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Isolation, Characterization, and Molecular Cloning of the cDNA Encoding a Novel Phytase from *Aspergillus niger* 113 and High Expression in *Pichia pastoris*

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Phytases catalyze the release of phosphate from phytic acid. Phytase-producing microorganisms were selected by culturing the soil extracts on agar plates containing phytic acid. Two hundred colonies that exhibited potential phytase activity were selected for further study. The colony showing the highest phytase activity was identified as *Aspergillus niger* and designated strain 113. The phytase gene from *A. niger* 113 (*phyI1*) was isolated, cloned, and characterized. The nucleotide and deduced amino acid sequence identity between *phyI1* and *phyA* from NRRL3135 were 90% and 98%, respectively. The identity between *phyI1* and *phyA* from SK-57 was 89% and 96%. A synthetic phytase gene, *phyI1s*, was synthesized by successive PCR and transformed into the yeast expression vector carrying a signal peptide that was designed and synthesized using *P. pastoris* biased codon. For the phytase expression and secretion, the construct was integrated into the genome of *P. pastoris* by homologous recombination. Over-expressing strains were selected and fermented. It was discovered that ~4.2 g phytase could be purified from one liter of culture fluid. The activity of the resulting phytase was 9.5 U/mg. Due to the heavy glycosylation, the expressed phytase varied in size (120, 95, 85, and 64 kDa), but could be deglycosylated to a homogeneous 64 kDa species. An enzymatic kinetics analysis showed that the phytase had two pH optima (pH 2.0 and pH 5.0) and an optimum temperature of 60ºC.

Keywords: Synthetic *phyI1* gene, Fermentation, Phytase production, *Pichia pastoris*, Recombinant DNA

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Introduction

Phytases are found naturally in plants and microorganisms, particularly fungi (Wodzinski *et al.*, 1996). As 3-phytases (EC 3.1.3.8) or 6-phytases (EC 3.1.3.26) (Ullah *et al.*, 1987), most phytases belong to the family of histidine phosphatases (Mitchell *et al.*, 1997). These enzymes catalyze the hydrolysis of phytic acid (*myo*-inositol hexakisphosphate) to mono-, di-, tri-, tetra-, and pentaphosphates of *myo*-inositol and inorganic phosphate. The salt form, phytate, is the major storage form of phosphorus and accounts for more than 80% of the total phosphorus in cereals and legumes. Monogastric animals have very low or no phytase activities in their digestive tracts, such as pigs, poultry, and fish. They are incapable of utilizing the phosphorus bound in phytate. Furthermore, phytate acts as an antinutrient by chelating divalent cations and preventing the uptake of minerals (Graf, 1983; Lei *et al.*, 1993). Inorganic phosphorus has to add to the feed in order to meet the phosphorus requirements of animals. However supplementation with inorganic phosphate imposes environmental problems and pollution. Thus, phytases are used as a cereal feed additive that enhances the phosphorus and mineral uptake in monogastric animals and reduces the level of phosphorus output in their manure. A number of phytase genes have been isolated from plants (Reddy, *et al.*, 1982; Gibson *et al*., 1988; Hegeman *et al.*, 2001), bacteria (Greiner *et al*., 1993; Kerovuo *et al.*, 1998; Rodriguez *et al.*, 1999), and fungi (Pasamontes *et al.*, 1997a, b; Berka *et al*., 1998).

P. pastoris is a kind of methylotrophic yeast. It can grow with methanol as the sole carbon and energy source (Cregg *et al.*, 1993). *P. pastoris* grows to a very high cell density in simple defined media, and an extremely high yield of intracellular protein using the methanol-controlled alcohol oxidase promoter (Waterham *et al.*, 1997). Using this system, many proteins have been produced with varying degrees of success (Sreekrishna *et al.*, 1997).

In this report, our objectives were to isolate a novel phytase

gene from soil. The phytase gene was then constructed into a high secretion expression vector of *P. pastoris*. We isolated two hundred strains, which exhibited the potential of producing active extracellular phytase from ten different soil samples. From those strains, we obtained a phytase gene named *phyI1*. In order to obtain a high expression in *P. pastoris* by using successive PCR (Yao *et al*., 1999; Peng *et al*., 2001; Roytrakul *et al*., 2001; Peng *et al*., 2002), we synthesized the mating factor α (α-factor) prepro-leader of S*accharomyces cerevisiae* (Kurjan *et al*., 1982; Brake *et al*., 1989; Romanos *et al*., 1992; Hollenberg *et al*., 1997) and the 1347 bp phytase gene *phyI1s*. To encourage over-expression, the synthesized gene and signal sequence included the optimum expression base pair for *P. pastoris* (Sharp *et al*., 1986; Zhao *et al*., 2000). Moreover, to optimize the translational efficiency of the heterologous proteins, the synthetic gene that included the 5'-UTR of the signal-mRNA was adjusted to be identical to that of AOX1-mRNA (Xiong *et al*., 2003).

Materials and Methods

Chemicals, enzymes, and strains Phytic acid, dodecasodium salt, phytic acid, and calcium salt were purchased from Sigma Chemical Co., Ltd (St. Louis, USA). The enzymes for the molecular biology (*Taq* DNA polymerase, T4 DNA ligase, and restriction endonucleases) were purchased from Promega (Madison, USA). The IPTG, RT-PCR kit, and T-vector were purchased from Takara Co., Ltd (Dalian, China). The DNA sequencing kit was purchased from the Applied Biosystems (Foster City, USA). The protein markers were purchased from Watson Bio-Tech Co., Ltd (Shanghai, China). The *P. pastoris* strain GS115 (his-4) was purchased from Invitrogen Corporation (San Diego, USA). Endoglycosidase H (Endo Hf) was purchased from New England Biolabs, Beverly, USA).

Isolated strains producing phytase from soil Ten different soil samples were obtained from the rice fields of the *Shanghai Academy of Agricultural Sciences* (Shanghai, China). These soil samples were dissolved with sterilized water and plated onto a selected plate (0.1% phytic acid calcium salt, 0.3% glucose, 0.5% NH_4NO_3, 0.05% KCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.03% $MnSO_4 \cdot 4H_2O$, 0.03% $FeSO_4 \cdot 7H_2O$, 1.5% Agar, pH 5.5). It was incubated 3 d at 30°C. The strains that had a clear zone were selected.

Analysis of phytase activity The selected strains were picked using a sterile toothpick that was put into shake flasks containing a grown medium (1.5% glucose, 0.3% peptone, 0.2% yeast-extract, 0.2% NH_4SO_4, 0.05% KCl, 0.003% $MnSO_4 \cdot 4H_2O$, 0.003% $FeSO_4 \cdot 7H_2O$, pH 5.5), then incubated 3 d at 30°C in a shaking incubator (200 rpm). Then the phytase activities of the strains were analyzed. One phytase unit was defined as the activity that releases 1 μmol of inorganic phosphorus (*Pi*) from sodium phytate per minute at 37°C (Heeft, 1995). One strain, named 113, was selected because of its high activity. Through microbial taxonomy methods, the 112 strain was identified as *A. niger.*

Preparation of mRNA from *Aspergillus niger 113* *Aspergillus niger* 113 was grown in a 250-ml baffled flask containing a 50 ml medium. After 3 days, the extraction of RNA and the isolation of mRNA were performed using a poly(dT) column (Promega), according to the manufacturer's instructions.

Isolation of phytase cDNA by RT-PCR, cloning and sequencing the cDNA The cDNA was synthesized from mRNA by RT-PCR, according to the manufacturer's instructions. For the isolation of the phytase cDNA, the PCR primers were synthesized, based on the phytase genes that were reported previously (*Aspergillus niger*, GenBank accession no. AB022700, *Aspergillus terreus* 9A1, GenBank accession no. U59805, *Aspergillus fusigatus*, GenBank accession no. U59804, *Emericella nidulans*, GenBank accession no. U59803, *Myceliophthora thermophila*, GenBank accession no. U59806.). *Pfu* DNA ploymerase was used for amplification. The PCR product was ligated into a T-vector overnight at 4°C using T4 ligase. The ligation mixture was transformed into competent *E.coli* DH5α. The transformants were grown in Luria-Bertani plates that were supplemented with ampicillin (100 μg/mL) and X-Gal (50 μg/mL). The cultures were induced by the addition of 0.5 mM IPTG, and recombinant white colonies were selected. The clone that contained the PCR product was verified by restriction enzyme digestion, agarose gel electrophoresis, and sequencing.

Chemical synthesis of the phytase gene Based on the sequence of the *phyI1* gene (GenBank accession no. AY150806) from *A. niger* 113, we designed and synthesized twenty oligonucleotide primers (Table 1). The twenty primers were joined in a single reaction step to synthesize the *phyI1s* gene. PCR reactions contained 10 ng of each inner primer and 100-200 ng of each outer primer (Fig. 1). Errors found upon confirmatory sequencing were corrected using the site-directed mutagenesis technique. The nucleotide sequence of the synthetic *phyI1s* gene was confirmed on a DNA sequencer (ABI377, PE Applied Bio Systems, USA). The condition of PCR that synthesizes the *phyI1s* gene was as follows: first 94°C 30 s, 65°C 40 s, 72°C 1 min 30 s; 5 cycles and then 94°C 30 s, 70°C 40 s, 72°C 1 min 30 s; 25 cycles.

Construction of the high expression vector for *P. pastoris* In order to obtain a high expression, a 357 bp α-factor prepro-leader MF4I (GenBank accession no. AY145833) was chemically synthesized with *P. pastoris*-preferred codon usage using successive PCR technique (Xiong *et al.*, 2003). For optimizing the translational efficiency of heterologous proteins, a 10-residues spacer peptide (EEAEAEAEPK) was inserted between the prepro-leader and the endoprotease processing site of the mating α−factor prepro-leader (Kjeldsen *et al.*, 1999). The first 3 residues (AIP) of MF4I were the same as the AOX1 protein in *P. pastoris* (Koutz *et al*., 1989). The 5-UTR of the MF4I-mRNA was also chemically synthesized. The nucleotide sequence was adjusted to be identical to that of the AOX1-mRNA. When compared to the original vector pPIC9 (GenBank No. Z46233), the new vector pYPX88 used our synthesized signal peptide, which was cloned into the *Hin*d III and *Xho* I sites. Moreover, the deletion of the *Bam* HI site, between the AOX1 promoter and signal, put the promoter in direct conjunction with the signal (Fig. 2a).

DNA manipulation was performed according to standard

Table 1. Primers for enzymatic synthesis of the *phyI1s* gene

Name	Oligonucleotides
Is1	AAA**CTCGAG**TTGGCTGTTCCAGCTTCTCGTAACCAATCTACTTGTGATACTGTTGATCAAGGTTAT CAATGTTTTTCTGAGACTTCTCA
Is2	TGTTTTTCTGAGACTTCTCATTTGTGGGGTCAATACGCTCCATTCTTCTCTTTGGCTAACAAATCT GCTATCTCTCCAGATGTTCCAGCT
Is3	TCTCTCCAGATGTTCCAGCTGGTTGTCAAGTTACTTTCGCTCAAGTTTTGTCTCGTCATGGTGCTC GTTATCCAACTGATTCTAAAGGTA
Is4	TCCAACTGATTCTAAAGGTAAGAAATATTCTGCTTTGATTGAGGAGATTCAACAAAATGCTACTA CTTTTAAGGAGAAATACGCTTTTTT
Is5	AAGGAGAAATACGCTTTTTTGAAAACTTACAACTATTCTTTGGGTGCTGATGATTTGACTCCAGA AGGTGAACAAGAATTGGTTAATTCT
Is6	AACAAGAATTGGTTAATTCTGGTGTTAAGTTTTACCAACGTTACGAATCTTTGACTCGTAATATTG TTCCATTTATTCGTTCTTCTGGTT
Is7	ATTTATTCGTTCTTCTGGTTCTTCTCGTGTTATTGCTTCTGGTAATAAATTCATTGAAGGTTTTCAAT CTACTAAATTGAAAGATCCACG
Is8	ACTAAATTGAAAGATCCACGTGCTCAACCAGGTCAATCTTCTCCAAAAATTGATGTTGTTATTTCT GAAGCTTCTTCTTCTAATAATACT
Is9	CTTCTTCTTCTAATAATACTTTGGACCCAGGTACTTGTACTGTTTTTGAAGATTCTGAATTGGCTG ATACTGTTGAAGCTAATTTTACTG
Is10	TGTTGAAGCTAATTTTACTGCTACTTTTGTTCCATCTATTCGTCAACGTTTGGAAAATGATTTGTCT GGTGTTACTTTGACTGATACTGA
Is11	AGTATCAAAAGAACACATGTCCATCAAGTAAGTAACTTCAGTATCAGTCAAAGTAACACCAGAC AAATCATTTTCCAAAC
Is12	AACAAATCACAAAATGGAGACAATTTAGTATCAACAGTAGAAGTAGAGATAGTATCAAAAGAAC ACATGTCCATCAAGTAAGTAACTTCA
Is13	ACCAGCACCATGACCGTAGTACTTTTTCAAAGATTGCAAATAATCATAATAATCCATTCATCATGA GTAAACAAATCACAAAATGGAGA
Is14	CTGGAGAATGAGTCAAACGAGCAATCAATTCATTAGCGTAACCAACACCTTGAGTTGGACCCAA TGGATTACCAGCACCATGACCGTAGT
Is15	AAAGTAGAATTCAATGGAAAAGTAGCTGGAGAAGAATCCAAAGTATGATTAGAAGAAGTATCAT CATGAACTGGAGAATGAGTCAAACGA
Is16	AGTACCATTGTACAAACCCAAAGCAAACAAAATAGAGATAATACCATTATCATGAGAGAAATCAG CGTACAAAGTAGAATTCAATGGAAA
Is17	GAACAGTCCAAGCAGAAGAAAAACCATCAGTTTGAGTGATATTTTCAACAGTAGTAGTAGACAA TGGTTTAGTACCATTGTACAAACCCA
Is18	AAAACACGAACCAATGGTTCTTGTTCAGCTTGACATTGCATCATTTCAACGTACAAACGAGAAG CTTCTGGAACAGTCCAAGCAGAAGAA
Is19	AGAATCACGAGTACAACGACCCAAAGCATCAACTGGACAACCATGCAATGGAACAACACGATC ATTAACCAAAACACGAACCAATGGTTC
Is20	AAA**GCGGCCGC**TTAAGCAAAGCATTCAGCCCAATCACCACCAGAACGAGCAAAAGACAAACCA CGAACAAAAGAATCACGAGTACAACGAC

The shadow and boxed regions were *Xho* I and *Not* I sites

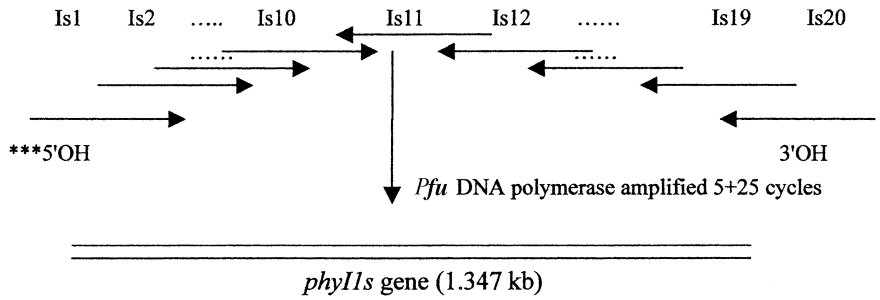

Fig. 1. *phyI1s* gene synthesis by successive PCR. Oligonucleotides of ~80-90 bp were assembled by one-step PCR using 10 ng of inner primers and 200 ng of external primers, which contained suitable restriction cleavage sites for cloning.

procedures (Sambrook *et al.*, 1989). The amplified PCR products were separated by 1% agarose gel electrophoresis. The gel slices containing the expected size band (1350 bp) were excised and extracted. The 1,350 bp PCR product (*phyI1s* gene) was inserted into the vector between the *Xho* I and *Not* I sites. The pYPX88 construct was then produced (Fig. 2b).

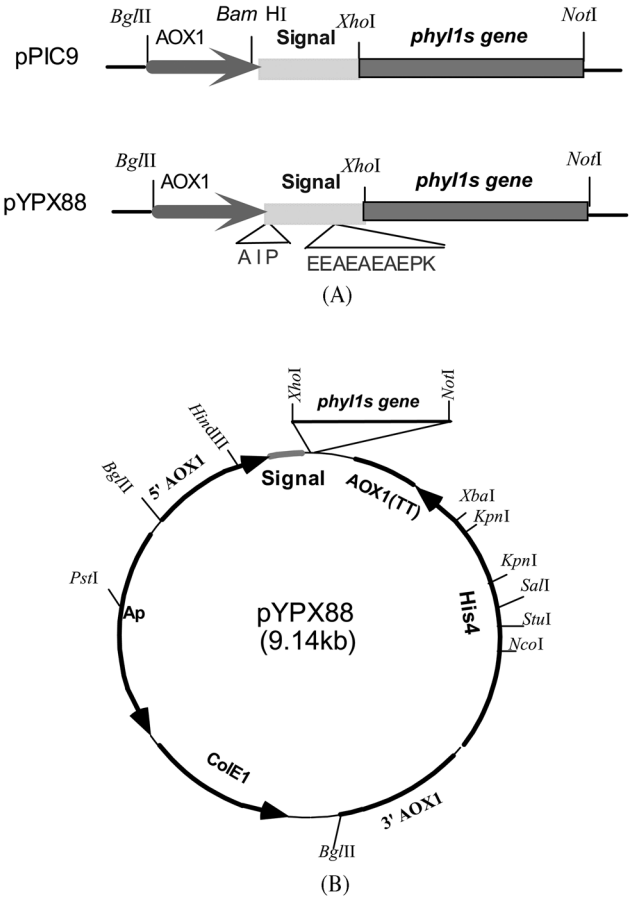

Fig. 2. Construction of the expression of pYPX88. (A) Comparison of expression unit between the synthetic MF4I signal sequence (GenBank accession no. AY145833) and the signal sequence of pPIC9 (GenBank accession no. Z46233). There exits three major changes: first, the *Bam*H I site between the AOX1 promoter and the signal was deleted; second, 3 residues (AIP) taken from the AOX1 protein in *P.pastoris* were added behind the ATG of synthetic signal; third, EEAEAEAEPK were added between the prepro-leader and the endoprotease processing site of synthetic signal. (B) The *phyI1s* gene was cloned into the *Xho* I and *Not* I site of the expression vector and the signal sequence was cloned into the *Hin*d III and *Xho* I site.

Transformation and screening The *P. pastoris* strain GS115 (his-4) was grown in 500 ml of a yeast extract-peptone-dextrose (YPD) medium for 18 h and prepared for transformation (Adams *et al.*, 1998). Two μg of pYPX88 DNA were linearized using a *Bgl* II restriction enzyme, then they were transformed into *P. pastoris* by electroporation (Bio-Rad Genepulser, Hercules, USA). After incubation for 30 min at 30°C in 1 M sorbitol, the cells were plated on selective plates [SD (Adams *et al.*, 1998), 18.6% sorbitol, 5% glucose, 2% agar]. For screening, the transformants were streaked in a regular pattern on both MM (1.34% YNB, 0.000004% biotin, 2% glucose, 2% agar) and MD [1.34% YNB (bacto-yeast nitrogen base without amino acids), 0.000004% biotin, 0.5% methanol, 2% agar) plates. The plates were incubated for 2 plus days at 30°C, then analyzed for transformants that grew normally on the MD plates but showed little or no growth on the MM plates. The selected clones were incubated in BMGY (1% yeast extract, 2% peptone, 1.34% YNB, 0.000004% biotin, 1% glycerol) at 30°C for two days. The cells were then pelleted (5,000 rpm for 3 min), resuspended in BMMY (SD, 0.000004% biotin, 0.5% methanol) to induce the phytase gene expression, and incubated at 30°C for two days before phytase activity was measured. The JM8 strain was selected for fermentation because of its high activity and expression.

Fermentation A 150 ml culture of JM8 was grown in a 500 ml shaking flask for 36 h in a YPD medium at 30°C until it reached OD_{600} = 3.0. The culture was then used to inoculate the B. Braun (Melsungen, Germany) 5 l fermentor containing 2 l of the BSM medium [H_3PO_4 (85 stock), 7.5 ml/l; $CaSO_4 \cdot 2H_2O$, 0.9 g/l; K_2SO_4, 18 g/l; Mg $SO_4 \cdot 7H_2O$, 14 g/l; $(NH_4)_2SO_4$, 5 g/l; Histidine, 1.5 g/l]. Next, 5% (v/v) glycerol [added as the sole carbon source, the pH was adjusted to 5.5 with 50% NH_4OH. 4 ml of biotin stock solution (0.2 g/l)] and 2 ml/l of the trace mineral mix PTM1 (Clare *et al.*, 1991) were added. The fermentation conditions were as follows: dissolved oxygen was maintained at 25% of saturated value; pH 5.0; initial 50% glycerol feed followed by methanol feed once the cell culture reached an OD_{600} = 200 (about 60 h). Fermentation took ~140 h to complete.

Enzyme activity and properties The *phyI1s* phytase was purified according to Wyss's procedure (Wyss *et al.*, 1999b). The optimum pH of the expressed phytase was determined (37°C) at every half pH between 1.5 and 6.5 by using buffers of 0.2 M glycine-HCl (pH 1.5 to 2.5), 0.2 M sodium citrate (pH 3.0 to 5.5), and 0.2 M Tris-HCl (pH 6.0 and 6.5). The activities were measured at 5-degree intervals between 20 and 80°C to determine the optimum temperature. Thermostability at 80°C was determined by heating phytase samples for increasing periods of time between 2 and 20 min, then chilling on ice and measuring the remaining activity at 37°C.

Protein analysis and deglycosylation of the expressed phytase The fermentation samples were loaded on 12% SDS-PAGE gels using the Mini-protein gel electrophoresis system (Bio-Rad Lab., Hercules, USA). After electrophoresis, the gels were stained for 30 min in a solution of 30% methanol, 10% acetic acid, and 0.05% Coomassie brilliant blue, and then destained in 30% methanol and 10% acetic acid (Sambrook *et al.*, 1989).

For Western blot analysis, the separated proteins were transferred onto a Protran nitrocellulose membrane with a Mini Trans-Blot cell (Bio-Rad Lab.). A rabbit polyclonal immunoglobulin G, raised against purified *phyI1s* phytase, was used as the primary antibody. It was diluted 1 : 5,000 prior to application. A goat anti-rabbit immunoglobulin G-horseradish peroxidase system (Bio-Rad Lab.) was used for the final colorimetric detection (Sambrook *et al.*, 1989).

Due to the heavy glycosylation, the expressed phytase was found to have molecular sizes of ~120, 95, 85, and 64 kDa. Endo Hf (New England Biolabs, Boston, USA) was used to deglycosylate the expressed phytase (Han *et al.*, 1999a, b). The reaction was carried out by incubating fermented samples with 0.3 IU of Endo Hf for 4 h at 37°C, according to the manufacturer's instructions.

Results

Isolation of the phytase cDNA and characterization of the deduced protein Using RT-PCR, cDNA was synthesized from isolated mRNA of *A. niger* 113. The putative phytase gene was amplified from the cDNA using primers based on previous sequences of phytases from the *A. niger* species. The primers used were 5' ATGGGTGTCTCTGCCGTTCTACTT CCTTTG 3' and 5' CTAAGCGAAACACTCCCCCCAATCA CCGCCAGATCT 3'. Amplification of the cDNA using the two primers produced an 1.4 kb fragment that was sequenced and compared to the sequences of previously isolated phytase genes. In a previous work, many phytase genes were cloned (GenBank accession numbers AB022700, U59803, U59805, U59804, and U59806). Through alignment of those sequences, it was found that the sequence of the *phyI1* gene (GenBank accession no. AY150806) showed a high homology with *phyA* genes from the *A. niger* strain NRRL3135 (Hartingsveldt *et al*., 1993) and SK-57 (GenBank accession no. AB022700). There was a 90% nucleotide identity and 98% deduced amino acid identity between the phytase from the *A. niger* strain 113 and NRRL3135. There was an 89% nucleotide identity and 96% amino acid identity between the phytase from the *A. niger* strain 113 and SK-57. The coding region of the *phyI1* gene from ATG started the codon to the TAG stop codon that had 1,404 bp. The cDNA open-reading frame encodes a putative pre-protein of 19 amino acids. The mature protein would, therefore, consist of 448 amino acids with a calculated molecular of mass of 49 kDa. There were nine putative N-glycosylation sites in the *phyI1* sequence. It was found that the highly conserved sequence, RHGARYPT, was the active-site sequence of the histidine phosphatases from microorganisms (Ullah *et al*., 1993; Kostrewa *et al*., 1997). Table 2 shows the variable amino acids of phytases among *A. niger* 113, NRRL3135 and SK-57. The four variable amino acid residues in phytase from *A. niger* 113 were Q53, K91, E92, and E384, respectively. The four sites were R53, E91, G92, F384 in NRRL3135 and H53, D91, G92, F384 in SK-57. Among 467 amino acid residues in every phytase from *A. niger* 113, NRRL3135 and SK-57, there existed nearly twenty variable amino acid residues between two of these three phytases. But, only four different amino acid residues were found between *phyI1* and the high specific activity phytases from *A. niger* NRRL3135 and SK-57. Table 3 compares the ORF, number, and homology of amino acids among phytases.

The synthetic *phyI1s* gene Using successive PCR, we synthesized a 1347bp DNA version of the phytase gene that we called *phy11s* (GenBank accession no. AF547224). DNA sequencing indicated the deletion of the signal sequence and intron. Sequencing showed a 75.4 % identity between the *phy11* gene and *phyI1s* gene. All of the codons were designed to be preferential to *P. pastoris* (Fig. 3).

Construction of the vectors for the expression of phytase in *P. pastoris* To study the expression of the *phyI1s* gene in *P. pastoris*, the expression plasmid pYPX88 (GenBank accession no. AY178045) was used. Plasmid pYPX88 is composed of the inducible promoter AOX1, the chemical-synthesized signal sequence (GenBank accession no. AY145833), and a termination transcription signal. Using the PCR method, we deleted the signal sequence and discovered that the *Xho* I site existed in the *phyI1* gene. The artificial *phyI1s* gene was cloned into the expression vector. After transformation and screening, the JM8 strain was obtained.

Expression of the phytase in *P. pastoris* JM8 was initially fermented with glycerol as the sole carbon source. After 60 h, the feeding was switched to methanol, which induced the phytase expression. As shown in Fig. 4, after 80 h of methanol induction (140 h total culture time), the fermentation culture achieved a final OD_{600} = 225. This culture yielded 4.2 g/l phytase with an activity of 39 U/ml (Fig. 4).

Due to heavy glycosylation, the expressed phytase had molecular sizes of ~ 120, 95, 85, and 64 kDa in SDS-PAGE (Fig. 5a). After deglycosylation by Endo Hf, the phytase had an apparent molecular size of 64 KD in SDS-PAGE. The Western blot is shown in Figs. 5b and 6. The results of the Western blot indicated that the protein from the fermentation fluid (Fig. 5a) was mainly phytase (Fig. 6).

Properties of the expressed phytase The activity of the expressed phytase was determined at different pHs, temperatures, and times, as described in Materials and Methods. The expressed phytase had a double pH optimum of

Table 2. The changes of amino acid positions among phytases of *A. niger* 113, NRRL3135 and SK-57

Phytase	Changes of amino acid										
A. niger 113	T11	A43	D47	**Q53**	**K91**	**E92**	V122	S186	T207	V208	
	T232	D269	K281	S326	T364	V367	**E384**	L388	A397	R432	A444
A. niger NRRL3135	**S11**	**V43**	**E47**	**R53**	**E91**	**G92**	**I122**	S186	T207	V208	
	T232	D269	K281	S326	T364	V367	**F384**	L388	A397	R432	A444
A. niger SK-57	T11	A43	D47	**H53**	**D91**	**G92**	V122	**T186**	**D207**	**I208**	
	S232	**E269**	**N281**	**N326**	**S364**	**A367**	**F384**	**M388**	**S397**	**K432**	**G444**

The shadow regions were four sites, which might be the key point of enzyme activity.

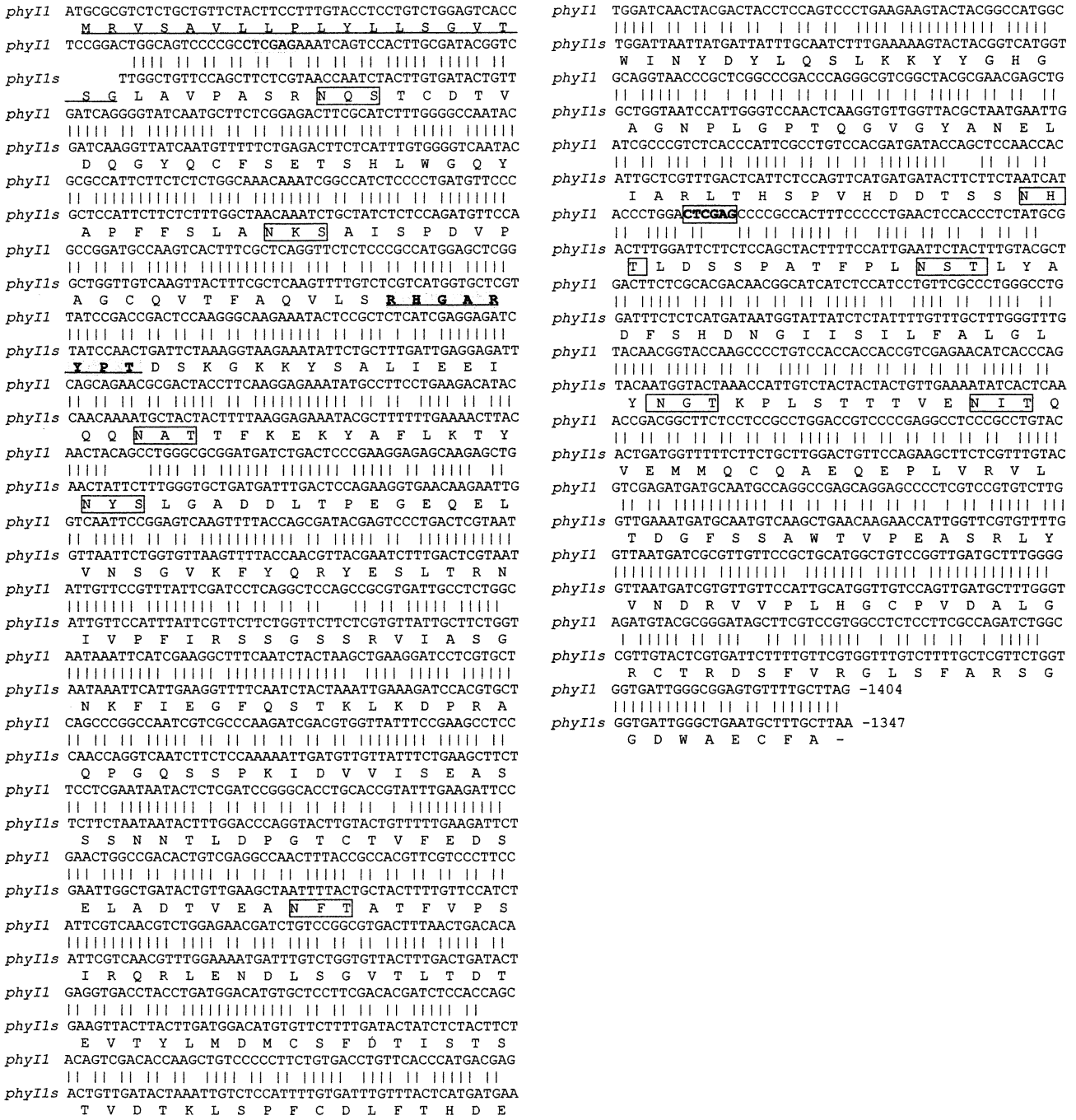

Fig. 3. Nucleotide sequences and deduced amino acid sequence of the *phyI1* gene and *phyI1s* gene. The predicted signal peptide is underlined and the nine putative N-glycosylation sites were boxed. The deletion *Xho* I site was shadowed and boxed. Active-site sequence of the phytase from microorganism was shadow, underline and bold. The entire ORF was 1,347 bp, and the two genes show 75.4 % Identity.

pH 5.0 and pH 2.0. The activity at pH 5.0 was 15% lower than that at pH 2.0 (Fig. 7a). The temperatures were tested (pH 2.0) at 25, 37, 45, 50, 60, 70, 80°C. It was discovered that 60°C was optimum (Fig. 7b). When the thermostability of the expressed phytase was tested, it was indicated that the enzyme retained 25% activity when heated at 80°C for 10 min, but that activity decreased significantly following heat treatment of 15 min or more (Fig. 7c).

Molecular properties and characteristics of phytases are presented in Table 4. The T_m value of phyI1 was obtained according to the general method (Lehmann *et al.*, 2000a). We obtained the isoelectric point of *phyI1* using PCGENE software. There was no significant difference among the phytases in temperature optimum, T_m, isoelectric point, and

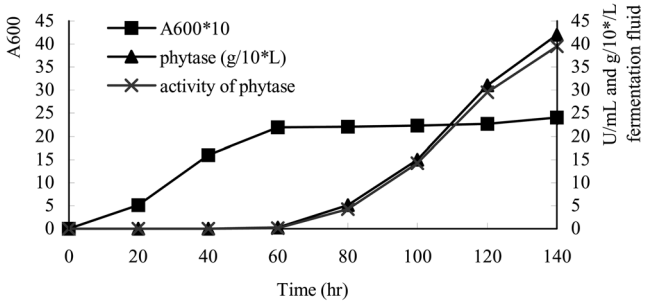

Fig. 4. Fermentation process of JM8. After fermentation 60 h, feeding was switched to methanol, which induced phytase expression, then activity and expression level of phytase were increased till fermentation 140 h.

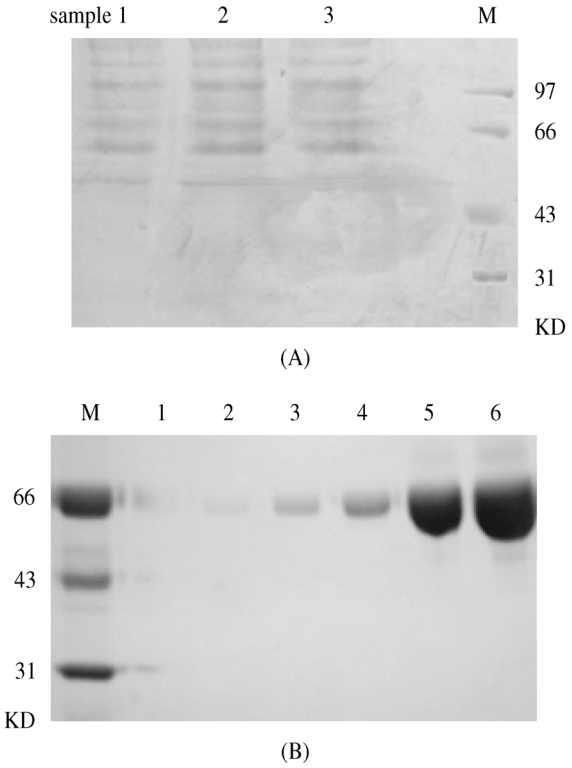

Fig. 5. Analysis of the expressed *phyI1s* gene in *P. pastoris.* (A) Expressed phytase ranging from 64 to 120 kDa due to glycosylation (B) Expressed phytase after deglycosylation by Endo Hf. Lane M, protein marker; Lanes 1-6, phytase expression after methanol induction of 0, 10, 20, 40, 60 and 80 h.

molecular sizes. The specific activity of *phyI1* was lowest among these phytases. The optimal pH was 2.0 and 5.0, which were significantly different from some other phytases (Wyss *et al.*, 1999a, b; Lehmann *et al.*, 2000b).

Discussion

The research indicated that the phytase was an acid phytase

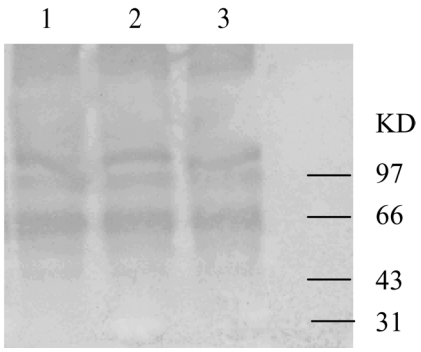

Fig. 6. Western blot analysis of the phytase protein expressed in JM8. The results of western blot indicated that the protein of fermentation fluid was phytase mainly.

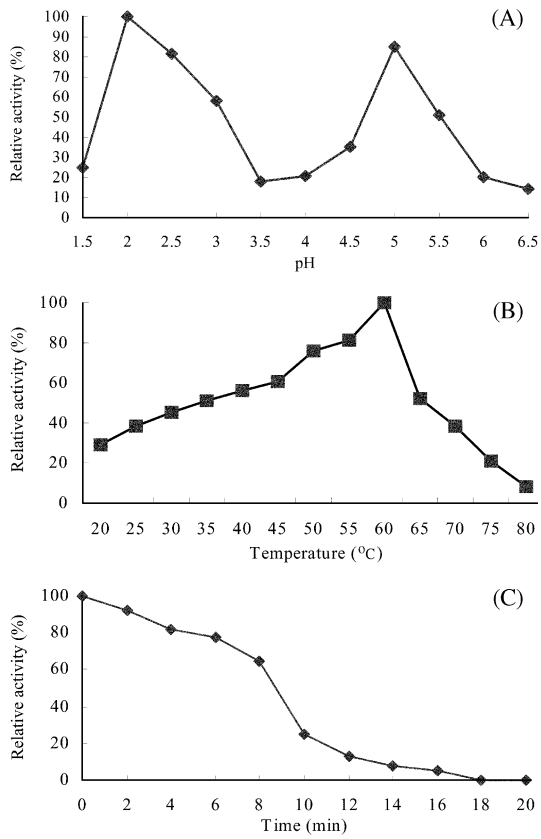

Fig. 7. The activity of expressed phytase was determined at different pHs, different temperatures, and different times. (A) pH dependence of enzyme activity. The expressed phytase optimum pHs were 5.0 and 2.0. The activity at pH 5.0 was 15% lower than that at pH 2.0. (B) Temperature dependence of enzyme activity. The 60ºC was found to be the phytase optimum temperature. (C) Heat stability of enzyme activity. The phytase retained 25% activity when heated at 80ºC for 10 min.

and its specific activity was lowest among all of the published phytases. The phytase had a double pH optimum and the optimal pH was 2.0. This characterization showed a potential of a good additive supplement in monogastric animals, such

Table 3. The ORF, Genbank accession number, and homology of amino acids among phytases

Phytase	Genbank acceeion no. Or reference	ORF (bp)	Number of amino acid	Homology of amino acid sequences
A. niger (ficuum) NRRL3135	Hartingsveldt *et al.*, 1993	1506	467	100
A. niger SK-57	AB022700	1515	467	95
A. niger 113	AY150806	1404	467	97
A. terreus 9A1	U59805	1449	467	60
A. terreus CBS	U60412	1452	467	62
A. fumigatus	U59804	1454	465	65
E. nidulans	U59803	1446	463	63
M. thermophila	U59806	1521	487	48
T. thermophilus	U59802	1455	466	61
B. subtilis	AF029053	1152	383	6

Table 4. Characterizations among those phytases of temperature optimum, T_m, isoelectric point, and molecular sizes

Phytase	Temperature optimum (°C)	T_m (°C)	Specific activities (U/mg)	Optimal pH	pI	Molecular sizes determined by	
						Sequence analysis	SDS-PAGE
A. niger (Natuphos)	55	63.3	102.5	5.5	4.78	48	66
*A. niger*NRRL3135	55	63.3	100.0	2.5 and 5.5	ND	ND	ND
A. niger SK-57	50	ND	158.0	2.5 and 5.5	ND	55	60
A. niger 113	60	61.5	9.5	2.0 and 5.0	4.63	49	64
A. terreus 9A1	49	57.5	141.6	5.5	5.08	49	61
A. terreus CBS	45	58.5	195.8	5.3	5.50	49	82
A. fumigatus	60	67.0	26.5	4.0 to 7.3	7.28	49	72
E. nidulans	45	58.5	28.6	6.5	5.27	49	66
M. thermophila	55	ND	41.8	5.5	4.95	51	63
T. thermophilus	45	ND	ND	ND	5.23	50	128
E. coli	ND	ND	811.2	2.5 to 7.0	7.01	46	47
B. subtilis	55	ND	88.0	7.0	ND	ND	43

pI was calculated by using amino acid sequence of mature protein.
ND, not determined.

as poultry and pigs. Many of the phytase genes have recently been isolated from plants, bacteria, and fungi. Although several phytase gene sequences are available, only a few phytase have been widely used in industry. Finding the novel phytase genes that had high activity and other characterizations (e.g. temperature stability, wide pH optima, etc.) was necessary for the commercial utilization.

The methylotrophic yeast *P. pastoris* has recently been recognized as an efficient host for high levels of the heterologous expression. *P. pastoris* can grow on methanol as the sole carbon and energy source. Using this system, a wide variety of proteins have been produced with varying degrees of success (Gellissen *et al.*, 2000). In this research, the phytase yield was improved by the modification of the signal peptides that were used for directing the secretion of the synthetic *phy11s* gene. The *S. cerevisiae* mating factor α (α-factor) prepro-leader has been used for the secretory expression of numerous heterologous proteins in both *S. cerevisiae* and *P. pastoris* (Kjeldsen *et al.*, 1999; Treerattakool *et al*., 2002). The synthetic ten-amino acid sequence EEAEAEAEPK was added between the prepro-leader and the endoprotease-processing site of the mating factor α. The *Bam* HI site between the AOX1 promoter and the signal sequence was deleted. These 3 residues (AIP), which were taken from the AOX1 protein in *P. pastoris*, were added behind ATG of the mating factor α prepro-leader. These changes proved to be effective in increasing the phytase secretion (Xiong *et al.*, 2003). After these improvements, it was possible to highly express phytase for commercial applications.

In this paper, we describe the molecular cloning and sequencing of a novel phytase cDNA that was cloned from *A. niger* 113, which was isolated from a soil sample in a rice field. The phytase gene was then highly expressed in *P. pastoris*. A nucleotide sequence analysis indicated that the length of the open-reading frame of the *phyI1* gene was 1,404 bp. The deduced amino acid sequence indicated that there were 19 amino acids pre-leader and 448 amino acids mature protein, as well as the highly conserved sequence

RHGARYPT that is the active-site sequence of the histidine phosphatases of the microorganism (Kostrewa *et al*., 1997). This active site motif was totally conserved in all fungal phytase and was also present in the *E. coli* phytase. There were only four major different amino acid residues that were found among the three phytases from *A. niger* 113, NRRL3135, and SK-57 (Table 3). The results demonstrated that the four amino acids were related to phytase specific activity. Moreover, the two G92 and F384 residues were the same between the two phytase with high specific activity from *A. niger* NRRL3135 and SK-57. The results further indicated the importance of the two amino acid residues in determining specific activity. These changes may modulate the domain flexibility, and thereby, the catalytic efficiency of the enzymes (Ullah *et al*., 1998; Mullaney *et al*., 2000). Through site-directed mutagenesis of these sites (Rodriguez *et al*., 2000), it was easy to obtain new mutant phytase genes with enhanced specific activities. This will be helpful in determining the phytase structure-function relationship. In addition, using the *phyI1* gene as a template, it will be easy to obtain many phytase mutants with improved characterization using *in vitro* molecular evolution by DNA shuffling. The key points of specific activity were not among the other different sites of these phytase, because there was a difference between the phytase of *A. niger* NRRL3135 and SK-57.

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