Cloning, Expression and Characterization of Three Novel Histidine acid Phytases from *Aspergillus niger*, *Aspergillus oryzae* and *Penicillium* sp

Junjun Liu¹, Honglei Liu¹, Qianqian Zhao, Liping Jiang, Xiao zhang and Yuzhen Zhang*

State Key Laboratory of Microbial Technology, Shandong University, Jinan - 250 100, China.

In the present study, a strategy for cloning three novel phytase genes (phyA) from different filamentous fungi strains by using combined utilization of Consensus-Degenerate Hybrid Oligonucleotide Primer (CODEHOP) PCR and Thermal Asymmetric Interlaced PCR (TAIL-PCR) was developed. Based on the conserved regions recognized by ClustalW online, CODEHOP primers were designed to amplify a partial sequence of the phytase gene. TAIL-PCR was then employed to clone the 5' and 3' flanking sequences. Using this strategy, three novel phytase genes of Aspergillus niger, Aspergillus oryzae and Penicillium sp. were cloned. After expression in Pichia pastoris, the properties of the three recombinant phytase were characterized. Sequence analysis and characterization of the recombinant enzymes revealed that they belonged to the family of histidine acid phosphatases. The results showed that combination of CODEHOP PCR and TAIL-PCR was an efficient strategy for cloning unknown genes from potential species.

Key words: Phytase, CODEHOP, TAIL-PCR, *Aspergillus niger*, *A. oryzae*, *Penicillium* sp.

Phytate (myo-inositol hexaphosphate), the major source of inositol and the main storage form of phosphorus in plant seeds, serves as an antinutritional factor in monogastric animals as well as a significant source of phosphorus pollutants in animal manures (Lei and Porres, 2003; Haefner *et al.,* 2005; Rao *et al.,* 2009). Phytase (EC3.1.3.8), a subfamily of histidine acid phosphatases (HAPs), can hydrolyze phytate to liberate inositol and inorganic phosphorus (Berka *et al.,* 1998; Bohn *et al.,* 2008; Amol *et al.,* 2012). Because of its desirable catalytic properties, considerable efforts have been made to produce economical phytase enzymes as

feed additives. A number of sources including plants, animals and microorganisms (especially of fungal and bacterial origin) were screened for the ability to secrete phytase (Pandey *et al.,* 2001; Kaur *et al.,* 2007). Many phytase genes have been isolated (van Hartingsveldt et al. 1993; Mitchell *et al.,* 1997; Rodriguez *et al.,* 1999; Lassen *et al.,* 2001; Shao *et al.,* 2008 Huang *et al.,* 2011a), and some strains containing two phytase genes have been found, such as *Aspergillus niger* (van Hartingsveldt *et al.,* 1993; Ehrlich *et al.,* 1993), *Escherichia coli* (Rodriguez *et al.,* 1999) and *Ceriporia* sp. (Lassen *et al.,* 2001).

At present, many of the reported phytase genes were isolated by screening DNA libraries or cDNA libraries. A hybridization probe is usually designed either by amplifying a fragment of the target gene or by determining partial amino acid sequence of the protein (van Hartingsveldt *et al.,*

* To whom all correspondence should be addressed. Tel.: +86-531-88362258; Fax: +86-531-88365610; E-mail: yzzhang1949@yahoo.com

1993; Mitchell *et al.,* 1997; Pasamontes *et al.,* 1997). However, this method is comparatively tedious and time-consuming. To date, many studies focus on isolating new phytase genes encoding phytases with preferable characteristics, such as high specific activity, excellent thermostability and appropriate pH optima (Lei and Porres, 2003). Thus, a simple and efficient method for cloning phytase genes is essential.

In recent years, some powerful tools, such as Thermal Asymmetric Interlaced PCR (TAIL-PCR) & Consensus-Degenerate Hybrid Oligonucleotide Primer PCR (CODEHOP PCR), were established to facilitate gene cloning. TAIL-PCR, an effective tool for cloning segments flanking known sequences, uses a set of nested sequences–specific primers plus a shorter arbitrary degenerate primer with a lower melting temperature (Liu and Whittier, 1995; Nakayama *et al.,* 2001, Zhang *et al.,* 2011a). Huang et al (2006b) tried to cloning the flanking sequences for a novel phytase from *Yersinia intermedia* (Huang *et al.,* 2006). Zhang et al (2011b) successfully amplified the complete phytase gene from *Serratia* sp. TN49. CODEHOP was developed to design primers with a 5' non-degenerate clamp region and a 3' degenerate core (Rose *et al.,* 1998; Rose *et al.,* 2003; Zlateva *et al.,* 2011).

In the present study, we applied a strategy for cloning phytase genes by combined utilization of CODEHOP PCR and TAIL-PCR. A pair of CODEHOP primers were designed and successfully applied to clone partial sequences from three filamentous fungi: *Aspergillus niger*, *Aspergillus oryzae* and *Penicillium* sp. Using the amplified sequences, TAIL-PCR was employed to isolate the 5' and 3'-flanking regions to obtain the complete sequences of the phytase genes. The phytase genes were expressed in *Pichia pastoris* GS115 and the recombinant enzymes were characterized. Theoretically, this strategy would be effective for cloning homologous genes if some homologous protein sequences were available.

MATERIALS AND METHODS

Strains, plasmids and chemicals

A. niger, A. oryzae and *Penicillium* sp. were isolated by our laboratory. The *P. pastoris* host strain GS115 and expression vector pPIC9 were kindly provided by Institute of Plant Physiology

and Ecology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences. All restriction enzymes, *rTaq* and PrimeStar™ were purchased from TaKaRa (Dalian) while Endo H_f was from NEB (New England Biolabs). All chemicals used in this study were commercially available and were of analytical grade unless specially noted.

Cloning the phytase genes from *Aspergillus niger*, *Aspergillus oryzae* and *Penicillium* sp.

Fungal mycelia of the three strains were obtained by inoculating spores (10^6 per ml) into medium containing 3% soluble starch, 0.5% peptone, 1% KNO_3, 0.015% $FeSO_4$, 0.07% $MgSO_4$ and 0.001% $MnSO_4$ at 28°C for 2 days. Genomic DNA was isolated from the filamentous fungi as described by Pasamontes et al (1997). Total RNA was isolated using the SV Total RNA Isolation System (Promega) according to manufacture's instructions. Reverse transcription PCR was performed with the SuperScript™ First-Strand Synthesis System for RT-PCR Kit (Invitrogen) according to manufacture's instruction. Total RNA as template plus oligo dT_{12-18} were used to produce the first-strand cDNA of the genes that were expressed in the fungal strains.

Ten fungal phytases belonging to the family of HAPs were selected and aligned by ClustalW (http://www.ebi.ac.uk/clustalW) (Thompson *et al.,* 1994). Block I and Block II (Figure 1) were chosen as the sequences for the design of the CODEHOP PCR primers CODE 1 (5'-CGTCCAGGTCATCCAG mgncayggngc-3') and CODE 2 (5'-GGTCAGGCGGGCCadnarytcrtt-3') (http://blocks.fhcrc.org/codehop.html). Minor revisions were made to the primers based on the codon usage of *A. niger.* The CODEHOP PCR system was optimized to the following conditions: 1×PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTP, 1 uM of each primer, 500 ng single-stranded cDNA template and 5 U r*Taq* in 100 ul reaction volume. A "touchdown" PCR program containing 40 cycles was performed to amplify a partial sequence of the phytase genes using the CODEHOP primers (Don *et al.,* 1991, Zlateva *et al.,* 2011). Single-stranded cDNA was first denatured for 5 min at 94°C to remove possible secondary structures. This was followed by 20 cycles with the same denaturation and elongation step (94°C 30 s and 72 °C 1 min, respectively) but different annealing steps (the

annealing temperature was decreased 0.5°C every second cycle from 70°C to 60°C). The last 20 cycles were set with a fixed annealing temperature of 60°C. After all the cycles were finished, the products were elongated at 72°C for 10 minutes. The PCR products were purified and sequenced. Based on the obtained partial sequences, three pairs of primers adjacent to the CODEHOP primers were designed and used to screen other *E. coli* transformants.

TAIL-PCR was used to isolate the 5' and 3' regions flanking the acquired partial sequence to isolate the full length phytase genes. Three nested insertion-specific primers as a group were designed and synthesized based on known sequences to isolate the 5' or 3' flanking sequences of the phytase genes from the three strains (Table 1). Arbitrary degenerate (AD) primers were designed and used to amplify the appropriate fragments containing the complete regions of interest (AD Primer Database for Microbes, ADDB, unpublished). The reaction system and procedure were optimized based on the study by Liu and Whittier (1995). The products of the TAIL-PCR were purified and sequenced. The sequences of the CODEHOP PCR and the TAIL-PCR product were assembled to obtain the full length genes.

Sequence analysis

The signal peptide was predicted using Signal P (http://www.cbs.dtu.dk/services/SignalP/). The theoretical M_w and pI of the deduced mature peptide were calculated with the compute pI/Mw tool in ExPASy (http://www.expasy.ch/tools/pi_tool.html). Searches for homology in nucleotide and amino acid sequences were done using the BLAST server. Potential N-glycosylation sites in the coding regions were identified. The nucleotide sequences of the cloned phytase genes from *A. niger*, *A. oryzae* and *Penicillium* sp. were deposited in GenBank with the accession numbers EF197825, EF197826 and EF197827.

Preparation of coding regions

Using single-stranded cDNA as templates, the coding regions (without the signal peptides) of the phytase genes were amplified with *EcoR*I and *Not*I sites generated in the 5' and 3' ends. Recombinant PCR was performed as described by Horton, et al. to acquire the putative cDNA of the *Penicillium* sp. phytase gene since the RT-PCR product still contained the intron

(Horton *et al.,* 1989). Primers P1 and P2, P3 and P4 were employed to amplify fragment 1 (exon 1 plus 5'-UTR) and fragment 2 (exon 2) for PCR splicing by overlap extension (SOEing) from *Penicillium* sp. genomic DNA. Primers UPen and DPen were used to amplify the coding region of the *Penicillium* sp. phytase gene using the SOEing reaction product as a template. All these PCR reactions for preparing coding regions were performed using PrimeStar™ to maintain the fidelity of the PCR products. All the primer sequences are shown in Table 1.

Yeast transformation and expression

The coding regions of the phytase genes were subcloned into an expressing vector pPIC9 at *EcoR*I and *Not*I sites. Thus, the phytase genes were controlled by an inducible promoter AOX1 and were preceded by the signal peptide á–factor in the constructed plasmids. The recombinant plasmids pPIC9-x (x=An*phy*A, Ao*phy*AÿPen*phy*A) and the empty vector pPIC9 (as a control) were linearized with *Stu*I and transferred into the *P. pastoris* host strain GS115 with the PEG 1000 transformation method. Single positive colonies of tranformants were first inoculated into 10 ml BMGY at 30°C until the cultures reached an OD_{600}=6. *P. pastoris* cells were harvested (3000 g, 5 min) and then diluted with 10 ml BMMY to a density of OD_{600}=1.0. Methanol (0.5%, v/v) was added to induce expression every 24 hours. Crude enzymes samples were collected at the following time points (hours): 12, 24, 36, 48, 60, 72, 96 and 108.

Enzyme assays and characterization of the recombinant phytases

The culture supernatant was prepared by centrifugation (8000 g for 5 min at 4°C) at different time points. Phytase activity was assayed as described by Han and Lei (1999). One unit was defined as the amount of enzyme that released 1 ìmol of phosphate per min at 37°C and pH 5.5.

The optimal pHs of the recombinant phytases were determined using different buffers from pH 1.5 to pH 6.5 (0.2M glycine-HCl pH 1.5-3.5, 0.2M sodium citrate pH 4.0-6.0, 0.2M Tris-HCl pH 6.5). The enzymes were incubated with sodium phytate (Sigma). The optimal temperatures were determined at pH 5.5. The assay was performed at different temperatures: 37, 45, 50, 55, 60, 70°C. To determine the thermostability of the recombinant phytases, the crude enzyme samples were incubated at

different temperatures for 15 min. After the heat treatment, the samples were immediately placed on ice. The remaining activity was measured and compared to samples without heat treatment.

Deglycosylation of the secreted phytase and SDS-PAGE analysis

Deglycosylation of the secreted recombinant phytases was performed using 0.3 IU of Endo H$_f$ according to the manufacture's instructions. Samples of the crude enzymes before and after treatment with Endo H$_f$ were analyzed by 15% SDS-PAGE.

RESULTS

Cloning the phytase genes from *A. niger*, *A. oryzae* and *Penicillium* sp.

Specific fragments of about 750 bp were amplified from cDNA templates of the three strains by CODEHOP PCR using primers CODE 1 and CODE 2 (Figure 2). The products were cloned into the pMD18-T vector (TaKaRa) and then transformed into *E. coli* DH5á. After sequencing, three pairs of primers adjacent to the CODE 1 and CODE 2 sites in the three obtained partial sequences were designed to screen other *E. coli* transformants.

As shown in Table 1, the arbitrary degenerate primers with high GC content provided by the ADDB were the most effective in the different TAIL-PCR reactions for cloning the target sequences in this study. The specific products of TAIL-PCR chosen for sequencing were more than 700 bp. Other AD primers that yielded shorter fragments are not shown. The assembly of each group of the three cloned fragments led to a sequence containing the full genomic nucleotide sequences of the phytase genes as well as the 5'-UTR and 3'-UTR sequences. The total lengths of the assembled sequences were 1934, 2128 and 1971 bp, containing phytase genes from *A. niger*, *A. oryzae* and *Penicillium* sp., respectively.

Sequence analysis and accession numbers for the three genes

The Met start codon of the three genes was identified according to previously cloned phytase genes and the results of BlastP. The length of the cloned phytase genes is shown in Table 2. An intron with the characteristic elements of fungal introns (Donor, Lariat, Acceptor sequences) was

found in the three cloned phytase genes (Rambosek and Leach, 1987). The length and locations of the introns varied in the different strains.

Signal peptide predictions of the deduced amino acid sequences by SignalP demonstrated that phytases from *A. niger* and *A. oryzae* were extracellular enzymes with a clear cleavable site. However, the *Penicillium* sp. phytase was a typical non-secreted protein, which is not ordinarily found in filamentous fungi. The number of potential N-glycosylation sites was different in the three phytase enzymes (Table 2). Blastn showed that the cloned phytase genes were highly similar to some *phy*A genes or cDNA of the same origin (99% homology). BlastP showed that all the three putative phytase proteins belonged to the family of HAPs.

Preparation of the coding regions

The reverse transcription PCR products were purified and sequenced, which revealed that the introns in *A. niger phy*A and *A. oryzae phy*A were absent while a putative intron still existed in the *Penicillium* sp. *phy*A. For the purpose of expression, recombination PCR was performed to remove the putative intron. Since exon 1 was too short, exon 1 together with the 5'-UTR were amplified as fragment 1 for an effective SOEing reaction. The whole ORF was cloned using primers UPen and DPen. Sequencing of the recombination PCR product confirmed that the putative intron was removed and the ORF was correct.

Expression of the cloned phyA genes in *P. pastoris*

The positive *P. pastoris* transformants containing pPIC9-x or the pPIC9 empty plasmid were screened and analyzed for their phytase activity after methanol induction. For every recombinant plasmid, 20 transformants were screened. Investigation of phytase activity showed that the phytase activities varied over a broad range (Data not shown). As a control, the pPIC9 transformant showed no significant activity (<0.008). The transformants of each recombinant plasmid with the highest activity were chosen and crude enzyme samples were taken at different time points and further assessed.

Characterization of the recombinant phytases
In the range of pH from 4.5 to 6.0, the recombinant phytases (rPhyAs) showed high activity (>60% of

Table 1 Primers used in cloning the phytase genes and the coding regions

Primer[a]	Sequencee5'-3'	Length	Position[b]
Primers for screening CODEHOP PCR product			
Ao-U	CGGTATCCAACGAAGAGCAAGTC	23	318-340
Ao-D	AGCGAAGCCGATGCCCTGC	19	1007-1025
An-U	CGGTATCCGACCGACTCCAAG	21	355-375
An-D	AGCGTAGCCGACGCCCTGC	19	1044-1062
Pen-U	CGGTATCCCACGGCGTCCA	19	341-359
Pen-D	GATGAATCCCACGCCTTGGG	20	1029-1048
Primers for TAIL-PCR			
Ao-5sp1	TGATCCCCGAATCCACCAACTG	22	479-500
Ao-5sp2	GTTCTCTCCAAAAGGCGTAAGGTC	24	455-478
Ao-5sp3	CTTGGCGTACTTCTCAGACTTGCT	24	344-367
Ao-3sp1	ATTCGACACCATCGCCTTGACC	22	871-892
Ao-3sp2	CCTTCTGCGCTTTGTTCACCCA	22	915-936
Ao-3sp3	GCATCGGCTTCGCTAACGAGC	21	1023-1043
Pen-5sp1	CACATTGATGGCTGCGTGAT	20	663-682
Pen-5sp2	AAAGAGCGGATAAAGGGGACT	21	550-570
Pen-5sp3	ATCTGGTGCTCGCCAAAGGAG	21	475-495
Pen-3sp1	CCGACGACCAAGTCATCTACCTC	23	846-868
Pen-3sp2	GACACCGTCGCAACCACCCCAG	22	887-908
Pen-3sp3	CCCTCGGCAAATACTACGGTTAC	23	981-1003
An-5sp1	TCGTAACGCTGGTAGAACTTGATGC	25	521-545
An-5sp2	AACCCAGGCTGTAGTTGTATGTCTTC	26	450-475
An-5sp3	GCCCTTGGAGTCGGTCGGATA	21	358-378
An-3sp1	CACTGAAGTGACCTACCTCATGGAC	25	864-888
An-3sp2	CACCAACCTGTCGCCCTTCTG	21	927-947
An-3sp3	CGTCGGCTACGCTAACGAGC	20	1050-1069
Ao-AD1	SAWGGAWGWCCAGG	14	/
Ao-AD2	SAWGWGAWGACGGA	14	/
An-AD3	SAWGGAWGWACACC	14	/
An-AD4	SAWGWGAWGTGCCC	14	/
Pen-AD5	SAWGGAWGWGCGCC	14	/
Pen-AD6	SAWGGAWGWGCGCC	14	/
Primers for Recombinant PCR[c]			
P1	AGGGATCCAGATCTCCAGTCTTGAA	25	/
P2	*GAACACGATGTGAGAC*CCCGCCACGAGCAGGACCA	35	/
P3	*GTCTCACATCGTGTTC*GGAC	20	/
P4	TCACTTTGAAGAAACGCCAC	20	/
Primers for amplification of the coding regions[d]			
U-Ao	CGGAATTCACTCCGGTGACCAGCCCG	26	/
D-Ao	CCGCGGCCGCTAAGCAAAGCACTCTCCCCAGT	32	/
U-An	CGGAATTCCTGGCAGTCCCCGCCTCGAG	28	/
D-An	CCGCGGCCGCCTAAGCAAAACACTCCGCCC	30	/
U-Pen	CGGAATTCATGTTTGACATTGCTCTTCGCTC	31	/
D-Pen	CCGCGGCCGCTCACTTTGAAGAAACGCCAC	30	/

[a] Ao, An, Pen represent *A. niger*, *A. oryzae* and *Penicillium* sp., respectively. AD1, AD3 and AD5 were used to clone the 5' flanking sequences, while AD2, AD4 and AD6 were used to clone the 3' flanking sequences. All the specific primers had a melting temperature of approximately 61°C. T_m was calculated by the formula T_m=69.3+0.41×GC%-650/Length. U is short for upstream while downstream is abbreviated as the character D.
[b] The position of the primers was identified and marked in the phytase gene.
[c] The italics in P2 and P3 are reverse complementary to each other to lead an SOEing reaction.
[d] The *Eco*RI and *Not*I sites are underlined.

Table 2. Characteristics of *phy*A and PhyA by sequence analysis

*phy*A/ PhyA	Size (bp)	Intron Location[a]	Total resi- due (aa)[b]	M_w[c] (kDa)	Signal peptide	pI value	N-glycosy- lation sites
A. niger	1506	45-146	467	51.14	21[d]	4.88	10
A. oryzae	1480	46-124	466	51.35	19	5.07	8
Penicillium sp.	1503	46-160	461	50.42	none	6.10	10

[a] Location of the introns is marked in the *phy*A sequence.

[b] AA represents amino acid while bp means base pair.

[c] molecular weight

[d] The predicted signal peptide of *A. niger* phytase did not agree with a previous study, which experimentally identified the N-terminal sequence as the first 19 amino acids(aa) (van Hartingsveldt, 1993). In the present study, the *A. niger* functional *phy*A was amplified without the first 19 aa sequence.

maximum). The optimal temperature for the rPhyAs was approximately 55°C. As shown in Figure 3, after heat treatment for 15 minutes at more than 55°C, the *Penicillium* sp. PhyA lost most of its activity, while *A. niger* PhyA and *A. oryzae* PhyA maintained 50% of their initial activities.

Fig. 1. A multiple sequence alignment of the selected 10 protein sequences produced by ClustalW. An: *A. niger*, AAA16898 ; At: *A. terreus*, AAB52507.1; Af: *A. fumigatus*, AAB96872.1; En: *Emericella nidulans*, AAB96871.1; Tther: *Talaromyces thermophilus*, AAB96873.1; Th: *Thielavia heterothallica*, AAB52508.1; Trap: *Trametes pubescens*, CAC48234.1; Cer: *Ceriporia* sp., CAC48163.1; Agp: *Agrocybe pediades*, CAC48160.1; Penl: *Peniophora lycii*, CAC48195.1;. Block I was chosen to design the upstream primer CODE 1, while the downstream primers CODE 2 were derived from Block II.
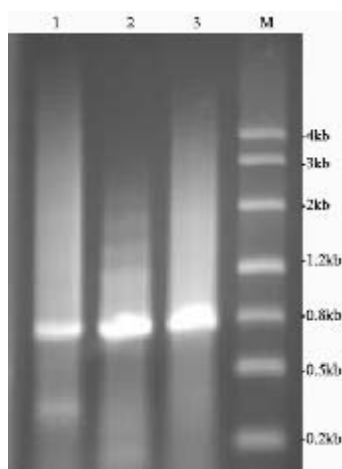

Fig. 2. Cloned partial sequences of the phytase genes using the CODEHOP primers and single-stranded cDNA templates. Lane 1: *A. niger* cDNA template; Lane 2: *A. oryzae* cDNA template; Lane 3: *Penicillium* sp. cDNA template; M: DNA marker
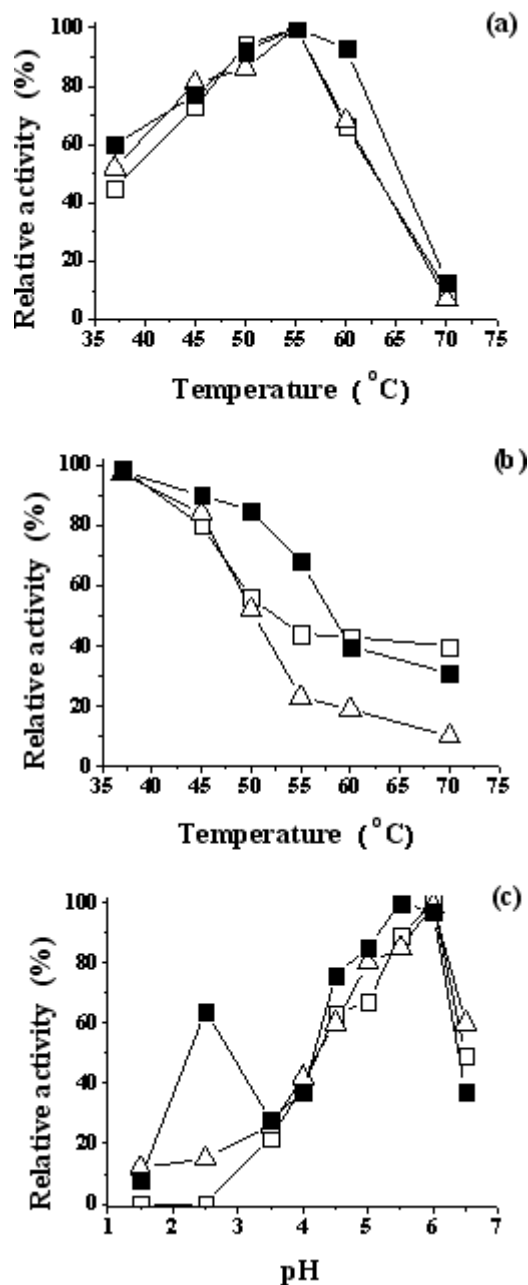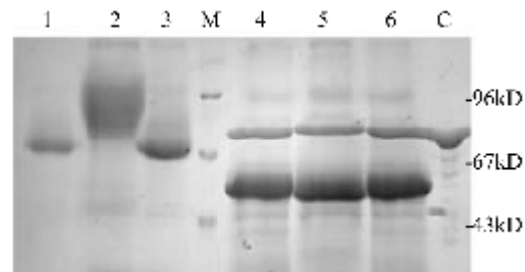
Fig. 4. SDS-PAGE analysis of the recombinant phytases before and after deglycosylation by Endo H$_f$. Samples of medium supernatant were collected after induction for 108 h. Line 1 to Line 3 were samples of *Penicillium* sp. recombinant phytase (rPhyA), *A. niger* rPhyA and *A. oryzae* rPhyA. Line 4 to Line 6 were samples of *Penicillium* sp. rPhyA, *A. niger* rPhyA and *A. oryzae* rPhyA treated by Endo H$_f$. Line M, SDS-PAGE marker; Line C, Endo H$_f$control

Deglycosylation of the secreted phytase and SDS-PAGE analysis

SDS-PAGE analysis showed that the molecular weight of *A. niger* rPhyA varied from 70kDa to 90 kDa while the other rPhyAs were approximately 70kDa in size. However, after deglycosylation by endoglycosidase H$_f$, the molecular weights of the three rPhyAs decreased to approximately 50 kDa, which was in accordance with the calculated molecular sizes shown in Table 2 and Fig. 4.

DISCUSSION

Three homologous genes from different filamentous fungi strains have been cloned by combining CODEHOP PCR and TAIL-PCR. Unlike other methods in which a DNA library or cDNA library need to be screened with various protocols (e.g. northern blotting, expression, PCR amplification), this strategy only requires the selection and alignment of amino acid sequences of homologous proteins for the purpose of acquiring proper blocks to design CODEHOP primers. If partial sequences of the target genes are obtained, several methods can be applied to clone the flanking segments, including inverse PCR, hemispecific PCR, TAIL-PCR and RACE (Nakayama *et al.,* 2001). As a powerful and efficient tool, TAIL-PCR was used in our project. Previous studies have demonstrated its effectiveness in many species.

Fig. 3. (a) The effects of temperature on phytase activity; (b) Residual phytase activity of the recombinantly expressed phytases after incubation at different temperatures; (c) The profiles of relative activity of the recombinant phytases at different pH. *A. niger* PhyA (■), *A .oryzae* PhyA (□) and *Penicillium*. sp PhyA (r). All the values were the average of three measurements.

Noting that eukaryotic genome is more complex than prokaryotic genome, single-stranded cDNA obtained by reverse transcription PCR was used as the template in the performance of CODEHOP PCR to decrease non-specific products. Testing of the CODEHOP PCR template showed that high-quality total RNA is a significant factor for cloning target sequences and obtaining reproducible results. When genomic DNA was used as the template, no specific product could be acquired. The "touchdown" PCR procedure was also effective in inhibiting mismatches and thus contributed to amplification of specific products. The CODEHOP PCR product could be a mixture even though there was only one band in the agarose gel after electrophoresis since DNA fragments cannot be fully separated if their lengths are similar. Therefore, after transferring the CODEHOP PCR product into *E. coli* DH5α and sequencing, we designed another pair of specific primers adjacent to the CODEHOP primers based on the acquired sequence for each fungus *phy*A gene. This was to identify other *E. coli* transformants. In our strategy, if screening with the specific primers did not lead to a specific product, the transformant was then sequenced to determine whether it was a new gene or not. In this study, a total of 20 *E. coli* transformants were screened for each CODEHOP product. We did not isolate more than one phytase gene from the strains. This may be due to the experimental conditions since more than one *phy*A gene can exist in certain species.

The genes encoding the phytases from three different fungi, *A. niger, A. oryzae* and *Penicillium* sp., were isolated by the described strategy. Unlike most characterized fungal phytases, the *Penicillium* sp. phytase was a typical nonsecreted protein based on SignalP analysis. All of the three phytase enzymes contained the conserved motif RHGXRXP and HD, which are the consensus sequences for the family of HAPs. The *phy*A genes of fungal origin were expressed in *P. pastoris*. As shown in figure 3, the expression level of *Penicillium* sp. rPhyA is very low compared with that of *A. niger* and *A. oryzae* rPhyAs. This may be due to the limited number of recombinant *P. pastoris* clones screened by us. We also detected the intracellular phytase enzymes and found that more than 86% all three rPhyAs were secreted (Data not shown). The expression levels were not high, but there are many methods to improve production. However, this aspect was not our major concern in this study. Sequence analysis and characterization of the recombinant phytases demonstrated that they all belong to the family of HAPs.

ACKNOWLEDGMENTS

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