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A Thermostable Phytase from *Neosartorya spinosa* BCC 41923 and Its Expression in *Pichia pastoris*

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A phytase gene was cloned from *Neosartorya spinosa* BCC 41923. The gene was 1,455 bp in size, and the mature protein contained a polypeptide of 439 amino acids. The deduced amino acid sequence contains the consensus motif (RHGXRXP) which is conserved among phytases and acid phosphatases. Five possible disulfide bonds and seven potential *N*-glycosylation sites have been predicted. The gene was expressed in *Pichia pastoris* KM71 as an extracellular enzyme. The purified enzyme had specific activity of 30.95 U/mg at 37°C and 38.62 U/mg at 42°C. Molecular weight of the deglycosylated recombinant phytase, determined by SDS-PAGE, was approximately 52 kDa. The optimum pH and temperature for activity were pH 5.5 and 50°C. The residual phytase activity remained over 80% of initial activity after the enzyme was stored in pH 3.0 to 7.0 for 1 h, and at 60% of initial activity after heating at 90°C for 20 min. The enzyme exhibited broad substrate specificity, with phytic acid as the most preferred substrate. Its K_m and V_{max} for sodium phytate were 1.39 mM and 434.78 U/mg, respectively. The enzyme was highly resistant to most metal ions tested, including Fe^{2+}, Fe^{3+}, and Al^{3+}. When incubated with pepsin at a pepsin/phytase ratio of 0.02 (U/U) at 37°C for 2 h, 92% of its initial activity was retained. However, the enzyme was very sensitive to trypsin, as 5% of its initial activity was recovered after treating with trypsin at a trypsin/phytase ratio of 0.01 (U/U).

***Keywords*:** phytase, *Neosartorya spinosa*, *Pichia pastoris*, gene cloning, enzyme characterization

Phytic acid or phytate (*myo*-inositol-1,2,3,4,5,6-hexakisphosphate; IP6) is the major form of phosphorus storage, representing 60-90% of the total phosphorus in cereal grains and legumes (Reddy *et al.*, 1982). Cereal grains and soybeans are the major ingredients of animal feed. Phytate in the feed is not utilizable as a source of phosphorus for monogastric animals (e.g., pigs and poultry) since their digestive tracts lack sufficient phytase activity. Commercial animal feeds thus require expensive inorganic phosphorus supplementation. Moreover, undigested phytate in the feed can interfere with absorption of divalent cations in the gut and pollute the environment when excreted. These problems can be overcome if animal feed is supplemented with phytase (Oh *et al*., 2004).

Phytases (*myo*-inositol hexakisphosphate 3-phosphohydrolases; EC 3.1.2.8 and EC 3.1.2.26) belong to the family of histidine acid phosphatases which catalyze phytic acid hydrolysis in a stepwise manner to lower inositol and inorganic phosphate (Pasamontes *et al.*, 1997). The enzyme is produced by a wide range of organisms including plants, animals, and more particularly microorganisms (Nakamura, 2000; Pandey *et al.*, 2001). Phytase is considered to be one of the most important sources of phosphorus for monogastric animals because it significantly releases phosphorus from phytate and lowers the anti-nutritional effect of phytate (Graf, 1983; Lei *et al*., 1993). In addition, phytase is used to improve the bioavailability of iron and zinc in foods for certain human population groups (Andlid *et al*., 2004).

To be useful as a feed supplement for poultry and swine industries, the enzyme should: (i) possess certain properties, including being highly active at the digestive tract pH which ranges from 2 to 6.5; and (ii) be resistant to acid, heat, and gut proteases. The requirement for acid resistance is obvious for maintaining its activity in the animal gut. Heat resistance is required if the enzyme is to be added before pelletization; that is, it should be able to withstand high temperatures (60-95°C) for at least 30 sec during the pelleting process. The best known heat-stable fungal phytases are: *A. fumigatus* ATCC 34625 phytase, which showed 90% of its initial activity remaining after being heated for 20 min at 100°C (Pasamontes *et al*., 1997); and *A. fumigatus* WY-2 phytase, which showed 43.7% of its initial activity after being exposed to 90°C for 15 min (Wang *et al*., 2007). Regarding protease resistance, *Aspergillus* phytases were generally more resistant to trypsin but less resistant to pepsin and pancreatin than those of *E. coli* (Rodriguez *et al*., 1999). Thus, characteristics of phytases must be taken into consideration when being applied in animal diets.

The primary nucleotide sequences of several fungal phytases suggest that they have evolved from histidine acid phosphatases containing the RHGXRXP sequence motif (Mitchell *et al.*, 1997). *A. fumigatus* WY-2 phytase (Wang *et al*., 2007), *A. fumigatus* ATCC 34625 phytase (Pasamontes *et al*., 2007) and *A. niger* NRRL 3135 phytase (Ullah *et al*., 2000) contain

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the RHGARYP sequence motif. On the other hand, *Debaryomyces castellii* contains the RHGERYP motif (Ragon *et al*., 2008), and a bacterium, *Klebsiella pneumoniae* subsp. *pneumoniae* XY-5, contains RHGIRYP (Wang *et al.*, 2004). In terms of deduced amino acid identities, phytase of *A. fumigatus* WY-2 showed 91% and 66% in comparison to those of *A. fumigatus* ATCC 34625 (GenBank accession no. U59804) and *A. ficuum* NRRL 3135 (GenBank accession no. M94550), respectively.

The phytase enzymes of *A. ficuum* NRRL 3135 and *A. fumigatus* ATCC 34625 show bi-hump pH optima: the former at pH 2.5 and 5.5, and the latter at pH 4.0 and 6.0-6.5 (Ullah and Gibson, 1987; Pasamontes *et al*., 1997). In contrast, *A. fumigatus* WY-2 phytase shows only one pH optimum at 5.5 (Wang *et al*., 2007). In this study, the *phyN* gene from *N. spinosa* BCC 41923 was cloned and expressed in *P. pastoris*, and the recombinant phytase produced was characterized.

The aim of this study was to search for fungi-producing phytases with better properties (e.g., active at physiological pH and temperature in chicken and swine guts; pH stability; resistance to proteases and metal ions; and thermostability) for use in animal feed. Even though a number of phytases are on the market, none has yet met the ideal properties; therefore, the search for phytases with better properties is continuing. In addition, the advantage of choosing fungi as the source of phytases is that fungi can be used in solid fermentation as well as liquid fermentation. Under certain conditions, such as those found in some developing countries, solid fermentation may be preferred.

Materials and Methods

Strains, plasmid and growth conditions

Neosartorya spinosa BCC 41923 was isolated from soil near a hot spring in Thailand, and was used as a phytase gene donor strain. The organism was generally cultured in potato dextrose agar (PDA) at 30°C or potato dextrose broth (PDB) at 30°C with 200 rpm shaking. *Pichia pastoris* KM71 (Invitrogen, USA) was used as a gene expression host; it was ordinarily cultured in yeast extract-peptone-dextrose (YPD) medium or buffered glycerol-complex medium (BMGY: 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base w/o amino acid, 4×10^{-5}% biotin and 1% glycerol) at 30°C with 200 rpm shaking. Growth during induction conditions (i.e., for continuous expression) was done in buffered methanol-complex medium+1% sorbitol (BMMY: 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base w/o amino acid, 4×10^{-5}% biotin and 0.5% methanol+1% sorbitol) at 30°C with 200 rpm shaking. pPIC9K plasmid (Invitrogen) was used as the vector for gene expression.

Genomic DNA isolation and purification

Genomic DNA of *N. spinosa* BCC 41923 was extracted using the cetyl trimethyl ammonium bromide (CTAB) method, with some modifications (Möller *et al.*, 1992). One mycelial plug of the fungus previously grown on PDA at 30°C for 3-4 days was transferred into 20 ml of PDB; the culture was then grown under a shaking condition (200 rpm) at 30°C for 2 days. The mycelium mass was harvested by centrifugation, washed with 20 ml distilled water followed by 20 ml Tris-EDTA (pH 8.0) containing 2% SDS (TES), and then used for isolation of genomic DNA. Five hundred microliter of TES buffer was added in a 2 ml microcentrifuge tube to approximately 0.5 g of wet cell weight, which corresponded to approximately 0.04 g dry cell weight. The mycelium suspension was boiled for 3 min and then frozen in liquid nitrogen for 2 min. This step was repeated 3 times. After the suspension reached room temperature, 100 µl of 1 mg/ml proteinase K was added, and the mixture incubated at 37°C for 30 min. Four hundred microliter of 5 M NaCl (0.7 vol) and 300 µl of 10% CTAB (0.5 vol) were then added to the sample mixture, incubated at 65°C for 10 min, and centrifuged at 6,000×g for 10 min. The supernatant was transferred into a new 2 ml microcentrifuge tube and extracted with 0.5 vol of chloroform/isoamyl alcohol (24:1), followed by addition of 0.5 vol of phenol. After mixing by inverting for 30 min, the sample was centrifuged at 13,000×g for 20 min and the aqueous phase was recovered. Seven hundred microliter of the aqueous phase was transferred to a new 1.5 ml microcentrifuge. DNA was precipitated by addition of 0.6 vol of isopropanol, and kept at -20°C for 30 min. After centrifugation, the supernatant was discarded. The pellet was washed with 750 µl of ice-cold 70% ethanol and centrifuged for 5 min. This step was repeated twice. Ethanol was discarded and the pellet was air-dried for 30 min. The obtained DNA pellet was then dissolved in 20 µl TE buffer. Five microliter of RNase (1 mg/ml) was added, and the mixture was incubated at room temperature for 60 min. DNA was then subjected to ethanol precipitation as described above, and stored at -20°C for future use.

DNA amplification, gene cloning and sequencing

Genomic DNA of *N. spinosa* BCC 41923 used as the template was isolated from culture as previously described. Two primers (Fumi_F1 and Fumi_R1) were designed from consensus sequence of the published gene sequences of *A. fumigatus* phytases (GenBank accession no. U59804, XM_746871, AY745738, AJ419776) and were used in amplification of the phytase gene of *N. spinosa* BCC 41923. The forward primer Fumi_F1 and the reverse primer Fumi_R1 (Table 1) contained *Eco*RI and *Avr*II restriction sites (underlined), respectively. PCR product of approximately 1.5 kb was generated, cloned into the vector pPIC9K, and sequenced. The resulting nucleotide and deduced amino acid sequences were used to perform homology searches on the National Center for Biotechnology Information (NCBI) databases by a BLAST search.

Genome walking to obtain complete sequence of the phytase gene

To obtain a complete sequence of the *N. spinosa* phytase gene (*phyN* gene), the DNA walking approach using GemomeWalker™ Universal kit (Clontech, USA) was employed. The kit provided GenomeWalker™ Adaptor, Adaptor Primer 1 (AP1) and Nested Adaptor Primer 2

Table 1. Primers used in this study

Primer name	Primer sequence (5′→3′)
Fumi_F1	GGAATTCTCCAAGTCCTGCGATACGGTAGACCTC
Fumi_R1	TCGCCTAGGTCAACTAAAGCACTCTCCCCAGTTGC
AP1	GTAATACGACTCACTATAGGGC
AP2	ACTATAGGGCACGCGTGGT
pUp1	GGTATAGTTGTACGTCTTCAGAAAGGC
pUp2	ACTTGCCCTTGAACGAGGTAGCATTCC
pDow1	ATAGGATTCACCAACGAGTTGATTGCC
pDow2	TCTTCTTTGCCATGGGCCTGTACAATG
α-factor	TACTATTGCCAGCATTGCTGC
3′ *AOX*1	GCAAATGGCATTCTGACATCC

(AP2) (Table 1). The primers (i.e., pUp1, pUp2, pDow1, and pDow2) as shown in Table 1 were designed from known nucleotide sequences of the previous PCR product. pUp1 (outer primer) and pUp2 (nested primer) were designed from the upstream sequence, while pDow1 (outer primer) and pDow2 (nested primer) were designed from the downstream sequence. gDNA and *Taq* DNA Polymerase (New England Biolabs, USA) were used as the template and the amplifying enzyme, respectively. The primary PCR products were obtained using AP1/pUp1 or AP1/pDow1 in the amplifications, while the secondary PCR products (nested PCR) were obtained using AP2/pUp2 or AP2/pDow2. A second GemomeWalker™ cycle was performed to obtain the entire sequence of the gene. The final PCR products were extracted from the gel and purified using a GFX™ PCR DNA and Gel Band Purification kit (GE Healthcare, UK). The resulting PCR products were submitted for nucleotide sequencing. With this approach, the entire sequence of the *phyN* gene was obtained.

Plasmid construction and transformation

The *phyN* gene was amplified by PCR and inserted in fusion with α-factor secretion signal into the pPIC9K expression vector. At first, the *phyN* gene was amplified by PCR using *Pfu* DNA polymerase (New England Biolabs). Fumi_F1 and Fumi_R1 were used as the primers. The PCR product was subcloned into the expression vector pPIC9K at *Eco*RI and *Avr*II sites, and the plasmid was used to transform *E. coli* DH5α. The plasmid containing the *phyN* gene prepared from *E. coli* DH5α was linearized by restriction enzyme *Pme*I and transformed into *P. pastoris* KM71 by electroporation (Sambrook and Russell, 2001). The transformed cells were plated on minimal dextrose medium agar plates (MD: 1.34% yeast nitrogen base w/o amino acid, 4×10^{-5}% biotin, 2% dextrose, 1.5% agar) to screen for His^+ transformants. The plates were then incubated at 30°C until colonies appeared. Selected transformants were tested for the presence of integrated plasmid by PCR amplification with α-factor primer and 3′ *AOX*1 primer (Invitrogen) (Table 1), and for their abilities to produce extracellular phytase by growing in the induction media BMMY+1% sorbitol for 72 h.

Expression of recombinant phytase in *P. pastoris* KM71

Expression of recombinant phytase in *P. pastoris* was carried out according to the manufacturer's instructions (Invitrogen). A single colony of the recombinant *P. pastoris* was cultured in 3 ml YPD broth. The culture was inoculated in 100 ml of BMGY in a 1 L baffled flask. The yeast was grown at 30°C with 200 rpm shaking until the culture reached an OD_{600} of 3. The cells were harvested by centrifugation at 3,000×g for 10 min at 4°C. The cell pellet was resuspended in 100 ml of BMMY in a 500 ml baffled flask to induce expression. Resuspended cells were returned to the shaking incubator to continue growth and expression. At every 12 h of incubation, 100% methanol was added to obtain a 0.5% final concentration in order to maintain the induction for continuous expression. After 72 h of incubation and continuous induction, the culture was centrifuged at 5,000×g for 20 min at 4°C. The supernatant was collected and analyzed for proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Removal of the background phosphate and concentration of the enzyme by ultrafiltration

Removal of the background phosphate and concentration of the crude enzyme were done simultaneously using Amicon® Ultra centrifugal filter devices (Millipore, USA) containing a membrane with molecular weight cutoff of 30 kDa. The phosphate-free concentrated enzyme was then used for phytase activity assay.

Phytase assay

Phytase assay was performed as described in Engelen *et al*. (1994), with some modifications. The reaction mixture contained 0.1 ml of enzyme sample (in 0.2 M sodium acetate buffer, pH 5.5) and 0.2 ml of substrate solution (pH 5.5) at a final concentration of 5 mM. The reaction was carried out at 42°C for 30 min and stopped by adding 0.2 ml of freshly prepared color reagent. Fifteen minutes after adding the reagent, the color developed was determined at 415 nm using a spectrophotometer (Unicam Heλios Alpha, UK). The control was prepared as that of the enzyme reaction mixture, with the color reagent added immediately to stop the reaction without incubation. One unit of phytase activity was defined as the amount of enzyme able to hydrolyze phytate and give 1 μmol/min of inorganic phosphate (P_i) under the assay conditions.

Protein concentration assay

The protein content was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as the standard.

Deglycosylation analysis with SDS-PAGE

The purified recombinant phytase (2.85 μg) was deglycosylated by mixing with 1,000 U of endoglycosidase H_f (Endo H_f) for 2 h at 37°C, according to the manufacturer's instructions (New England Biolabs, USA). Both the deglycosylated and the untreated recombinant phytase were analyzed by SDS-PAGE (with a 5% stacking gel and a 12% separation gel) for the differences in sizes of the proteins before and after the enzyme treatment.

Effects of pH on enzyme activity and stability

Enzyme activity was determined at different pH values, ranging from 2.0 to 11.0, in various buffers: 0.2 M glycine-HCl for pH 2.0-3.5; 0.2 M sodium acetate for pH 3.5-7.0; 0.2 M Tris-HCl for pH 7.0-9.0; and 0.2 M glycine-NaOH for pH 9.0-11.0. The optimum pH was determined from the curve of relative enzyme activity versus pH. The pH stability of the purified enzyme was determined by assaying residual activity after incubating the enzyme at the respective pH values for 1 h at 25°C.

Effects of temperature on enzyme activity and stability

Enzyme activity was determined at different temperatures, ranging from 30 to 70°C, at pH 5.5. The optimum temperature was determined from the curve of relative enzyme activity versus temperature. Thermostability of the recombinant phytase was determined by assaying residual activity after incubating the enzyme in 0.2 M acetate buffer (pH 5.5) for 20 min at different temperatures ranging from 20 to 90°C.

The Michaelis-Menten parameters, K_m and V_{max}

K_m and V_{max} were determined by incubating the phytase enzyme (13 mU at pH 5.5) with various concentrations of sodium phytate at 37°C in 0.2 M sodium acetate buffer (pH 5.5) for 10 min. The phytate concentrations were 0.02, 0.04, 0.06, 0.08, 0.10, 0.12, 0.16, 0.20, and 0.22 mM. Data obtained were analyzed by creating: (i) a plot of initial velocities versus substrate concentrations (V_0 versus [S]); and (ii) a reciprocal plot of V_0 and [S] to make a Lineweaver-Burk plot for calculation of K_m and V_{max}.

Effects of pepsin and trypsin on phytase activity

The purified phytase was incubated with different amounts of pepsin and trypsin, following the manufacturer's instructions (Sigma, USA). A stock solution of each protease was prepared by dissolving 1 mg of pepsin (2,850 units/mg protein) in 1 ml of 10 mM HCl, pH 2.0, and 1 mg of trypsin (14,300 *N*-benzoyl-L-arginine ethyl ester (BAEE) units/mg protein) in 1 ml of 80 mM ammonium bicarbonate, pH 7.5. Pepsin and trypsin activities of the stock solutions were determined against hemoglobin and BAEE, respectively, according to the modified method of Worthington (1982). One unit of pepsin was defined as 0.001 absorbance change at 280 nm/min at 37°C under the specified conditions. One unit of trypsin was defined as a 0.001 absorbance change at 253 nm per min at pH 7.6 and 25°C, with BAEE as the substrate.

To determine the resistance to proteolytic activity of the phytase enzyme, the purified phytase (0.1 U/ml) was incubated at 37°C for 2 h with pepsin or trypsin at protease/phytase (U/U) ratios ranging from 0.001 to 0.2 in a final volume of 500 µl. Then the reaction

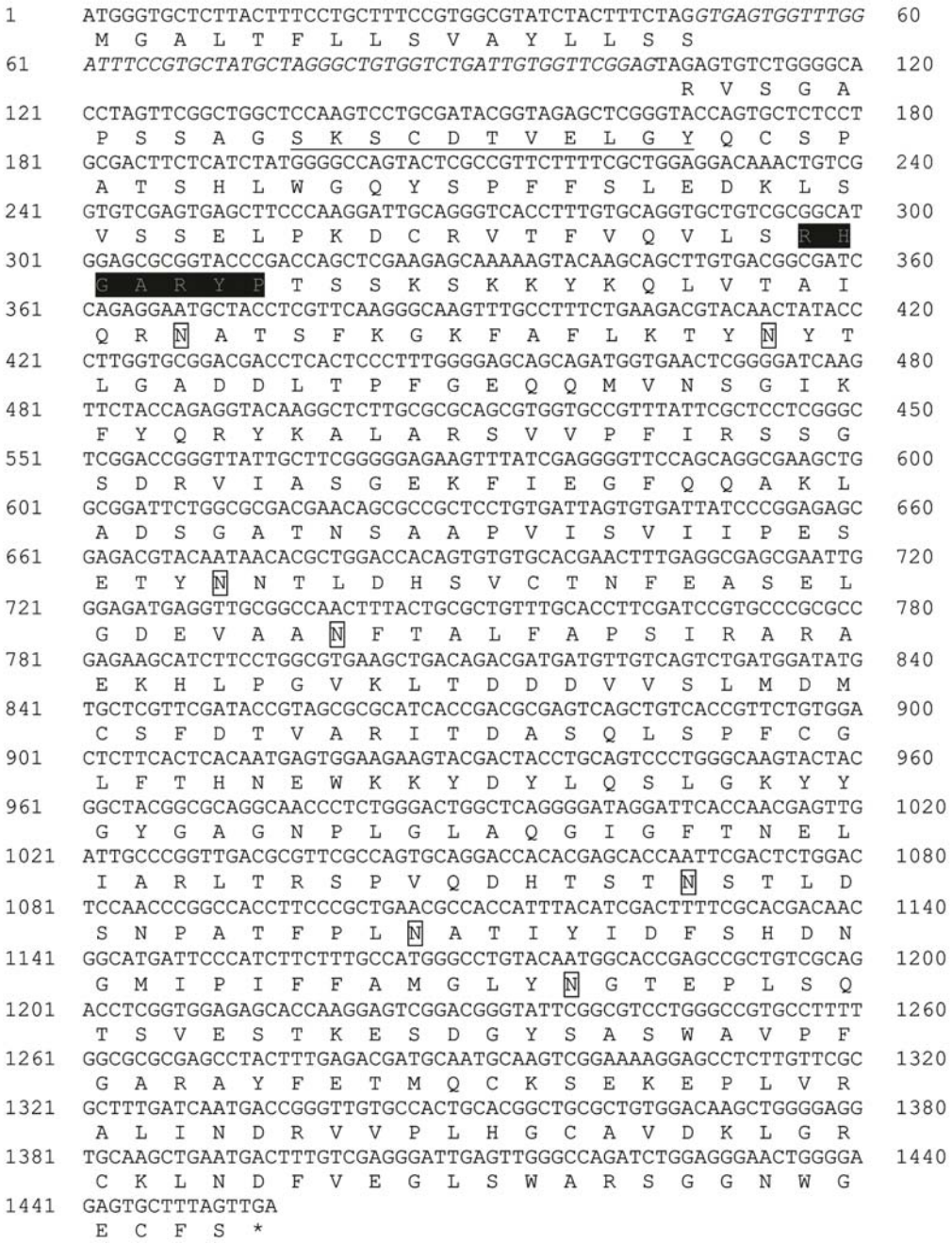

Fig. 1. Complete nucleotide and deduced amino acid sequence of *N. spinosa* phytase. The underlined amino acids are the beginning of the predicted mature protein. The conserved sequence is shown in a gray box. Potential *N*-glycosylation sites are boxed. The intron is indicated by italic letters. The proposed intron donor and acceptor sites are in accordance with the GT-AG rule of Breathnach *et al.* (1978).

mixture was immediately used as the enzyme source for phytase activity assay.

Substrate specificity

Substrate specificity was examined by measuring the phytase activity with different phosphate substrates: sodium phytate, *p*-nitrophenyl phosphate (*p*NPP), ATP, β-glycerophosphate, DL-α- glycerophosphate, fructose 1,6-bisphosphate, 1-naphthyl phosphate, and glucose 6-phosphate. Enzyme activity was determined after incubating the enzyme with 5 mM of each substrate in 0.2 M sodium acetate buffer (pH 5.5) at 42°C for 30 min.

Effects of metal ions on phytase activity

Effects of metal ions on phytase activity were determined by pre-incubation of the enzyme in 0.2 M sodium acetate buffer (pH 5.5) containing 1 mM metal ion (either Co^{2+}, Mg^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, K^{+}, Ca^{2+}, Zn^{2+}, Mn^{2+} or Al^{3+}) for 15 min at 30°C before performing the enzymatic activity assay.

Results and Discussion

Cloning and sequence analysis

The phytase gene from *N. spinosa* BCC 41923 was amplified by PCR, yielding a 1,234 bp DNA fragment which was found to be an internal sequence of the gene. To obtain the whole phytase gene, GenomeWalker™ was used to determine the upstream and downstream portions of the gene. The upstream fragment was obtained from *Stu*I libraries and the downstream fragment was obtained from *Eco*RV libraries in the second PCR cycle. As such, a 2,443 bp fragment was reconstituted. Using the Gene Runner 3.05 program to determine an open reading frame (ORF) in the obtained sequence, the ORF of the phytase gene was found to contain a 1,455 bp. The phytase gene was composed of two exons (1-47 bp and 105-1,455 bp), separated by an intron (57 bp), which encoded a polypeptide of 439 amino acids with a calculated molecular mass of 48.2 kDa (Fig. 1). The corresponding amino acid sequence contained the RHGARYP motif, which matched the consensus RHGXRXP sequence found in the active sites of most high-molecular-weight acid phosphatases (Ullah *et al.*, 1991). The sequence also showed the C-terminal HD motif that was found to be well conserved in a wide variety of phytase sequences. The nucleotide and protein sequences were compared with the sequences of previously isolated phytases by the nucleotide blast (BLASTN) and protein blast (BLASTP) programs, respectively. The *phyN* gene and the mature *N. spinosa* phytase showed 91-96% identity with both nucleotide and amino acid sequences of phytases of various *A. fumigatus*, including those that were used to design the cloning primers, and *N. fischeri* NRRL 181. Sequence analysis using DiANNA software showed that 10 cysteine residues could theoretically form 5 disulfide bonds in *N. spinosa* phytase. This is similar to what was reported with phytase of *A. niger* N-J (Zhao *et al*., 2007). On the other hand, 7 potential *N*-glycosylation sites of *N. spinosa* phytase were predicted by the NetNGlyc 1.0 server, while 11 potential *N*-glycosylation sites were predicted for *A. niger* N-J phytase (Zhao *et al*., 2007).

Expression of phytase in *P. pastoris*

After 72 h of induction by methanol in BMMY+1% sorbitol, the crude enzyme was concentrated by tenfold with an Amicon centrifugal filter device containing a membrane with a molecular weight cutoff of 30 kDa. It was found to be free from phosphate, as determined by the phosphate determination of Engelen *et al*. (1994) (data not shown). The recombinant *P. pastoris* produced phytase at 141.06 U/ml (at 42°C) or 113.05 U/ml (at 37°C). The purified phytase showed specific activity of 38.62 U/mg (at 42°C) or 30.95 U/mg (at 37°C).

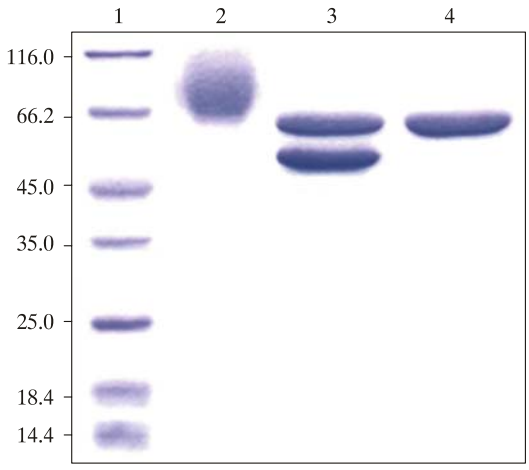

Fig. 2. SDS-PAGE analysis of recombinant phytase and deglycosylated phytase. Lanes: 1, protein standard; 2, glycosylated phytase; 3, deglycosylated phytase and Endo H_f; 4, Endo H_f.

Deglycosylation of recombinant phytase

The deglycosylated phytase, after being treated with Endo H_f, displayed a molecular weight of 52 kDa on SDS-PAGE (Fig. 2). This was slightly higher than the calculated value of the deduced amino acids (439 amino acids) which was 48.2 kDa. The difference may be explained by the incomplete removal of *N*-acetyl glucosamine or *O*-glycosyl residues by the endoglycosidase H.

The theoretical p*I* of *N. spinosa* phytase was 6.10, which was nearly equal to the 5.95 of *A. fumigatus* WY-2 phytase (Wang *et al*., 2007). However, the p*I* value for *A. fumigatus* ATCC34625 phytase was 7.04 (Pasamontes *et al*., 1997).

Effects of pH on enzyme activity and stability

Because phytase is intended for use as an animal feed supplement, its activity and stability will be affected by the pH, temperature, and the presence of metal ions and proteases in the animal's digestive tract. *N. spinosa* phytase had one optimum pH range of 5.5-6.0, similar to those reported for *A. oryzae* RIB40 *niaD*$^{-}$ (Uchida *et al*., 2006) and *A. fumigatus* WY-2 (Wang *et al*., 2007), but different from those of *A. fumigatus* (Pasamontes *et al*., 1997), *Mucor hiemalis* Wehmer (Boyce and Walsh, 2007) and *Penicillium simplicissimum* (Tseng *et al.*, 2000), whose optimum pH ranges are 6.0-6.5, 5.0-5.5, and 4.0, respectively (Fig. 3A). On the other hand, other fungal phytases showed two pH optima at 2.5 and 5.5: *A. ficuum* NRRL 3135, *A. niger* 307, *A. oryzae* AK9, and *A. niger* N-J (Ullah and Gibson, 1987; Gargova and Sariyska, 2003; Chantasartrasamee

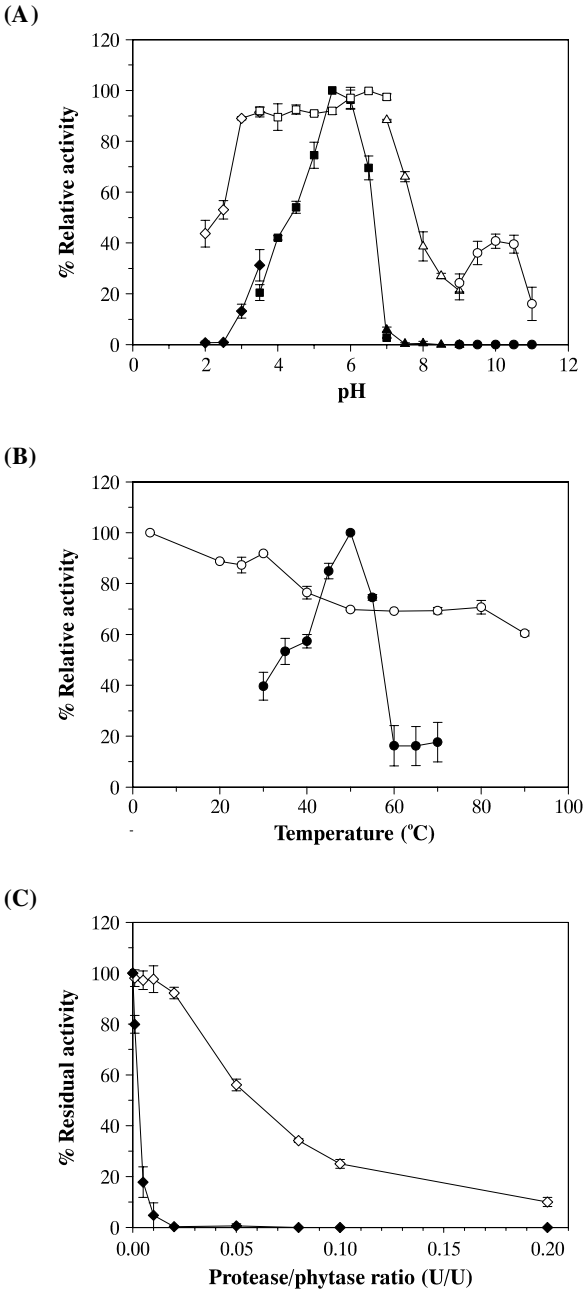

Fig. 3. Effects of pH, temperature and proteases on phytase activity. (A) Effect of pH on enzyme activity (closed symbol) and stability (open symbol). The recombinant enzyme activity was determined at 42°C and at various pHs ranging from 2-11. Buffers: pH 2.0-3.5, 0.2 M glycine-HCl (◆, ◇); pH 3.5-7.0, 0.2 M sodium acetate (■, □); pH 7.0-9.0, 0.2 M Tris-HCl (▲, △); and pH 9.0-11.0, 0.2 M glycine-NaOH (●, ○). For pH stability, the recombinant enzyme was kept for 1 h at 25°C in various pHs ranging from 2-11 before being assayed for activity. (B) Effect of temperature on enzyme activity (closed symbol) and stability (open symbol). The phytase activity was determined at pH 5.5 in 0.2 M acetate buffer at various temperatures ranging from 30-70°C. For thermostability, the recombinant enzyme was incubated at 20-90°C for 20 min in sodium acetate buffer (pH 5.5) before being assayed for activity. (C) Sensitivity of recombinant phytase to pepsin (◇) and trypsin (◆). Residual activity of the recombinant phytase enzyme was determined after being incubated with pepsin and trypsin at different protease/phytase ratios (U/U) for 2 h.

et al., 2005; Zhao *et al*., 2007). Furthermore *A. ficuum* NRRL 3135 phytase, which had two optimum pH values, contained the consensus sequence similar to that of *N. spinosa* and *A. fumigatus* phytases which had only one optimum pH. Therefore, even though the optimum pH for phytases of *N. spinosa*, *A. fumigatus* ATCC 34625 (GenBank accession no. U59804), and *A. ficuum* NRRL 3135 were different, their consensus motif sequences were the same.

At pH 7.0 or higher, PhyN phytase was completely inactivated, as was the case for most phytases in previous reports (Tseng *et al.*, 2000; Uchida *et al*., 2006; Wang *et al*., 2007). In acidic conditions, PhyN phytase was more stable. Its activity was reduced along with decreasing pH, and was completely inactivated at pH 2.5. In terms of pH stability, when the PhyN phytase was incubated at different pH for 1 h at 25°C, the enzyme was found to be quite stable at a pH range of 3.0-7.0, with over 80% of the maximum activity retained (Fig. 3A). This was a better performance than for most phytases reported, except for *P. simplicissimum* phytase which was shown to be highly stable in a pH range of 3.5-6.0 for 7 days (Tseng *et al.*, 2000).

Effects of temperature on enzyme activity and stability

Optimum temperature of PhyN phytase was 50°C, while those reported for some other fungi were slightly higher: 55°C for *P. simplicissimum* (Tseng *et al.*, 2000) and *A. fumigatus* WY-2 (Wang *et al*., 2007), and 60°C for another *A. fumigatus* (Rodriguez *et al*., 2000). The activity of PhyN phytase at 40°C was around 60% of the maximum activity (Fig. 3B). Thermostability is important since animal feed is usually produced by pelletization or extrusion, which exposes the feed to high heat. In terms of thermostability, PhyN phytase retained more than 60% residual activity after exposure to 90°C for 20 min (Fig. 3B). This was superior to the performance of several phytases in earlier reports, such as: (i) *A. fumigatus* WY-2, where only 43.7% of activity remained after 15 min at 90°C (Wang *et al*., 2007); (ii) *A. terreus* and *M. thermophila,* where 50% of activity remained after 20 min at 50-55°C (Pasamontes *et al.*, 1997; Wyss *et al*., 1998, 1999); and (iii) *A. niger* 113, where 25% of activity remained after 10 min at 80°C (Xiong *et al*., 2004). Hence, the PhyN phytase enzyme would be good for use with pelletization where high temperatures (60-95°C) are employed for 30-60 sec.

Kinetic parameters

The kinetic constants K_m and V_{max} of *N. spinosa* phytase were 1.39 mM and 434.78 U/mg (7,247.8 nKat/mg), respectively, as determined from the intercept values on a Lineweaver-Burk plot. The phytase activity was inhibited by the substrate, sodium phytate, at 0.25 mM. When compared to the K_m values of other recombinant phytases expressed in the same hosts (*P. pastoris*), it was larger than those of *A. fumigatus* phytase (30 μM) (Ullah *et al*., 2000), *A. fumigatus* WY-2 (114 μM) (Wang *et al*., 2007), *A. niger* N-J (0.196 mM) (Zhao *et al*., 2007) and *Penicillium oxalicum* PJ3 (0.37 mM) (Lee *et al.*, 2007). In contrast, the V_{max} value of *N. spinosa* phytase (434.78 U/mg or 7,247.8 nKat/mg) was, in general, larger than those of *A. fumigatus* phytase (435 nKat/mg), *A. ficuum* phytase (3,300 nKat/mg) (Ullah *et al.*, 2000), and *A. niger* N-J phytase (5,685 nKat/mg) (Zhao *et al*., 2007), but was slightly lower

Table 2. Substrate specificity of the recombinant phytase

Substrate (5 mM)	% Relative activity
Sodium phytate	100
*p*NPP	56
β-Glycerophosphate	47
DL-α-glycerophosphate	63
Glucose-6-phosphate	15
1-Naphthyl phosphate	97
Fructose 1,6-bisphosphate	47
ATP	14

Table 3. Effects of metal ions on phytase activity

Metal ions	% Residual activity
Control	100
Co^{2+} ($CoCl_2$)	97
K^{+} (KCl)	106
Ca^{2+} ($CaCl_2$)	106
Fe^{3+} ($FeCl_3$)	101
Fe^{2+} ($FeSO_4{\cdot}7H_2O$)	107
Mg^{2+} ($MgSO_4{\cdot}7H_2O$)	101
Cu^{2+} ($CuSO_4{\cdot}5H_2O$)	100
Zn^{2+} ($ZnSO{\cdot}7H_2O$)	90
Mn^{2+} ($MnSO_4{\cdot}H_2O$)	108
Al^{3+} ($Al_2(SO_4)_3$)	105

Phytase was assayed in 200 mM acetate buffer (pH 5.5) in the presence of metal ions at 1 mM concentration, as compared to the control with no metal ion.

than that of *Penicillium oxalicum* PJ3 phytase (526.3 U/mg) (Lee *et al.*, 2007).

Sensitivity of recombinant phytase to proteolytic enzymes

At a pepsin/phytase ratio of 0.001-0.02, more than 90% of the initial PhyN phytase activity was retained after 2 h of pepsin digestion. However, at a higher pepsin/phytase ratio (0.1) only 25% of the initial activity was retained (Fig. 3C). Its pepsin resistance was better than that of r-PhyA, which lost 58% and 77% of its original activity when mixed with pepsin at ratios of 0.005 and 0.01 w/w, respectively, for 2 h of incubation (Rodriguez *et al*., 1999), but lower than that of *A. fumigatus* WY-2 which was reported to resist pepsin at a pepsin/phytase ratio of 0.1 for 2 h of incubation (Rodriguez *et al*., 1999; Wang *et al*., 2007). In contrast, when trypsin was used at trypsin/phytase ratios of 0.001, 0.005, and 0.01 for 2 h, *N. spinosa* phytase activity retained 80%, 18%, and 5% of its initial activity, respectively (Fig. 3C), while r-PhyA was reported to retain more than 85% of its original activity when mixed with trypsin at a trypsin/phytase ratio of 0.001 (Rodriguez *et al*., 1999). But *A. fumigatus* WY-2 phytase was quite sensitive to trypsin, as only 32.6% of the initial activity remained after incubation with trypsin at a trypsin/phytase ratio of 0.005 (Wang *et al*., 2007). The proteolytic resistance is important for animals, since an effective phytase needs to resist hydrolytic breakdown by digestive proteinases in the digestive tract - and PhyN phytase seems to perform at a reasonable level.

Substrate specificity

The *N. spinosa* phytase enzyme exhibited broad substrate specificity, as shown in Table 2. Of the 8 substrates tested, phytic acid was the most preferred substrate, making it a true phytase. This finding was similar to that reported for *A. niger* 92 phytase (Dvoráková *et al.*, 1997) and *A. fumigatus* WY-2 phytase (Wang *et al*., 2007). On the other hand, *M. hiemalis* Wehmer phytases preferred *p*-nitrophenol phosphate and phenyl phosphate over sodium and calcium phytate by approximately a tenfold margin (Boyce and Walsh, 2007); while *Sporotrichum thermophile* displayed broad substrate specificity by hydrolyzing various organic phosphates (Singh and Satyanarayana, 2009).

Effects of metal ions on phytase activity

The results showed that all metals at the concentration tested showed no (or only slight) inhibitory or stimulatory effects (Table 3). This was quite different from the results reported for *A. fumigatus* WY-2 phytase, in that Zn^{2+}, Cu^{2+}, Fe^{3+}, and Al^{3+} showed strong inhibitory effects (Wang *et al*., 2007), and for phytases of *A. niger* 307 (Sariyska *et al*., 2005) in that Hg^{+}, Hg^{2+}, and Cu^{2+} showed strong inhibitory effects. Even though *N. spinosa* phytase had high amino acid sequence identity to *A. fumigatus* and *A. ficuum* phytases, some enzyme properties - such as substrate specificity and high resistance to various metal ions - were significantly different. Further investigations on the significance of individual or combinations of diverse amino acids by *in vitro* mutagenesis may explain their contribution to the differing properties, i.e. substrate specificity and high resistance to various metal ions. The knowledge gained may be useful for improving the properties of other enzymes.

Conclusion

A phytase gene was isolated from *N. spinosa* BCC 41923 and expressed in *P. pastoris* KM71. The enzyme was active at pH 3.0-7.0, with optimum activity at pH 5.5-6.0. It displayed high substrate specificity toward phytate, high thermostability, high resistance to acid and pepsin hydrolysis, high resistance to various metal ions, and was stable in acidic pH. The uniqueness of this phytase is: (i) it was resistant to all heavy metal ions tested, including the most unusual ones such as Fe^{2+}, Fe^{3+}, and Al^{3+}; and (ii) it was highly thermostable, i.e. it could withstand a temperature of 90°C for 20 min with 60% of its activity remaining - a result higher than those reported for most fungal phytases. These characteristics make it a good candidate for use as a supplement in animal feed.

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