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Biochemical characterization and *in vitro* digestibility assay of *Eupenicillium parvum* (BCC17694) phytase expressed in *Pichia pastoris*

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ABSTRACT

A mature phytase cDNA, encoding 441 amino acids, from *Eupenicillium parvum* (BCC17694) was cloned into a *Pichia pastoris* expression vector, pPICZαA, and was successfully expressed as active extracellular glycosylated protein. The recombinant phytase contained the active site RHGXRXP and HD sequence motifs, a large α/β domain and a small α-domain that are typical of histidine acid phosphatase. Glycosylation was found to be important for enzyme activity which is most active at 50 °C and pH 5.5. The recombinant phytase displayed broad substrate specificity toward *p*-nitrophenyl phosphate, sodium-, calcium-, and potassium-phytate. The enzyme lost its activity after incubating at 50 °C for 5 min and is 50% inhibited by 5 mM Cu^{2+}. However, the enzyme exhibits broad pH stability from 2.5 to 8.0 and is resistant to pepsin. *In vitro* digestibility test suggested that BCC17694 phytase is at least as effective as another recombinant phytase (r-A170) which is comparable to Natuphos, a commercial phytase, in releasing phosphate from corn-based animal feed, suggesting that BCC17694 phytase is suitable for use as phytase supplement in the animal diet.

Introduction

Phytate (*myo*-inositol hexakis phosphate) is the primary storage form of phosphorus and inositol in plant seeds and grains which serve as a major source of nutrients for the animals [1]. However, the bound phosphorus in phytate is poorly utilized in digestive tract of monogastric animals such as pig and chicken. The expensive inorganic phosphorus, therefore, is supplemented in animal feeds. Meanwhile, the excess inorganic phosphorus and unutilized phytate are excreted causing environmental pollution [2]. Furthermore, phytate in animal feeds can interfere with the absorption of divalent cations, carbohydrates and proteins in small intestine by forming complexes with these nutrients [3]. These problems can be solved if animal feeds are supplemented with phytase [4].

Phytase (*myo*-inositol hexakisphosphate phosphohydrolase) catalyzes the removal of phosphate group from phytate to give *myo*-insitol derivatives and inorganic phosphate. Based on biochemical and catalytic properties, phytases can be divided into two major classes: histidine acid phosphatase (HAP; EC 3.1.3.2)[1] and alkaline phosphatase [4]. Phytases are found naturally in plants and microorganisms, particularly fungi. At present, all commercial feed additive phytases are produced by recombinant strains of filamentous fungi such as Natuphos (BASF) from *Aspergillus niger*, Finase (AB Enzymes) from *A. awamori* and Bio-Feed Phytase (Novozymes) from *Peniophora lycii* [5].

Current estimate of global fungal biodiversity is about 1.5 million species [6]. Since Thailand is in the tropics, she is possibly being home to ~70,000–150,000 fungi species. Previously, through our initial screening of ~150 fungi, several fungal strains demonstrated active phytase at wide pH ranges. One of these is *Eupenicillium parvum* (BCC17694), which was shown to produce phytase that is active at both the pH 2 and 5.5, which are close to the pH of animal gut (pH 2.7) and small intestine (pH 5.9), respectively [7]. The enzyme also exhibited high level of phytase activity at 40 °C (approximately the temperature of animal stomach, crop and gizzard). This suggested the possibility of using our locally isolated phytase as a potential enzyme in animal feed industry. In this study, this novel phytase from *E. parvum* (BCC17694) is cloned, fused in-frame with α-factor within pPICZαA vector, an *Escherichia coli*/*Pichia pastoris* shuttle vector, and expressed *via* methanol-inducible promoter in *P. pastoris*, a methylotrophic yeast [8]. Finally, the recombinant phytase enzyme is purified, biochemically characterized, and preliminary tested for its suitability for animal feed application.

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[1] *Abbreviations used:* HAP, histidine acid phosphatase; BCC, BIOTEC culture collection; AP, adaptor primer; SOE, splice overlap extension; *p*NPP, *p*-nitrophenyl phosphate.

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Materials and methods

Strains, plasmids, culturing conditions, and primers

Eupenicillium parvum (BCC17694) was obtained from the BIOTEC Culture Collection (BCC), Thailand. It was isolated from soil in the central region of Thailand. The fungus was inoculated in 2% soluble starch medium (2% soluble starch, 3% glucose, 0.86% $NaNO_3$, 0.05% KCl, 0.05% $MgSO_4$, 0.01% $FeSO_4$) and grown at 25 °C with continuous shaking at 250 rpm. After 7 days, the mycelia were separated from the media by filtration through double-layered cheese cloth. *E. coli*, DH5α, was used as host for plasmid propagation and *P. pastoris* KM71 was used as host for phytase expression. All oligonucleotides (Table 1) were purchased from PROLIGO, Singapore.

Fungal genomic DNA extraction and touchdown nested-PCR

BCC17694 genomic DNA was extracted *via* SDS-based DNA extraction method [9] and used as template in touchdown (−0.5 °C/cycle) nested-PCR with degenerate primers (PhyI and PhyA380 for first PCR; PhyA120 and PhyA370 for the second PCR; Table 1) using 1 U DyNAzymeII (Finnzyme, Finland). The amplicon of the expected size was gel-purified using QIAquick gel extraction kit (Qiagen, Germany), cloned into pGEM-T Easy vector (Promega, USA) and sequenced (Macrogen Inc., Korea).

Total RNA isolation, 3′RACE, and Genome Walking

BCC17694 total RNA was isolated from mycelia using TRI Reagent (Molecular Research Center, USA). For 3′RACE, cDNAs were generated from 2 μg of total RNA using oligo(dT) adaptor (PM1) and SuperScript III reverse transcriptase (Invitrogen, USA). PCR was performed using a gene-specific primer (3′RacePhy) and PM2, an adaptor primer. See table 1 for details of the primers employed.

Since 5′RACE failed, the partial 5′ end cDNA of BCC17694 phytase was obtained by RT-PCR. Briefly, first-strand cDNAs were generated using partial-heat denaturation reverse transcription method [10] using 5′RacePhy2Nested, a gene-specific primer and Superscript III reverse transcriptase. PCR was performed using a degenerate primer (PhyI) and again 5′RacePhy2Nested primer.

The remaining 5′end was obtained by using Genome Walker Universal Kit (Clontech, USA). Briefly, BCC17694 genomic DNA was digested with *Pvu*II and purified using Wizard DNA clean-up system (Promega, USA). Then, a GenomeWalker adaptor was ligated to the digested genomic DNA. The DNA was then used as template in a nested-PCR. The first PCR was performed using 0.2 μM of adaptor primer (AP1) and a gene-specific primer (5′RacePhy3), 1 μl *Pvu*II-DNA library, 1.5 mM $MgCl_2$, 0.2 mM each of dNTPs and 2 U DyNAzyme II DNA polymerase. The second PCR condition was as the first except that it contained 0.2 μM of nested adaptor primer (AP2) and nested gene-specific primer (5′RacePhy4Nested), 1 μl of 1:50 dilution of the first PCR product as template.

Table 1
Oligonucleotides used in this study.

Primer names	Sequence (5′ → 3′)
PhyI	MGICAYGGIGMNMGITAYC
PhyA120	GCITTYYTIAARWSITAYAAYTA[a]
PhyA370	RTCRTGISWRAARTCIRCRTA[a]
PhyA380	CCRTTRTANARICCIARNGC[a]
PM1	CCGGAATTCAAGCTTCTAGAGGATCCTTTTTTTTTTTTTTT
PM2	CCGGAATTCAAGCTTCTAGAGGATCC
3′RacePhy	CATCGCCCGTTTAACCCACTC
5′RacePhy2Nested	CTTGGGTGTTGGTTGGTGGC
5′RacePhy3	GAACGCAGCATCTCCCTTGAAATC
5′RacePhy4Nested	CGGCATACCTCACGCTCTTC
Adaptor Primer 1 (AP1)	GTAATACGACTCACTATAGGGC
Adaptor Primer 2 (AP2)	ACTATAGGGCACGCGTGGT
ExPhy-F1	CCGCTCGAGAAAAGAATGCCCAGATACGGAAG
ExPhy-F2	CCGCTCGAGAAAAGAATGGGTGCCGTGATCC
ExPhy-R1	ATGCGGCCGCTTAAGAATAGCAACTCGCCC
MuXhoI-Phy-F	TGCCCAAAACTTGAGAACGACTCGCTCAGC
MuXhoI-Phy-R	TGAGCGAGTCGTTCTCAAGTTTTGGGCAGG
ExPhy-R2	CCGCTCTAGATTAAGAATAGCAATCGCCC
ExPhy-RPRA	CCGCTCGAGAAAAGACGTCCACGAGCCTGC

[a] For degenerate primer, the following abbreviations are used, (N = A, T, G, C; M = A, C; K = G, T; Y = C, T; R = A, G; W = A, T; S = C, G; H = A, C, T; D = A, G, T; V = A, C, G; I = Inosine).

Cloning and expression of BCC17694 phytase in P. pastoris

Gene-specific primers were designed to amplify the full-length phytase cDNA. First-strand cDNAs were reverse transcribed using Superscript III and oligo(dT) adaptor (PM1). Then, PCR was performed using ExPhy-F2 and ExPhy-R1 primers. The phytase gene was subcloned into pPICZαA, using the *Xho*I and *Xba*I sites. Since there is a XhoI site in the middle of the full-length gene, a silent mutation was generated using splice overlap extension (SOE) PCR technique [11]; at the same time, the codon usage of *P. pastoris* was also improved. The upstream part of XhoI site was changed by using ExPhy-F2 and MuXhoI-Phy-R primers while the downstream part of XhoI site was changed using MuXhoI-Phy-F and Exphy-R2 primers. The PCR fragments of both reactions were gel-purified and used as template in SOE-PCR.

The mature BCC17694 phytase cDNA was amplified using ExPhy-RPRA and ExPhy-R2 primers using the silent mutated full-length phytase gene as template. The PCR product was gel-purified and double-digested with XhoI and XbaI (Promega, USA) before it was ligated in-frame with the α-factor secretion signal present in pPICZαA. Next, the recombinant plasmids were linearized with BglII (Promega, USA) and electroporated into *P. pastoris*.

For small-scale expression, five integrants were randomly chosen and were grown in 5 ml BMGY media at 30 °C, 250 rpm, until OD_{600} reached 5–6. To induce expression, cells were pelleted and resuspended in 1 ml BMMY containing 2% (v/v) methanol. Absolute methanol was added every 24 h to maintain induction. The culture was collected everyday for 10 days. The secreted proteins were analyzed by using SDS–PAGE [12] and also tested for phytase activity. Scaled-up expression was carried out in 600 ml BMMY under the same conditions for 7 days.

Purification of recombinant BCC17694 phytase

The culture media containing secreted phytase was centrifuged twice: 4000*g*, 5 min and then 10,000*g* for 20 min at 4 °C. The secreted protein was concentrated by using Amicon Ultra-15 30 K (Millipore, USA). The concentrated proteins were changed into a new buffer (20 mM sodium acetate, pH 4.5) using HiTrap desalting column (Amersham Bioscience, USA). Next, the proteins were purified by using HiTrap SP XL, cation-exchange chromatography (Amersham Bioscience, USA), equilibrated with 20 mM sodium acetate, pH 4.5. Fractions containing phytase activity were pooled and further concentrated by Amicon Ultra-15 10K before further purified by gel filtration (Superdex 200, Amersham Bioscience, USA), equilibrated with 20 mM sodium acetate buffer, pH 4.5, containing 150 mM NaCl at room temperature and ran on AKTA Purifier (Pharmacia, USA). The enzyme was eluted with the same buffer. The active fractions of phytase activity were pooled and stored at 4 °C for further analysis.