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Molecular cloning, characterization, and expression of the phytase gene from marine yeast *Kodamaea ohmeri* BG3

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ABSTRACT

The extracellular phytase structural gene was isolated from the cDNA of the marine yeast, *Kodamaea ohmeri* BG3, using the switching mechanism at 5′-end of RNA transcript (SMART)™ rapid-amplification of cDNA ends (RACE) cDNA amplification kit. The gene had an open reading frame of 1389 bp and the coding region of the gene had no intron. It encoded 462 amino acid residues of a protein with a putative signal peptide of 15 amino acids. The protein sequence deduced from the extracellular phytase structural gene contained the consensus motifs (RHGXRX P and HD), which are conserved among histidine acid phosphatases, and six conserved putative N-glycosylation sites. According to the phylogenetic tree of the phytase, the phytase from *K. ohmeri* BG3 was closely related to *Candida albicans* (XP_713452) and *Pichia stipitis* (XP_001385108) phytase proteins and more distantly related to other phytases. The mature peptide encoding cDNA was subcloned into the pET-24a (+) expression vector. The recombinant plasmid [pET-24a (+)*PHY1*] was expressed in *Escherichia coli* BL21 (DE3). The expressed fusion protein was analysed by SDS-PAGE and Western blotting, and a specific band with a molecular mass of about 51 kDa was found. An enzyme activity assay verified the recombinant protein as a phytase. A maximum activity of 16.5 U mg^{-1} was obtained from the cellular extract of *E. coli* BL21 (DE3) harbouring pET-24a (+)*PHY1*. The optimal pH and temperature of the crude recombinant lipase were 5 and 65 °C, respectively, and the crude recombinant phytase had hydrolytic activity towards phytate.

Introduction

Phytic acid is the principal storage form of phosphorus in legumes, cereals, oil seeds, and nuts. Phytases (*myo*-inositol hexakisphosphate phosphohydrolase) are histidine acid phosphatases (HAPs), a subclass of phosphatases that catalyse the hydrolysis of phosphate moieties from phytic acid, thereby resulting in the loss of ability of phytic acid to chelate metal ions (Li *et al.* 2008a). The supplementation of animal feed with phytase increases the bioavailability of phosphorus in monogastric animals besides reducing phosphorus pollution. Thus, for both environmental and economic reasons, phytases and phytase-producing microbes are attracting significant industrial interest. Phytases become potential candidates for the production of special isomers of different lower phosphate esters of *myo*-inositol, some of which are considered to be pharmacoactive and important intracellular secondary messengers (Haefner *et al.* 2005). It is well known that microbial sources are more promising for the production of phytases on a commercial scale than any other sources (Li *et al.* 2008a). Of the various organisms reported, phytase production by *Aspergillus* sp. has been most commonly employed (Vats & Banerjee 2004). In recent years, it has been found that some of the yeasts are also good sources for phytase production (Li *et al.* 2008a). The yeast

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species that could produce phytase include *Arxula adeninivorans*, *Hansenula polymorpha*, *Schwanniomyces castellii*, *S. occidentalis*, *Saccharomyces cerevisiae*, *Candida tropicalis*, *C. boidinii*, *Rhodotorula gracilis*, *Torulopsis candida*, *Kluyveromyces fragilis*, *Debaryomyces castellii*, *C. brusei*, *Pichia anomala*, and *Kodamaea ohmeri* (Vats & Banerjee 2004; Li *et al.* 2008a). In general, phytases have an estimated molecular weight of 35–700 kDa depending upon the source of origin and are usually active within a pH range of 4.5–6 at 45–60 °C. They have identical sequences in the regions of the N-terminal motif (RHGXRXP) and the C-terminal motif (HD) (Vats & Banerjee 2004). So far, the gene encoding phytase has been cloned from *D. castelli*, *S. occidentalis*, *P. stipitis*, *L. elongisporus*, *P. guilliermondii*, *Kluveromyces lactis*, and *S. cerevisiae*. The phytase A gene (*phyA*), phytase B gene (*phyB*) from *Aspergillus niger* (ficuum) NRRL 3135, the ORF encoding the *Debaryomyces castellii* CBS 2923 phytase and *APHO1* gene encoding extracellular acid phosphatase from *Arxula adeninivorans* have been overexpressed (Kaur *et al.* 2007). In our previous study (Hirimuthugoda *et al.* 2006), we found that *Hanseniaspora uvarum* strain WZ1, *Yarrowia lipolytica* strain W2B, *K. ohmeri* BG3, *Candida* sp. strain N12C, *Issatchenkia orientalis* strain YF04C, *Candida* sp. strain MA6, *Y. lipolytica* strain YF08, *Candida* sp. strain YF12C, *Candida* sp. strain NY4E and *Candida* sp. strain MB2 isolated from different marine environments could produce phytase (Hirimuthugoda *et al.* 2006). Among them, *K. ohmeri* BG3 was found to produce the largest amount of extracellular phytase. In other studies (Li *et al.* 2008a; Li *et al.* 2008b), phytase production by *K. ohmeri* BG3 was optimized and the phytase produced was purified and characterized. In the present study, the gene encoding phytase in *K. ohmeri* BG3 was cloned and expressed in *Escherichia coli*. To our knowledge, this is the first time that the gene encoding phytase from *K. ohmeri* isolated from the marine environment has been cloned.

Materials and methods

Strains, plasmids, and media

The marine yeast strain *Kodamaea ohmeri* BG3, isolated from the gut of marine fish (*Hexagrammes otakii*) (Hirimuthugoda *et al.* 2006), was used as a source of phytase and as a DNA and RNA donor strain. *Kodamaea ohmeri* BG3 used in this study has been deposited in Marine Microorganism Culture of China (http://www.mccc.org.cn). *Escherichia coli* DH5α and BL21 (DE3) (Novagen, Madison, WI) were used as the host cells to amplify the plasmids carrying the cloned gene and express the target gene, respectively. Plasmid pMD19-T Simple Vector for cloning of PCR products was purchased from TaKaRa Biotechnology (Dalian, China). Expression vector pET-24a (+) was obtained from Novagen. The marine yeast strain used in this study was grown in YPD medium (10 g l^{-1} yeast extract, 20 g l^{-1} polypeptone, 20 g l^{-1} glucose). The *E. coli* transformants were grown in LB medium with 30 μg ml^{-1} kanamycin.

Isolation of DNA, restriction digestions, and transformation

DNA manipulations were carried out using standard methods as described by Sambrook *et al.* (1989). Bacterial plasmid DNA was purified using Perfectprep plasmid minikits (Eppendorf, Hamburg, Germany). Yeast genomic DNA for amplification of the extracellular phytase gene was isolated as described by Chi *et al.* (2007). Restriction endonuclease digestions and DNA ligations were performed according to the manufacturer's recommendations. *Escherichia coli* was transformed with plasmid DNA according to Sambrook *et al.* (1989). Transformants were plated out onto Luria-Bertani (LB) medium containing kanamycin (30 μg ml^{-1}).

DNA sequence and computer analysis

BLAST and ORF Finder programs at the National Center for Biotechnology Information (NCBI) were used for the nucleotide sequence analysis, deduction of the amino acid sequences, and database searches. Multiple sequence alignments of DNA and amino acid were carried out using the programs of DNAMAN 6.0 (http://www.lynnon.com) and Clustal X 1.8 (Thompson *et al.* 1997). A phylogenetic tree of the phytases was constructed by using PHYLIP 3.56 (Felsenstein 1995).

Amplification of the partial DNA fragment encoding the extracellular phytase

The partial DNA fragment encoding phytase from *Kodamaea ohmeri* BG3 was amplified by PCR using the genomic DNA obtained above as a template. The conserved motifs were usually used to design the degenerate primers to clone these homologues. In this case, amino acid sequences of extracellular phytase from different species of eukaryotic microorganisms were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/) and aligned (Fig 1). The reaction system (50 μl) was composed of 5 μl of 10× buffer, 4 μl (2.5 mM) of dNTPs, 1 μl (50 mM) of u1 (Table 1), 1 μl (50 mM) of u2 (Table 1), 1 μl Taq DNA polymerase, 2 μl (10 ng ml^{-1}) of template DNA, and 36 μl H_2O. The conditions for the PCR amplification were as follows: initial denaturation at 94 °C for 5 min, denaturation at 94 °C for 1 min, annealing temperature at 53 °C for 1 min, extension at 72 °C for 1 min, final extension at 72 °C for 10 min. PCR was run for 32 cycles and the PCR cycler was GeneAmp PCR System 2400 (PerkinElmer, Waltham, MA). The PCR products were cloned into pMD-19 T vector and sequenced. The amino acid sequence of the cloned DNA fragment was deduced and protein sequences were aligned using the programs of DNAMAN 6.0 (http://www.lynnon.com) and Clustal X 1.8 (Thompson *et al.* 1997).

Amplification of the open reading frame (ORF) encoding the extracellular phytase using RACE

Total RNA was extracted from *Kodamaea ohmeri* BG3 using RNA simple Total RNA Kit (TianGen, Beijing, China) according to the manufacturer's protocol. The concentration and quality of the total RNA were estimated by measuring the absorbance ratio of 260/280 nm and agarose gel electrophoresis, respectively.

To extend the phytase DNA sequence in the 3′ and 5′ directions, respectively, the four gene specific primers (sense primers: GSP2, NGSP2; anti-sense primers: GSP1, NGSP1; Table 1) were designed according to the sequence of the DNA fragment obtained above. The total RNA obtained above was

```
1      ATGGTTGCAATTTCTAAATTGATGAACAATGGCCTCCTCTTGGCTTCACAGTCTGTGTTT
1       M  V  A  I  S  K  L  M  N  N  G  L  L  L  A  S  Q  S  V  F
61     CAAGATTTGGCAACTCCAGAACAGGCTGCAGTAGAGCAGTACAACATCATCAACTTCTTG
21      Q  D  L  A  T  P  E  Q  A  A  V  E  Q  Y  N  I  I  N  F  L
121    GGTGGCTCTGCTCCATATATACAGCGCAATGGTGCGGGTATTTCAACTGACATTCCTCAA
41      G  G  S  A  P  Y  I  Q  R  N  G  A  G  I  S  T  D  I  P  Q
181    CAATGTACTTTAGAGCATGTGCAATTGTTCTCCAGACACGGTGAGAGGTACCCCGGACTT
61      Q  C  T  L  E  H  V  Q  L  F  S  R  H  G  E  R  Y  P  G  L
241    GATCTCGGTGGGACATTAGAAGATATCTACAAGAAATTTAAAGAGTACAACGGAACTTTC
81      D  L  G  G  T  L  E  D  I  Y  K  K  F  K  E  Y  N  G  T  F
301    AAAGGAGACTTGGCTTTCTTGAACGATTATACCTACTTTGCTGATAACAAGAACTTGTAC
101     K  G  D  L  A  F  L  N  D  Y  T  Y  F  A  D  N  K  N  L  Y
361    GAGAAGGAAACTACTCCAATGAACTCCGAGGGTCTTTTCTCTGGAACATCGGATGCTATG
121     E  K  E  T  T  P  M  N  S  E  G  L  F  S  G  T  S  D  A  M
421    AGACATGGTGCGGCATTCCGTGCCAAATATGGTTCTCTCTACAAAGAAAACACTACATTA
141     R  H  G  A  A  F  R  A  K  Y  G  S  L  Y  K  E  N  T  T  L
481    CCGGTGTTTTCATCTAGTTCCGGTCGTGTGTTCTTGACTGGTGAATATTTCACTAGAGGC
161     P  V  F  S  S  S  S  G  R  V  F  L  T  G  E  Y  F  T  R  G
541    TTCTTTGGTGAAGAGTACTCCGATGAAACTCACAAGTACGTTATTGTTGACGAAGATCCA
181     F  F  G  E  E  Y  S  D  E  T  H  K  Y  V  I  V  D  E  D  P
601    CTGATGGGAGGAAACTCATTGACACCTAGTAATGGATGCACTGCATTTGACTGGTATGCT
201     L  M  G  G  N  S  L  T  P  S  N  G  C  T  A  F  D  W  Y  A
661    AGCGATAAGTTGTTAGAGGCTTACGACACTTCGTATTTAGATGACATCGCTGATCGTTTC
221     S  D  K  L  L  E  A  Y  D  T  S  Y  L  D  D  I  A  D  R  F
721    AACAATGCCAACAAAGGTCTCAACATCTCCTCCACAGAGGTCTCGCATTTATTTGACTGG
241     N  N  A  N  K  G  L  N  I  S  S  T  E  V  S  H  L  F  D  W
781    TGTGCATACGAGCTCAACGTGCGTGGAGCGTCACCTTTCTGTGATATCTTCACCAACGAG
261     C  A  Y  E  L  N  V  R  G  A  S  P  F  C  D  I  F  T  N  E
841    GAGTTCATTCGTGCTTCTTATGCACAAGATTTGTTATACTACTACAGCAATGGTCCTGGA
281     E  F  I  R  A  S  Y  A  Q  D  L  L  Y  Y  Y  S  N  G  P  G
901    AACAATGACAGTGCGCTGGTGGGTTCACCGATTTTGGAAGCTTCATTAAAGTTACTTAAA
301     N  N  D  S  A  L  V  G  S  P  I  L  E  A  S  L  K  L  L  K
961    GACACCGAAGCTAAAAATCAGTTGTGGTTATCATTCACCCACGACTTTGAGATCGAATTT
321     D  T  E  A  K  N  Q  L  W  L  S  F  T  H  D  F  E  I  E  F
1021   TTCCATGCTGCTTTGGGTCTTTTGTCACCAAAAGAGCATTTACCTCTTGGATACATCCCC
341     F  H  A  A  L  G  L  L  S  P  K  E  H  L  P  L  G  Y  I  P
1081   GTACCAAACCCATATGTCCATGCTTCGATAGTTCCCCAGGGTGCCCGGATTTACATCGAG
361     V  P  N  P  Y  V  H  A  S  I  V  P  Q  G  A  R  I  Y  I  E
1141   AAGTATGGATGTGGCAATGACTCGTACGTACGTATCATCGTCAATGATGCTGTCGTCCCC
381     K  Y  G  C  G  N  D  S  Y  V  R  I  I  V  N  D  A  V  V  P
1201   ATTGAAAAGTGCTTTTCTGGACCAGGATTTTCCTGTAAGATTGCGGACTATGAGAAAATT
401     I  E  K  C  F  S  G  P  G  F  S  C  K  I  A  D  Y  E  K  I
1261   ATCAACGATAGACTCAACCAGGAAAACTATCGCCAACACTGTAAAATCGCAATGAATACC
421     I  N  D  R  L  N  Q  E  N  Y  R  Q  H  C  K  I  A  M  N  T
1321   CCCGATCACATTAGTTTTTATTGGAACTACATGAACACGACTTACAACGCTCCACTCGGA
441     P  D  H  I  S  F  Y  W  N  Y  M  N  T  T  Y  N  A  P  L  G
1381   AACTATTAA
461     N  Y  *
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Fig 1 – Nucleotide sequence of the phytase gene of *Kodamaea ohmeri* BG3 and its deduced amino acid sequence. The signal peptide is underlined. The six N-linked glycosylation sites are shaded in grey. The N-terminal and C-terminal motifs RHGXRX P and HD are boxed.

Table 1 – Sequences of the primers used in this study

Primers	Sequences
Primers for DNA fragment PCR	
u1	5′-AGACAYGGTGARAGRTAYCC-3′
u2	5′-ACAWGAGAAWCCWGGWCC-3′
Primers for 3′ RACE PCR	
GSP2	5′- CGTGCGTGGAGCGTCACCTTTCTGT -3′
NGSP2	5′- GGAAACAATGACAGTGCGCTGGTGG -3′
Primers for 5′ RACE PCR	
GSP1	5′- TTCTCGATGTAGATCCGGGCACCCTG -3′
NGSP1	5′- GCATGGACATATGGGTTTGGTACGGG -3′
Primers for genomic DNA PCR	
lxyu	5′- ATGGTTGCAATTTCTAAATTGA -3′
lxyd	5′- TTAATAGTTTCCGAGTGGAGCG -3′
Primers for expression	
ypet1	5′-CCGGAATTCTCACAGTCTGTGTTTCAAG-3′
ypet2	5′- ACGCGTCGACATAGTTTCCGAGTGGAG-3′

reversely transcribed into the first-strand cDNA using Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas Life Sciences, Lithuania). The first-strand cDNA obtained was used as a template. Amplification of the ORF encoding the extracellular phytase was conducted using RACE according to SMART™ RACE cDNA Amplification Kit User Manual. Amplification reactions of the 3′ and 5′ RACE were performed with a thermal cycling profile of 94 °C for 5 min and 32 cycles at 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 3 min, followed by an extension at 72 °C for 10 min. The PCR products were cloned into pMD-19 T vector and sequenced. The amino acid sequence of the cloned cDNA fragment was deduced and protein sequences were aligned using the programs of DNAMAN 6.0 (http://www.lynnon.com) and Clustal X 1.8 (Thompson *et al.* 1997). The extracellular phytase gene cloned from cDNA was named cDNA*PHY1* (the accession number: EU082006).

Cloning of the DNA sequence encoding the extracellular phytase from genomic DNA

To obtain the DNA sequence encoding the extracellular phytase from genomic DNA of *Kodamaea ohmeri* BG3, the gene specific primers lxyu and lxyd listed in Table 1 were designed according to the cDNA*PHY1* sequence obtained above. The PCR reaction was performed in a total volume of 50 μl PCR mixture containing 5 μl of 10× Taq buffer, 4 μl of 2.5 mM dNTPs, 1 μl of 50 μM each primer (lxyu and lxyd), 2 μl (10 ng ml^{-1}) template DNA, 36.5 μl sterile, deionized water and 0.5 μl Taq DNA polymerase. The conditions for the PCR amplification were as follows: initial denaturation at 94 °C for 5 min, denaturation at 94 °C for 1 min, annealing temperature at 55 °C for 1 min, extension at 72 °C for 90 s, final extension at 72 °C for 10 min. PCR was run for 32 cycles. The PCR products were cloned into pMD-19 T vector and sequenced. The amino acid sequence of the cloned DNA fragment was deduced and protein sequences were aligned using the programs of DNAMAN 6.0 (http://www.lynnon.com) and Clustal X 1.8 (Thompson *et al.* 1997). The extracellular phytase gene cloned from the genomic DNA was named *PHY1*.

Phytase expression in Escherichia coli

The cDNA*PHY1* gene without signal sequence was amplified with the primers of ypet1 and ypet2 (Table 1) by PCR. The fragment amplified was ligated into pET-24a (+) with restriction sites of *Eco*R I and *Sal* I to generate construct pET-24a (+)*PHY1*. The expression construct was used to transform *Escherichia coli* BL21 (DE3) for expression of *PHY1*. The transformants with plasmid pET-24a (+) were used as the control. The transformants were screened on LB broth supplemented with 30 μg ml^{-1} kanamycin and cultured by shaking at 37 °C until OD_{600nm} reached 0.4–0.6. Then, IPTG (final concentration 1 mM) was added for induction. The bacterial cells were further cultured at 20 °C overnight.

Preparation of the crude recombinant phytase

The positive transformant cells that had been induced were harvested and washed by centrifugation at 8000*g* and at 4 °C for 5 min with 0.2 M sodium acetate (pH 5). Then, the washed cell pellets were suspended in 5 ml of 0.2 M sodium acetate (pH 5) and submitted to repeated (27) seven s-cycles of ultrasonication using an ultrasonic homogenizer (400 W model, Sonics & Material, Newtown, PA) at maximum output in the ice (Chi *et al.* 2005). After disruption by ultrasonication and removal of cell debris by centrifugation at 14 000*g* and at 4 °C for 20 min, the supernatant obtained was used as the crude recombinant phytase preparation. The lysate from the transformants with plasmid pET-24a (+) was used as the control.

SDS–PAGE analysis and Western blotting

The presence of the recombinant phytase in the crude recombinant phytase preparation from *Escherichia coli* cells obtained above was confirmed in non-continuous denaturing SDS-PAGE (Laemmli 1970) with a two-dimensional electrophoresis system (Amersham Biosciences, Piscataway, NJ) and stained by Coomassie Brilliant Blue R-250 (George & Diwan 1983). The molecular mass standards for SDS-PAGE comprised β-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35 kDa), restriction endonuclease *Bsp*981 (25 kDa), β-lactoglobulin (18.4 kDa), and lysozyme (14.4 kDa).

To confirm the recombinant phytase expression as a His-tagged fusion protein, Western blot analysis was carried out using monoclonal mouse-anti-His-Tag antibody (TianGen, Beijing, China) as the primary antibody and goat anti-mouse IgG antibody conjugated with horseradish peroxidase (HRP; TianGen) as the secondary antibody. After SDS-PAGE, the proteins were transferred to nitrocellulose membrane for 2 h at 17 mA. The membrane was blocked with 5 % skimmed milk in phosphate-buffered saline containing 0.05 % Tween-20 (PBS/T) for 2 h at 37 °C, and then was incubated with the mouse-anti-His-Tag antibody at a dilution of 1:1500 for 2 h at 37 °C and washed three times for 15 min in PBS/T. Subsequently, the membrane was incubated with goat anti-mouse IgG antibody conjugated with horseradish peroxidase (HRP) (TianGen) at a dilution of 1:200 for 2 h at 37 °C. After washing in PBS/T four times and PBS (without Tween-20) once, the

membrane was applied with 3,3-diaminobenzideine (DAB) reagents (TianGen) according to manufacturer's instruction.

Determination of recombinant phytase activity

Phytase activity was assayed as follows: 0.8 ml sodium phytate solution (5 mM sodium phytate in 0.2 M sodium acetate, pH 5) was pre-incubated at 65 °C for 5 min and 0.2 ml of the crude recombinant phytase preparation was added and mixed well. The mixture was incubated 65 °C for 30 min. The reaction was stopped by addition of 1 ml of 50 g l^{-1} trichloroacetic acid (TCA). The inorganic phosphate liberated was quantitatively determined spectrophotometrically at 700 nm *via* the ammonium molybdate method (Chi *et al.* 1999). One unit of phytase activity was defined as the amount of enzyme causing the release of 1 nM inorganic phosphate min^{-1} under assay conditions. Specific phytase activity was units per milligram of protein. Protein concentration was measured by the method of Bradford, and bovine serum albumin served as the standard (Bradford 1976).

Effects of pH and temperature on the crude recombinant phytase activity and stability

The effect of pH on crude recombinant phytase activity was determined by incubating the crude recombinant enzyme between pH 3 and 9 using the standard assay conditions. The buffers used were 0.2 M acetate buffer (pH 3–6) and 0.2 M $Na_2B_40_7.10H_2O$–H_3BO_3 buffer (pH 7–10). pH stability was tested *via* 6 h pre-incubation of the crude recombinant enzyme in appropriate buffers that had the same ionic concentrations at different pH values ranging from 3–10 at 0 °C. The remaining activities of the crude recombinant phytase were measured immediately after this treatment with the standard method as mentioned earlier (Chi *et al.* 2008).

The optimal temperature for activity of the enzyme was determined at 30–70 °C in the same buffer as described above. The temperature stability of the crude recombinant enzyme was tested by pre-incubating the enzyme at different temperatures (20–80 °C) for 1 h, the residual activity was measured immediately as described above. The pre-incubated sample at 0 °C was used as a reference to calculate the residual activity (Chi *et al.* 2008).

Phytate hydrolysis by the crude recombinant phytase

Sodium phytate solution (0.8 ml; 5 mM sodium phytate in 0.2 M sodium acetate, pH 5) was pre-incubated at 65 °C for 5 min, and 0.2 ml of the crude recombinant phytase preparation (final concentration 65 U ml^{-1}) was added and mixed well. The mixture was incubated at 65 °C for 3 h and sampled every hour for determination of degradation. The end products of phytate hydrolysis after 3 h at 65 °C were withdrawn and identified to ascertain the extent of hydrolysis by ascending tlc (Silica gel 60, Merck, Darmstadt, Germany) with the solvent system of 16:6:5.2:4.8:2.8 of chloroform: acetone: methanol: glacial acetic: water. The spots were located by exposing the plates to iodine vapour. Inositol from Sigma was used as the standard during the tlc.

Results

Cloning of extracellular phytase gene from Kodamaea ohmeri *BG3*

The conserved motifs were used to design the degenerate primers to clone the partial gene encoding the extracellular phytase in *Kodamaea ohmeri*. In this case, amino acid sequences of extracellular phytases from different species of eukaryotic microorganisms were downloaded from GenBank and aligned (Fig 1). The PCR-generated fragments were sequenced. Analysis of the sequence by BLAST program indicated that the fragment (1023 bp, accession number EU009483) of the putative phytase gene was isolated as the fragment containing the N-terminal and C-terminal motifs RHGXRX P and HD, which are common characteristics for histidine acid phosphatases (Vats & Banerjee 2004). Then, the new primers (Table 1) were designed according to the sequence of the fragment (EU009483) of the putative phytase gene and used for amplification of the ORF encoding the phytase from cDNA of *K. ohmeri* by RACE as described in Materials and methods. Finally, the cDNA*PHY1* fragment with 1389 bp was obtained and sequenced (Fig 1). The new primers (lxyu1 and lxyu2; Table 1) were designed according to the sequence of cDNA*PHY1* fragment and used for amplification of the *PHY1* gene encoding the phytase from genomic DNA of *K. ohmeri* by PCR. After the sequencing of the PCR products, it was found that the sequence of *PHY1* gene from genomic DNA of *K. ohmeri* BG3 was the same as that of cDNA*PHY1* (Fig 1). This means that there was no intron in *PHY1*gene. After the sequence of *PHY1* gene cloned in this study was aligned with the known sequences of extracellular phytase genes from other fungi by using Nucleotide–**nucleotide** BLAST (blastn; at http://www.ncbi.nlm.nih.gov/Blast), it was found that the sequence of *PHY1* gene had high similarity to that of phytase genes from *Candida albicans* (XM_708385) and *Pichia stipitis* (XM_001385071) (Jones *et al.* 2004; Jeffries *et al.* 2007), confirming that the extracellular phytase gene of *K. ohmeri* BG3 was isolated.

Analysis of Kodamaea ohmeri *BG3 extracellular phytase protein deduced from cDNAPHY1 gene*

The deduced protein from cDNA*PHY1* gene showed 61 and 58 % identity with *Candida albicans* (XP_713452) and *Pichia stipitis* (XP_001385108) phytase proteins (Jones *et al.* 2004; Jeffries *et al.* 2007), respectively. The topology of the phylogram confirms that the amino acid sequence deduced from the cDNA*PHY1* gene was also closely related to the *C. albicans* and *P. stipitis* phytase proteins (Fig 2) (Jones *et al.* 2004; Jeffries *et al.* 2007) and more distantly related to other phytases. The predicted protein from the cDNA*PHY1* gene consisted of 462 amino acids with a calculated molecular mass of 51.9 kDa. Signal peptide analysis of the protein deduced from cDNA*PHY1* gene at http://cbs.dtu.dk/services/SignalP/showed that the signal peptide had 15 amino acids. The peptide bond between the 15th and 16th amino acids would be cleaved by signal peptidase (Fig 1).

N-glycosylation sites of the protein were also analysed at http://cbs.dtu.dk/services/NetNGlyc and the results indicated that there were six potential N-linked glycosylation sites of

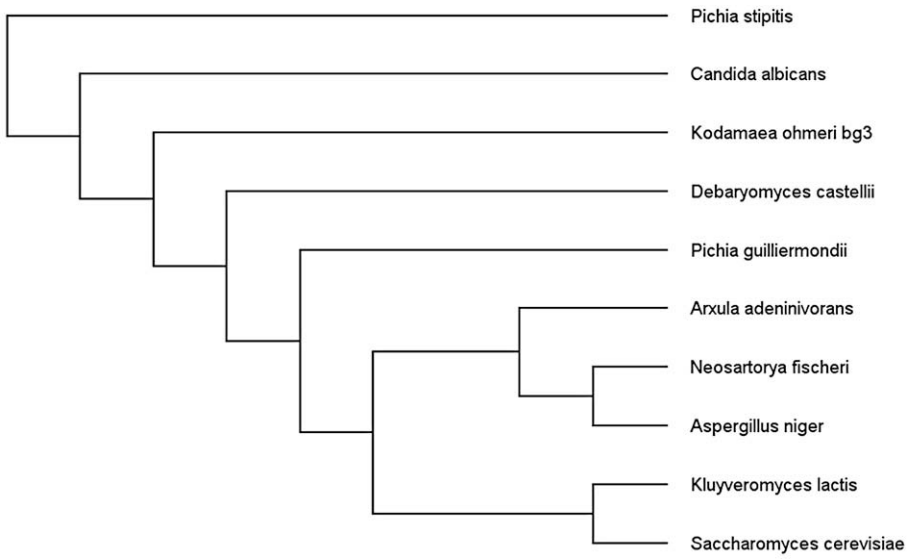

Fig 2 – Phylogenetic tree showing the evolutionary relatedness and levels of homology between the phytase amino acid sequences. The sequences are of the following proteins: *Arxula adeninivorans* (CAJ77470); *Aspergillus niger* (ABC39588); *Candida albicans* (XP_713452); *Debaryomyces castellii* (ABN04184); *Kluveromyces lactis* (CAA83964); *Neosartorya fischeri* (XP_001267206); *Pichia guilliermondii* (CAL69849); *P. stipitis* (XP_001385108); *Saccharomyces cerevisiae* (EDN64708).

the protein: -N-G-T-(97), -N-T-T-(157), -N-I-S-(248), -N-D-S-(302), -N-D-S-(386), -N-T-T-(452), respectively.

Similar to phytases from other eukaryotic microorganisms, the deduced 462-amino-acid protein sequence from the cDNA*PHY1* gene also contained the consensus motifs (RHGXRX PT and HD) which are conserved among histidine acid phosphatases (Fig 1).

Expression of the phytase in Escherichia coli

The phytase gene amplified with the primers ypet1 and ypet2 (Table 1) was cloned into the expression vector pET-24a (+) and the recombinant plasmid containing *PHY1* gene was transformed into BL21 (DE3). The positive transformant BL21 (DE3)/pET-24a (+)*PHY1* obtained was induced by IPTG. After disruption by ultrasonication and removal of cell debris of the induced cells of BL21 (DE3)/pET-24a (+)*PHY1*, the crude recombinant phytase activity in the supernatant obtained was determined and the cell free extract from the transformants with only plasmid pET-24a (+) was used as the control. The results show that specific phytase activity in the cell-free extract from the induced cells of BL21 (DE3)/pET-24a (+)*PHY1* was $16.5 \pm 0.3\ \mathrm{U\ mg^{-1}}$, whereas no phytase activity was detected in the cell-free extract from BL21 (DE3)/pET-24a (+). This means that the cloned phytase gene from *Kodamaea ohmeri* BG3 has been expressed in the *Escherichia coli* cells.

The results of SDS–PAGE analysis show that cell free extracts from the induced cells of *E. coli* BL21 (DE3) harbouring pET-24a (+)*PHY1* exhibited one specific band with a molecular mass of about 51 kDa (lane 2 in Fig 3A–B), which was a fusion hybrid protein and was a similar size (51.9 kDa) to the deduced amino acid sequence of the cDNA*PHY1* gene. It was also found that a large amount of the recombinant phytase existed as inclusion bodies in *E. coli* cells (lane 3 in Fig 3A–B). However, the specific band of the recombinant phytase was not detected in the controls (lane 1 in Fig 3A–B). The results of Western blotting (Fig 3B) demonstrate that the specific band with a molecular mass about 51 kDa (lanes 2 and 3 in Fig 3B) was indeed the 6× His-tagged fusion protein of the recombinant phytase.

Optimum temperature and thermal stability of the crude recombinant phytase

The crude recombinant phytase activity measured as a function of temperature from 30–70 °C shows that the activity was the highest at 65 °C (Fig 4). The thermostability was investigated by pre-incubating the enzyme in the same buffer, as described above, for 1 h and the remaining activity was determined. As shown in Fig 4, the residual phytase activity still kept 93 % of the control after the treatment at 60 °C for 1 h. Fig 4 also reveals that the enzyme was inactivated rapidly at temperatures higher than 65 °C and was inactivated totally at 80 °C within 1 h. From these results, the recombinant phytase seemed to have considerable thermostability.

Optimum pH and pH stability of the crude recombinant phytase

Crude recombinant phytase activity was measured at various pHs in buffers with the same ionic concentrations. Our results (Fig 5) show that the maximum activity was observed at pH 5. pH stability was tested by 6 h pre-incubation of the crude recombinant phytase in appropriate buffers that had the same ionic concentrations at different pH values ranging from 3–10 at 0 °C. The remaining activities of phytase were measured immediately after this treatment with the standard

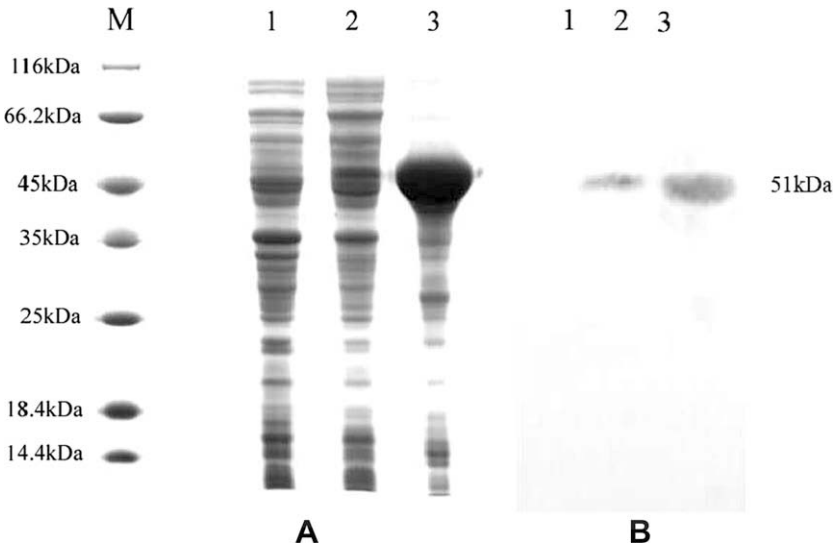

Fig 3 – SDS–PAGE analysis of phytase expression (A) and Western blot analysis of the recombinant phytase/His-Tag (B). Lane M, molecular protein markers; lane A1, the whole cell extract of the induced BL21 (DE3)/pET-24a (+); lane A2, the supernatant of induced BL21 (DE3)/pET-24a (+)*PHY1*; lane A3, the pellet of the induced BL21 (DE3)/pET-24a (+)*PHY1*; lane B1: Western blot of the whole cell extract of the induced BL21 (DE3)/pET-24a (+); lane B2: Western blot of the supernatant of induced BL21 (DE3)/pET-24a (+)*PHY1*; lane B3: Western blot of the pellet of the induced BL21 (DE3)/pET-24a (+)*PHY1*.

method as mentioned above. It can be seen from the results in Fig 5 that the activity profile of the enzyme was stable in the pH range from 3–8.

Phytate hydrolysis by the crude recombinant phytase

It can be noted from Fig 6 that most of phytate in the reaction mixture could be converted into different sizes of hydrolysis products by the action of the crude recombinant phytase within 3 h. This means that the crude recombinant phytase could actively hydrolyse phytate within a short period.

Discussion

The extracellular phytase structural gene (cDNA*PHY1*) isolated from cDNA of the marine yeast *Kodamaea ohmeri* BG3 had an ORF of 1389 bp (Fig 1). The coding region of the *PHY1* gene had no intron. It encoded 462 amino acid residues of a protein with a calculated molecular mass of 51.9 kDa and had a putative signal peptide of 15 amino acids. However, in our previous studies (Li *et al.* 2008b), we found that the molecular mass of the purified phytase from the marine yeast strain was estimated

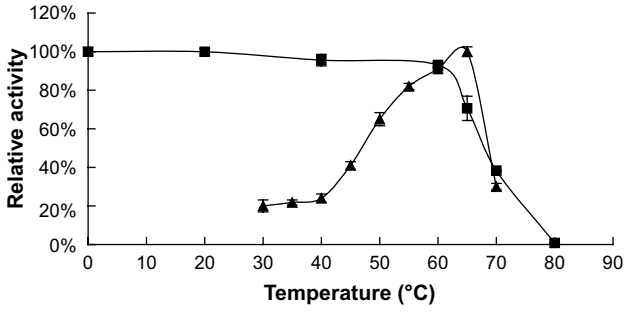

Fig 4 – Effect of temperature on activity of the crude recombinant phytase. (■) Thermal stability of the recombinant phytase; (▲) optimal temperature. Temperature stability of the crude recombinant phytase was tested by pre-incubating the enzyme at different temperatures (0, 20, 40, 60, 65, 70, and 80 °C) for 1 h, the residual activity was measured as described above immediately. Here, pre-incubated sample at 0 °C was used a reference to calculate the residual activity. Data are given as means ± S.D., $n = 3$.

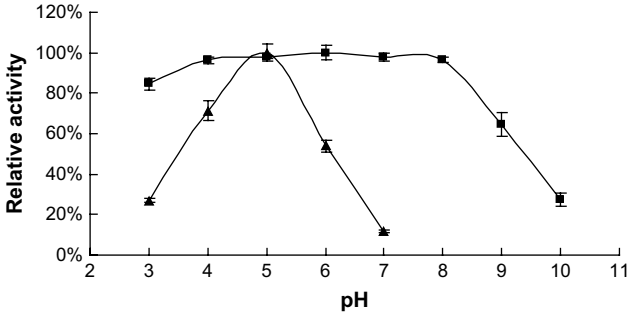

Fig 5 – Effect of pH on activity of the crude recombinant phytase. (■) pH stability of the crude recombinant phytase and (▲) optimal pH. pH stability was tested by 6 h pre-incubation of the crude recombinant enzyme in appropriate buffers that had the same ionic concentrations at different pH values ranging from 3–10 at 0 °C. The remaining activities of phytase were measured immediately after this treatment with the standard method as mentioned in the text. Values are given as mean ± S.D., $n = 3$.

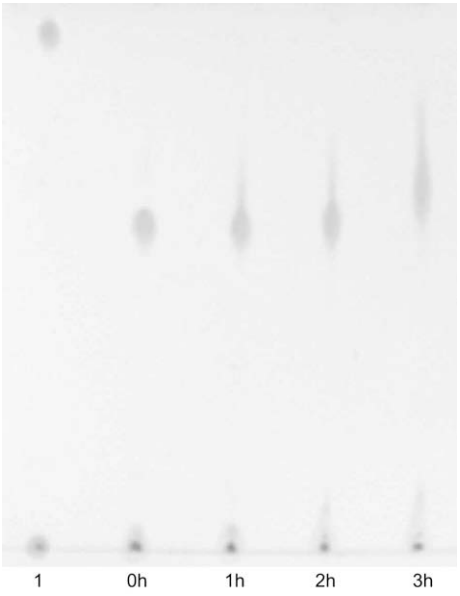

Fig 6 – Thin-layer chromatogram of the hydrolysis products of phytate with the crude recombinant phytase. Lanes numbered left to right: lane 1, Inositol; lane 2, phytate + the crude recombinant phytase for 0 h; lane 3, the hydrolysis products for 1 h; lane 4, the hydrolysis products for 2 h; lane 5, the hydrolysis products for 3 h. The end products of phytate hydrolysis were analysed by using a tlc plate with the solvent system 160 ml chloroform, 60 ml acetone, 52 ml methanol, 48 ml glacial acetic acid, and 28 ml water. The spots were located by exposing the plates to iodine vapour.

to be 92.9 kDa. As discussed below, there were many N-glycosylation sites in the enzyme. Therefore, the phytase may undergo many N-glycosylations and other processes during secretion in the cells of the wild-type so that the molecular mass of the produced phytase was much higher than calculated molecular mass of the phytase. The signal peptide of phytase produced by *Emericella nidulans* comprises the first 22 amino acids of the enzyme whereas the first 14 amino acids of the enzyme corresponds to the putative signal peptide of phytase produced by the thermophilic fungus *Talaromyces thermophilus* (Pasamontes *et al.* 1997). The sequence of the extracellular acid phosphatase encoded by the *APHO1* gene includes an N-terminal secretion sequence of 17 amino acids (Kaur *et al.* 2007). From the results, it can be seen that a shorter signal peptide of the phytase produced by *T. thermophilus* occurs than that of the phytase produced by the marine yeast *K. ohmeri* BG3. The protein sequence deduced from the extracellular phytase structural gene (cDNA*PHY1*) contained the consensus motifs, RHGXRX P and HD, which are conserved among all the histidine acid phosphatases (Fig 1) and six conserved putative N-glycosylation sites (Fig 1). According to the phylogenetic tree of the phytases, the phytase from *K. ohmeri* BG3 was closely related to *Candida albicans* and *Pichia stipitis* phytases and more distantly related to other phytases (Jones *et al.* 2004; Jeffries *et al.* 2007), respectively (Fig 2). The *APHO1* gene encoding extracellular acid phosphatase from *Arxula adeninivorans* gene harbours an ORF of 1449 bp encoding a protein of 483 amino acids with a calculated molecular mass of 52.4 kDa (Kaur *et al.* 2007). The deduced amino acid sequence from *APHO1* gene exhibits 54 % identity to phytases from *Aspergillus awamori*, *A. niger* and *A. ficuum* and a more distant relationship to phytases of the yeasts *C. albicans* and *D. hansenii* (36–39 % identity) (Kaur *et al.* 2007). The 461-amino-acid sequence deduced from the ORF encoding the *Debaryomyces castellii* CBS 2923 phytase corresponded to a 51.2 kDa protein (Ragon *et al.* 2008). No signal sequence cleavage site was detected. Nine potential N-glycosylation sites have been predicted. The protein shared 21–69 % sequence identities with various phytases of yeast or fungal origin. This suggests that the deduced amino acid sequence from the cDNA*PHY1* gene obtained in this study also had a distance relationship to APHO1 and phytase from *D. castellii*. All of these results demonstrate that PHY1 produced by the marine yeast *K. ohmeri* BG3 was greatly different from the phytases from other yeasts.

The cloned phytase gene from *K. ohmeri* can be expressed in *Escherichia coli* cells. It has been reported that the gene (*phyA*) for the *A. niger* phytase with optima at pH 5.5 and 2.2 was expressed in *E. coli* under the control of the T7*lac* promoter (Phillippy & Mullaney 1997). Heterologous expression of the *D. castellii* CBS 2923 phytase in the methylotrophic yeast *P pastoris* was tested and maximum production levels obtained were 476 U ml^{-1} (Ragon *et al.* 2008). The recombinant phytase produced by the cells of *E. coli* BL21(DE3) harbouring pET-24a (+)*PHY1* had a molecular mass of about 51 kDa (lane 2 in Fig 3A–B), which was a fusion hybrid protein and was the similar size to the deduced protein (51.9 kDa) from the cDNA*PHY1* gene. However, a large amount of the recombinant phytase existed as inclusion bodies in *E. coli* cells. Therefore, the expression of cDNA*PHY1* gene in *E. coli* cells needs to be further improved.

We found that the activity of the crude recombinant phytase was the highest at 65 °C and the enzyme was stable up to 65 °C (Fig 4). In our previous study (Li *et al.* 2008b), the activity of the purified phytase from *K. ohmeri* BG3 was also found to be the highest at 65 °C and was stable up to 60 °C. This means that the optimal temperature and stability of the crude recombinant phytase from *E. coli* cells was similar to those of the native phytase from *K. ohmeri* BG3. Phytases, in general, show high activity in the temperature range of 50–70 °C, while the temperature for their maximal activity is mostly between 45 and 60 °C (Vats & Banerjee 2004). However, the phytase produced by *D. castellii* exhibited the activity at high temperatures, with maximal activity at 77 °C and thermostability up to 74 °C (Pandey *et al.* 2001; Segueilha *et al.* 1992).

The optimal pH of the recombinant phytase was 5 and the phytase was stable in the pH range 3–8 (Fig 5). In our previous study (Li *et al.* 2008b), the maximum activity of the purified phytase from *K. ohmeri* BG3 was observed at pH 5, and the enzyme was very stable in the pH range 3–9. This indicates that the optimal pH and stability of the recombinant phytase from *E. coli* cells were also similar to those of the native phytase from *K. ohmeri* BG3. Generally, the phytases from fungi had the optimum pH range of 2.5–6, and the stability of phytases decreased dramatically above pH 7.5 and below pH 3, with a few exceptions of lower pH optima of 2 (Han *et al.* 1999; Pandey *et al.* 2001; Vats & Banerjee 2004). For example, the optimum pH of the phytase produced by *D. castellii* was 4.4 (Segueilha *et al.* 1992).

The recombinant phytase obtained in this study could actively hydrolyse phytate, but it could not fully hydrolyse the 6-phosphates binding of the myo-inositol hexakisphosphate (Fig 6). It has been reported that a novel phytase from the yeast *D. castellii* is able to fully hydrolyse the 6-phosphates binding of the myo-inositol hexakisphosphate (Ragon *et al.* 2008). The use of phytase has been shown to be very effective in producing different inositol phosphate species (Vats & Banerjee 2004). Therefore, phytases that do not fully hydrolyse the 6-phosphates binding of myo-inositol hexakisphosphate may have potential applications in producing different inositol phosphate species.

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