (0.1 mg/mL) and 80 mM ammonium bicarbonate, pH 7.5 (0.1 mg/mL), respectively, and 30 μg enzyme was incubated with different amount of pepsin or trypsin at ratios (protease/phytase, wt/wt) ranging from 0.001 to 0.1, at 37°C, pH 2.0 or 7.5 for 2 h; then the reaction was stopped on ice and the pH of mixture was adjusted to 5.5 for the phytase activity assay.

Results and Discussion

Isolation and Characterization of *A. fumigatus* WY-2 Phytase Gene. The phytase gene from *A. fumigatus* WY-2 was amplified by PCR. A 1.5-kb DNA fragment was generated, cloned, and sequenced. As shown in Fig. 1, the gene (GenBank accession no. AY745738) is composed of two exons (1–47 bp and 109–1459 bp), which are separated by an intron (61 bp), encoding a polypeptide of 465 amino acids with a calculated molecular mass of 49 kDa. Like all the other fungal phytases, the phytase gene from *A. fumigatus* WY-2 was found to have the active site septa-petide RHGXRXP and catalytically active dipeptide HD. In comparison with the sequences of previously isolated phytases by BLAST search, the deduced amino acid sequence of *A. fumigatus* WY-2 phytase showed 91% and 66% identities to *A. fumigatus* ATCC34625 phytase (GenBank accession no. U59804) and *A. ficuum* NRRL3135 phytase (GenBank accession no. M94550), respectively. Based on the analysis of the deduce amino acid sequence, the mature *A. fumigatus* WY-2 phytase had a theoretical pI value 5.95 that was much lower than the pI value of the *A. fumigatus* ATCC34625 phytase (7.04).

Overexpression of the Phytase in *P. pastoris* GS115. Directed by the α-factor secretion signal peptide of expression vector pPIC9K, the recombinant phytase was secreted to the extracellular space. After 60 h of induction by methanol, the recombinants produced the maximum phytase activity (16 U/mL) in the culture medium. The phytase activity yield was increased 114-fold by heterogeneous expression in comparison with that of the wild-type *A. fumigatus* WY-2 (0.14 U/mL). The recombinant phytase in fermentation liquid was

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   1 atggtgactctgactttcctgctttcggtggcgtatctgctatctgggtgagtggctctatcttgttgctatggtcgggctgtacagggtt  91
   1  M  V  T  L  T  F  L  L  S  V  A  Y  L  L  S  G                                            16
  92 ctgattgtggtacgggagtagagtgtctggggcaccgagttcggcaggctccaagtcgtgcgatacggtagagctcgggtaccagtgttct 181
  17                R  V  S  G  A  P  S  S  A  G  S  K  S  C  D  T  V  E  L  G  Y  Q  C  S   40
 182 cctgcgacttctcatctgtggggccagtactcgccgttcttttcgctcgacgacgagctgtcggtgtcgagtgagcttcccaaggattgc 271
  41  P  A  T  S  H  L  W  G  Q  Y  S  P  F  F  S  L  D  D  E  L  S  V  S  S  E  L  P  K  D  C   70
 272 cgggtcacctttgtgcagatgctgtcgcggcatggagcgcggtacccgaccagctccaagagcaagaagtacaagcagctagtgacggcg 361
  71  R  V  T  F  V  Q  M  L  S  R  H  G  A  R  Y  P  T  S  S  K  S  K  K  Y  K  Q  L  V  T  A  100
 362 ctccagaggaatgccacctcattcaagggcaagtttgcgtttctgaagacgtacaactacaccctgggtgcggacgacctcactccctttt 451
 101  L  Q  R  N  A  T  S  F  K  G  K  F  A  F  L  K  T  Y  N  Y  T  L  G  A  D  D  L  T  P  F  130
 452 ggggagcagcagatggtgaactcgggaatcaagttctaccagaagtacaaggctcttgcgcggagcgtggtgccgtttattcgctcctcg 541
 131  G  E  Q  Q  M  V  N  S  G  I  K  F  Y  Q  K  Y  K  A  L  A  R  S  V  V  P  F  I  R  S  S  160
 542 ggctcggaccgcgtcattgcttctggggagaagttcatcgaggggttccagcaggcgaagctggcggattctggcgccacgaaccgcgcc 631
 161  G  S  D  R  V  I  A  S  G  E  K  F  I  E  G  F  Q  Q  A  K  L  A  D  S  G  A  T  N  R  A  190
 632 gctccggtgatcagtgtgattatcccggagagcgagacgttcaacaacacgctggaccacagtgtgtgcacgaactttgaggcgagcgaa 721
 191  A  P  V  I  S  V  I  I  P  E  S  E  T  F  N  N  T  L  D  H  S  V  C  T  N  F  E  A  S  E  220
 722 ttgggggatgaggttgcggctaacttcacggcgctctttgcacctgcgatccgtgctcgcgccgagaagcatctccctggtgtgaagctg 811
 221  L  G  D  E  V  A  A  N  F  T  A  L  F  A  P  A  I  R  A  R  A  E  K  H  L  P  G  V  K  L  250
 812 acagacgatgacgtcgtcagcctgatggacatgtgctcgttcgatactgtagcacgcaccagcgacgcgagccagctgtcgcccttctgt 901
 251  T  D  D  D  V  V  S  L  M  D  M  C  S  F  D  T  V  A  R  T  S  D  A  S  Q  L  S  P  F  C  280
 902 gcactcttcactcacaacgagtggaagaagtacgactacctgcagtccctgggcaagtactacggctacggcgcaggcaacgctctggga 991
 281  A  L  F  T  H  N  E  W  K  K  Y  D  Y  L  Q  S  L  G  K  Y  Y  G  Y  G  A  G  N  A  L  G  310
 992 ccggctcagggcataggattcaccaacgagctgattgcccggttgacccgttcgccagtgcaggaccacacgagcaccaactcgactcta 1081
 311  P  A  Q  G  I  G  F  T  N  E  L  I  A  R  L  T  R  S  P  V  Q  D  H  T  S  T  N  S  T  L  340
1082 gactccaacccggccaccttcccgttgaacgccaccatttacgttgatttttcgcacgacaacggcatgattcccatcttctttgccatg 1171
 341  D  S  N  P  A  T  F  P  L  N  A  T  I  Y  V  D  F  S  H  D  N  G  M  I  P  I  F  F  A  M  370
1172 ggcctgtacaatggcaccgagcccctgtcgctgacctcggtggaaagcacaaaggactcggacgggtattcggcgtcctgggcggtgcct 1261
 371  G  L  Y  N  G  T  E  P  L  S  L  T  S  V  E  S  T  K  D  S  D  G  Y  S  A  S  W  A  V  P  400
1262 tttgccgcgcgagcctacttcgagacgatgcaatgcaagtcggaaaaggagcctcttgtccgcgctttgatcaatgaccgggttgtgcca 1351
 401  F  A  A  R  A  Y  F  E  T  M  Q  C  K  S  E  K  E  P  L  V  R  A  L  I  N  D  R  V  V  P  430
1352 ctgcatggctgcgctgtggacaagctggggagatgcaagctgaatgactttgtcgagggattgagttgggccaggtctggaggcaactgg 1441
 431  L  H  G  C  A  V  D  K  L  G  R  C  K  L  N  D  F  V  E  G  L  S  W  A  R  S  G  G  N  W  460
1442 ggagagtgctttagttga                                                                        1459
 461  G  E  C  F  S  *                                                                          465
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Fig. 1. Nucleotide sequence and deduced amino acid sequence of *Aspergillus fumigatus* WY-2 phytase gene. The intron sequence is dot-underlined. Asterisk (*) means a termination codon.

very stable without obvious loss of activity after 7-day storage at 4°C (data not shown).

Purification of the Recombinant Phytase. The recombinant enzyme was isolated from the culture medium by ammonium sulfate fractionation and purified by anion exchange chromatography. After purification, the enzyme was found to show a specific activity of 51 U/mg, which is much higher than 20 U/mg for *Bacillus* sp. DS11 and 26 U for *A. fumigatus* ATCC34625 phytases, respectively [4, 20]. The purified recombinant phytase displayed a single protein band on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and its molecular mass was approximately 88 kDa (Fig. 2). The value is similar to the size described by Peng et al. and Ullah et al. [12, 17], but slightly bigger than the data reported by Rodriguez et al. [14]. This could be caused by the different glycosylation levels. Wyss et al. previously reported that glycosylation was a highly variable process, which not only depended on the expressed system used, but also varied among different batches produced in the same express system [21].

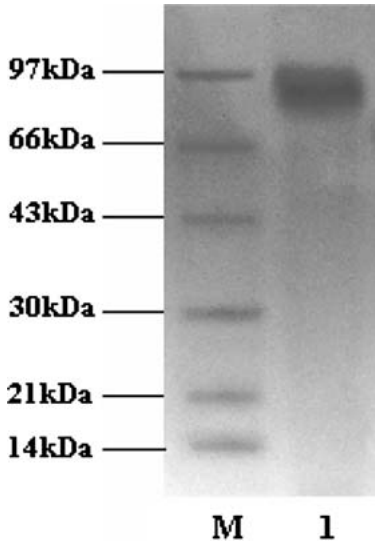

Fig. 2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the purified recombinant phytase. Lane M: protein marker; Lane 1: the purified recombinant phytase (MW = 88 kDa).

Effect of pH and Temperature on Phytase Activity and Stability. The recombinant phytase was found to show high enzyme activity in a pH range of 4–7 and display the maximum activity at pH 5.5 (Fig. 3A). In contrast to the phytase from *A. fumigatus* ATCC34625 [11, 14, 20], the *A. fumigatus* WY-2 phytase displayed a sharp decline in the activity as the pH moved toward the

neutral range, and maintained negligible activity beyond pH 7.0. The difference might have resulted from the changes in primary sequence. The theoretical pI of *A. fumigatus* WY-2 phytase was 5.95, whereas the pI value for *A. fumigatus* ATCC34625 phytase was 7.04. Nevertheless, the recombinant enzyme was very stable at the low pH, losing no activity when incubated at pH 2.0 for 4 h (data not shown). The optimum temperature of the enzyme was found to be at 55°C (Fig. 3B), but there was no significant difference between 55°C and 60°C, which is in close agreement with studies performed by several other researchers [11, 14]. It also showed a high ability to resist heat inactivation (Fig. 3C). After being exposed to 90°C for 15 min, the recombinant enzyme in crude and purified form retained 42% and 43.7% of the initial enzyme activity, respectively. Wyss previously reported that *A. fumigatus* phytase in crude form showed a low thermal stability because of protease attack at a high temperature [22]. In our study, the recombinant was found to produce very low protease activity with the optimized medium A/B for fermentation (data not shown).

Kinetic Parameters, Substrate Specificities, and Effect of Metal Ion on Phytase. In order to investigate the kinetic parameters of the recombinant phytase, the initial rate of enzyme reaction was measured under different sodium phytate concentrations. The K_m, K_{cat}, and K_{cat}/K_m were determined to be 114 μM, 102 s^{-1}, and 8.9×10^5 $M^{-1}s^{-1}$, respectively. These data generally fall in the range previously reported from microbial phytases [18]. Substrate specificity studies illustrated that the enzyme could dephosphorylate a range of phosphorylated substrates, and sodium phytate was the most specific substrate (Fig. 4A). This catalytic characteristic is quietly different from that of *A. fumigatus* ATCC34625 phytase, which displays a much higher phosphatase activity with *ρ*-nitrophenyl phosphate as substrate than phytase activity with sodium phytate as substrate [20]. Analysis of the effects of metal ions on activity of the recombinant phytase revealed that K^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, and Co^{2+} had a slight stimulatory effect, whereas Zn^{2+}, Cu^{2+}, Fe^{3+}, and Al^{3+} had an inhibitive effect (Fig. 4B).

Effect of Proteolysis on Phytase Activity. The recombinant phytase showed a strong resistance to pepsin (Fig. 5). After 2 h of pepsin digestion, the phytase activity almost remained unchanged at the ratio (pepsin/phytase) of 0.1. In contrast to strong resistance to pepsin, the enzyme was very sensitive to trypsin. The residual phytase activity was only 32.6% of the initial activity at the ratio (trypsin/phytase) of 0.005 after 2 h of trypsin digestion. These characterizations are similar to those of *A. fumigatus* ATCC34625 phytase [14]. Susceptibility of feed enzymes to proteolytic degradation is crucial because the rate and site of

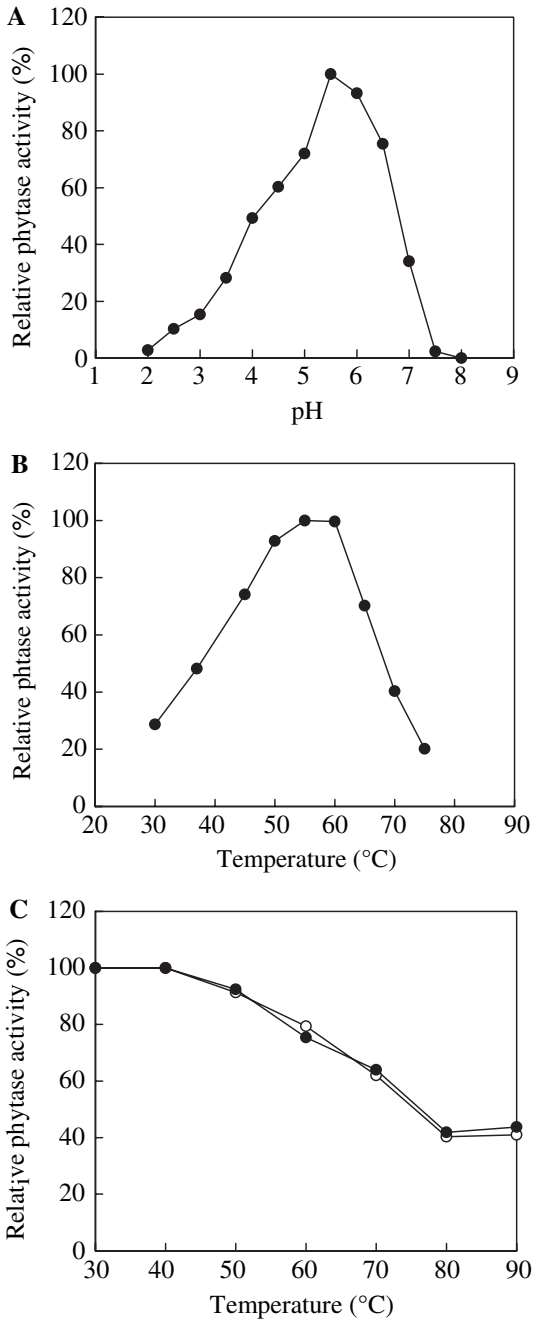

Fig. 3. Effect of pH and temperature on phytase activity. **(A)** pH versus activity profile of the recombinant phytase at 37°C. Buffers: 200 mM glycine–HCl [pH 2.0–3.5]; 200 mM NaAc–HAc [pH 4.0–5.5]; 200 mM imidazole–HCl [pH 6.0–6.5]; 200 mM Tris–HCl [pH 7.0–8.0]. **(B)** Temperature versus activity profile of the recombinant phytase at pH 5.5. **(C)** Thermal stability of the recombinant phytase in purified form (•) and crude form (○) after exposure for 15 min to the indicated temperature in 200 mM sodium acetate buffer at pH 5.5.

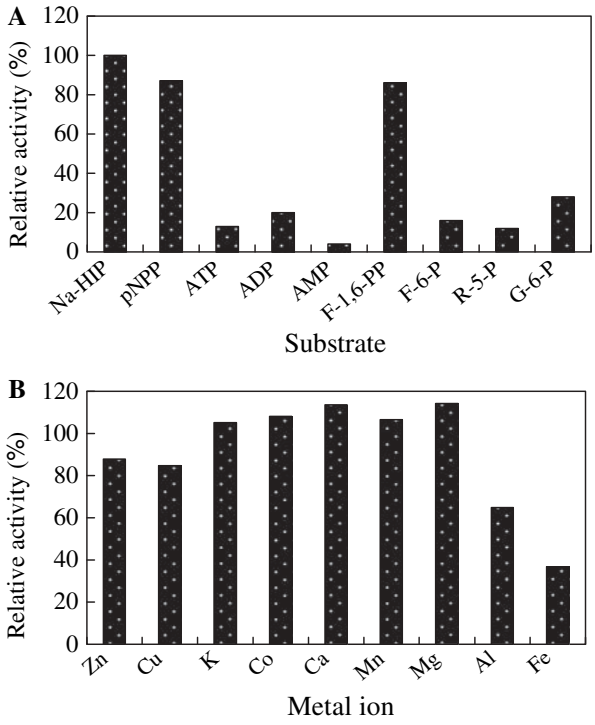

Fig. 4. Substrate specificity and effect of metal ion on phytase activity. **(A)** Relative activity of the recombinant phytase on various substrates. **(B)** Effects of various metal ions.

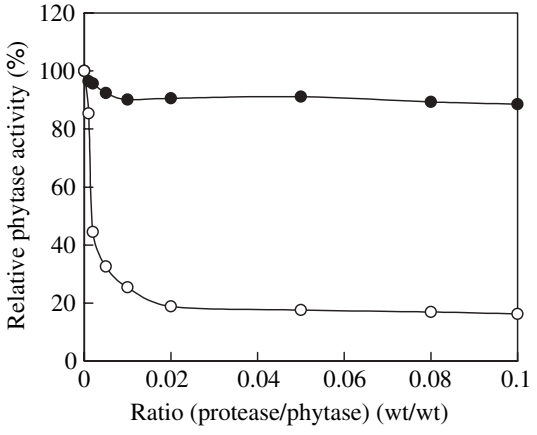

Fig. 5. Residual phytase activity of the recombinant phytase after being incubated with pepsin (●) and trypsin (○) at different protease/phytase (wt/wt) ratios.

inactivation of an enzyme are determined by this property. Because the pH range of the fungal phytases is narrow and falls in the pH range present in the stomach or crop, the inactivation rate at acidic pH by pepsin is an important limiting factor for their effectiveness [15]. From this applied perspective, strong resistance to pepsin gives this present enzyme an appealing property as an effective feed additive.

Conclusion

A novel phytase gene was isolated from *A. fumigatus* WY-2 and expressed in *P. pastoris* GS115. Compared with the reported *A. fumigatus* phytase, the enzyme in the present study is novel with respect to its molecular characterization, specific activity, pH-activity profile, and substrate specificity. It also displays a high thermostability and a strong resistance to pepsin. These properties give it a potential application in animal nutrition. The high identity in amino acid sequence between the two *A. fumigatus* phytase genes would be helpful in the investigation of the structure, evolution, and reaction mechanisms of the enzyme. This gene is also useful in computing the modifications of the enzyme for improving its properties by using the protein-engineering method.

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