J. Microbiol. Biotechnol. (2008), **18**(7), 1221–1226

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Cloning, Expression, and Characterization of a New Phytase from the Phytopathogenic Bacterium *Pectobacterium wasabiae* DSMZ 18074

Shao, Na, Huoqing Huang, Kun Meng, Huiying Luo, Yaru Wang, Peilong Yang, and Bin Yao*

Department of Microbial Engineering, Feed Research Institute, Chinese Academy of Agricultural Sciences, No. 12 Zhongguancun South Street, Beijing 100081, P. R. China

Received: December 21, 2007 / Accepted: February 1, 2008

The soft rot bacterium *Pectobacterium wasabiae* is an economically important pathogen of many crops. A new phytase gene, *appA*, was cloned from *P. wasabiae* by degenerate PCR and TAIL-PCR. The open reading frame of *appA* consisted of 1,302 bp encoding 433 amino acid residues, including 27 residues of a putative signal peptide. The mature protein had a molecular mass of 45 kDa and a theoretical pI of 5.5. The amino acid sequence contained the conserved active site residues RHGXRXP and HDTN of typical histidine acid phosphatases, and showed the highest identity of 48.5% to PhyM from *Pseudomonas syringae*. The gene fragment encoding the mature phytase was expressed in *Escherichia coli* BL21 (DE3), and the purified recombinant phytase had a specific activity of 1,072±47 U/mg for phytate substrate. The optimum pH and temperature for the purified phytase were pH 5.0 and 50°C, respectively. The K_m value was 0.17 mM, with a V_{max} of 1,714 μmol/min/mg. This is the first report of the identification and isolation of phytase from *Pectobacterium*.

Keywords: Histidine acid phosphatase, *Pectobacterium wasabiae*, phytase, phytopathogenic bacteria

Phytate (*myo*-inositol hexakisphosphate), the major storage form of phosphorus in cereal grains, legumes, pollens, and oil seeds [17, 18, 24], is an antinutrient in animal feed. Monogastric animals such as poultry and pigs are incapable of digesting phytate phosphorus because they lack phytase [24]. Undigested phytate not only reduces the feed quality, but also leads to environmental pollution. Phytase (*myo*-inositol hexakisphosphate phosphohydrolase; E.C. 3.1.3.8 or 3.1.3.26) is an enzyme that initiates the hydrolysis of phytate to various lower-phosphate *myo*-inositol derivatives and inorganic phosphates [17]. It has been applied broadly as an additive in the feed industry to improve uptake of phosphorus and other nutrients, and to reduce phosphorus pollution of animal waste.

Currently, four classes of phosphatase enzymes are known to degrade phytic acid: histidine acid phosphatase (HAP), cysteine phytase (CPhy), purple acid phosphatase (PAP), and β-propeller phytase (BPP) [6, 16]. However, most phytases with relatively higher specific activity and better catalytic properties belong to the histidine acid phosphatase (HAP) family [25]. Several bacterial phytase genes of the HAP family have been cloned, such as from *Citrobacter amalonaticus* [15], *Escherichia coli* [5, 20, 26], *Klebsiella* sp. [21], *Obesumbacterium proteus* [27], *Pseudomonas* sp. [4, 10, 11], and *Yersinia intermedia* [9], most of which are from *Enterobacteriaceae*.

In this study, a new phytase gene was cloned from *Pectobacterium wasabiae*, which causes soft rot in stored grains and blackleg or stem rot in economically important crops [7, 19]. The gene was heterologously expressed in *E. coli*, and the recombinant phytase was purified and characterized. The superior biochemical properties of the recombinant phytase suggest that it may be an attractive enzyme for application in the animal feed industry.

Materials and Methods

Strains, Plasmids, and Chemicals

Pectobacterium wasabiae, exhibiting phytase activity, was purchased from the German Resource Centre for Biological Material (DSMZ, Accession No. 18074). The strain was cultivated by shaking at 250 rpm at 30°C in LB medium (yeast extract, 5 g/l; NaCl, 10 g/l; and tryptone, 10 g/l). *E. coli* JM109 (TaKaRa, Japan) and vector pGEM-T Easy (Promega, U.S.A.) were used for cloning and sequencing. *E. coli* BL21 (DE3) and vector pET-22b(+) (Novagen, U.S.A.) were used for gene expression. All chemicals were of analytical grade, commercially available.

Cloning and Sequencing of Phytase Gene

To clone the phytase gene, we used a two-step PCR that included degenerate PCR and TAIL-PCR [9, 13, 15]. Based on the two amino

*Corresponding author
Phone: 86-10-68975126; Fax: 86-10-68975127;
E-mail: yaobin@caas-bio.net.cn

Table 1. Primers used in this study.

Primer name	Primer sequence	Size (bases)
FI	5'-AGCCGCCATGGNGTRCGNYCNCC-3'	23
RI	5'-TWKGCMAKRTTRGTATCATG-3'	20
usp1	5'-CGTGTGTGGCAACACCGCAACCGG-3'	24
usp2	5'-CATATAAGCCTGTGCGGTTGCGCGA-3'	25
usp3	5'-CCGTGGCGGCATAGCCATGTCCG-3'	23
dsp1	5'-CGGTTCGACTTCTATCAGTGGCCTGAATACGC-3'	32
dsp2	5'-GCTGGAATATAGCGAAAACCTGCCGCTAG-3'	29
dsp3	5'-CAGCGCGGTGGTTCGCTATTGATG-3'	24
AD10	5'-TCTTICGNACITNGGA-3'	16
AD6	5'-CAWCGICNGAIASGAA-3'	16
PmF[a]	5'-GATGAATTCGCAGGATCGCTATCTGCTGGAA-3'	31
PmR[a]	5'-GACGCGGCCGCTTATGGCGCGTAATGCACCGTTGCTAAC-3'	39

[a]The EcoRI (PmF) and NotI (PmR) restriction sites are underlined.

acid sequence motifs (RHGXRXP and HDTN) conserved among HAP phytases from *Enterobacteriaceae*, we designed degenerate primers (FI, RI; Table 1) using the preferred codons in *Enterobacteriaceae*. To obtain the core region between the two conserved blocks, degenerate PCR was performed with an annealing temperature of 48°C and *P. wasabiae* DSMZ 18074 genomic DNA as template. The PCR product was cloned into vector pGEM-T Easy for sequencing. TAIL-PCR was used to identify the DNA sequences that flank the core region [13]. The nested insertion-specific primers for TAIL-PCR were designed and named as usp1-3 (upward special primers) and dsp1-3 (downward special primers), respectively (Table 1). The nucleotide sequence for *appA* from *P. wasabiae* was deposited in GenBank under Accession No. EU203663.

Nucleotide Sequence Analysis

Sequence data were assembled and analyzed by Vector NTI 7.0. Multiple alignments of protein sequences were carried out using CLUSTALW (http://www.ebi.ac.uk/clustalW/). The promoter and signal peptides were predicted using BPROM (http://www.softberry.com/berry.html) and SignalP (http://www.cbs.dtu.dk/services/SignalP), respectively. Homology searches in GenBank were performed using the BLAST server.

Expression of Recombinant Phytase

The gene *appA* lacking the signal peptide coding sequence was amplified by PCR from the genomic DNA of *P. wasabiae* using primers PmF and PmR (Table 1) and inserted into vector pET-22b(+) with EcoRI and NotI. The resulting plasmid was transformed into *E. coli* BL21 (DE3). Positive transformant cells were grown at 37°C in 1,000 ml of LB medium containing 100 μg/ml ampicillin up to OD_{600}=0.6. Phytase expression was induced by addition of 1 mM IPTG, and the cultures were further incubated at 30°C for 4 h. The cells were removed by centrifugation, and the phytase activity in the supernatants was measured as described below. The recombinant protein produced in *E. coli* by *appA* was named r-APPA.

Phytase Activity Assay

Phytase activity was determined by the modified ferrous sulfate-molybdenum blue method [8, 9]. Briefly, 50 μl of enzyme solution was incubated with 950 μl of substrate solution (1.5 mM sodium phytate in 0.25 M sodium acetate buffer, pH 5.0) at 37°C for 15 min. The reaction was stopped by adding 1 ml of 10% (w/v) trichloroacetic acid (TCA). The released inorganic phosphate was analyzed by adding 2 ml of a coloring reagent C, including 1% (w/v) ammonium molybdate, 3.2% (v/v) sulfuric acid, and 7.2% (w/v) ferrous sulfate, and the optical density was measured at 700 nm. One unit (U) of phytase activity was defined as the amount of enzyme that released 1 μmole of phosphate per minute at 37°C. All phytase activity determinations were performed in triplicate.

Purification of the Recombinant Enzyme

Ammonium sulfate was added to the cell-free supernatant to 40% of saturation, followed by centrifugation at 10,000 ×*g* for 15 min at 4°C. The precipitate was then resuspended in buffer A (20 mM Tris-HCl, pH 8.0) and dialyzed against the same buffer. The dialysate (5 ml) was then applied to a 5-ml HiTrap Q Sepharose XL FPLC column (Amersham Pharmacia, Sweden). The phytase was eluted with a linear salt gradient of 0–1.0 M NaCl in buffer A at a flow rate of 1.0 ml/min. Fractions containing phytase activity (~0.25–0.32 M NaCl) were pooled, and the protein concentration was determined by the Bradford assay [1] using bovine serum albumin as a standard.

Characterization of the Purified r-APPA

The optimum pH of the purified r-APPA was determined by measuring the activity over the pH range of 2.0–8.0. The buffers were 0.1 M glycine-HCl, pH 2.0–3.5; 0.1 M sodium acetate, pH 3.5–6.0; 0.1 M Tris-acetate, pH 6.0–7.0; and 0.1 M Tris-HCl, pH 7.0–8.0. The phytase was diluted in these buffers that contained 0.05% bovine serum albumin and 0.05% (w/v) Triton X-100. The effect of pH on r-APPA stability was estimated by measuring the residual enzyme activity under standard conditions (pH 5.0, 37°C, 15 min) after incubating the phytase in the various buffer systems for 1 h.

The optimum temperature for r-APPA activity was determined under standard conditions but over the range of 20–70°C at 5° interval. Thermal stability was measured by assessing enzyme activity under standard conditions after incubation of the enzyme in 0.1 M sodium acetate (pH 5.0) at 60°C or 70°C for 2, 5, 10, or 20 min, respectively.

The effect of metal ions and chemical reagents on the activity of the purified enzyme was determined by measuring phytase activity

at the presence of 1 mM or 5 mM Na^+, K^+, Ca^{2+}, Li^+, Co^{2+}, Cr^{3+}, Ni^{2+}, Cu^{2+}, Mg^{2+}, Fe^{3+}, Mn^{2+}, Zn^{2+}, Pb^{2+}, Ag^+, Hg^{2+}, SDS, or ethylenediaminetetraacetic acid (EDTA). The test system without addition of irons or reagents was used as the control. Data are shown as the mean of three independent experiments.

To determine resistance to proteolysis, the residual enzyme activity was determined after incubating protease (either pepsin or trypsin) in the purified phytase solution at 1:10 ratio at 37°C for 5, 10, 20, 30, 60, and 120 min, respectively.

Substrate Specificity and Kinetic Analysis of r-APPA

Substrate affinity of the purified enzyme was determined by replacing phytic acid in the standard assay mixture with an equal concentration (1.5 mM) of the following phosphorylated compounds: 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), *p*-nitrophenyl phosphate, and glucose 6-phosphate. The kinetic parameters (K_m and V_{max}) were determined from Lineweaver-Burk plots with different concentrations of sodium phytate (0.0625–2 mM).

RESULTS

Cloning and Sequencing of the Phytase Gene

The core region of *appA* was amplified by degenerate PCR with primers FI and RI and sequenced. Based on the core region sequence (857 bp), nested insertion primers were designed to clone the 5' and 3' flanking regions of the core region by TAIL-PCR. Two PCR products from the 5' and 3' flanking regions (about 1,000 bp and 620 bp, respectively) were obtained using the primers AD10 and AD6 (Table 1), respectively. Finally, the upstream and downstream sequences were assembled with the core region sequence. One open reading frame was evident from 179–1,480 bp (Fig. 1), which encoded 433 amino acid residues with a calculated protein molecular mass of 48 kDa. The N-terminal 27 residues constituted a signal peptide with a putative processing site (QAQ-DR). The conserved active site residues RHGXRXP and HDTN (as found in other HAP family phytases) were identified in the *appA* sequence. Comparison of the amino acid sequence with that of other functional phytases revealed that r-APPA is most identical (48.5%) to the phytase PhyM from *P. syringae* [4], suggesting that *appA* encodes a new phytase.

Expression and Purification of *appA* in *E. coli*

The recombinant plasmid (pET-*appA*) was constructed and transformed into *E. coli* BL21 (DE3). When the positive clones were induced by IPTG, the r-APPA activity was

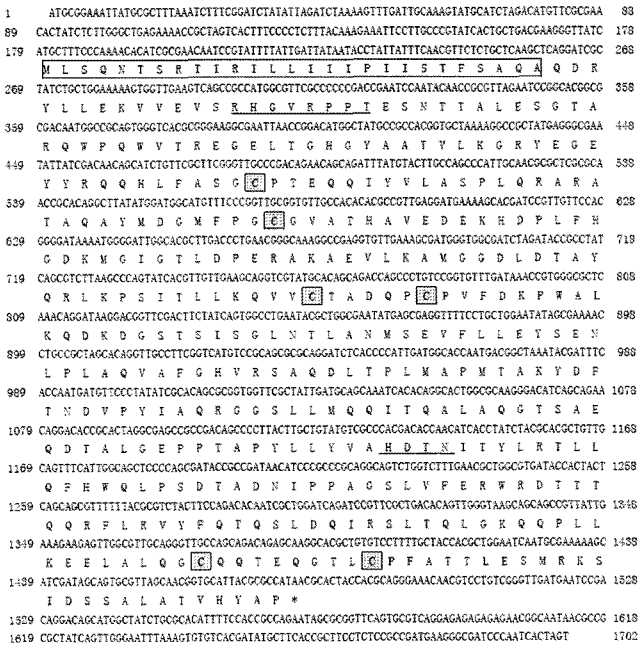

Fig. 1. Nucleotide and deduced amino acid sequences of *appA*. The deduced amino acid sequence is shown under the nucleotide sequence. Two conserved sites are underlined. The putative signal peptide is boxed. The cysteine residues are marked in grey boxes. The asterisk indicates a stop codon.

detected in the extracellular space and cell lysate. After 4 h of induction by IPTG, the maximum phytase activity in the culture medium reached 2.86 U/ml.

The recombinant enzyme was purified from the culture medium by ammonium sulfate fractionation and anion-exchange chromatography (Table 2). As a result, the specific activity of the purified r-APPA was 1,072±47 U/mg at pH 5.0 and 37°C after 43-fold purification, with a final activity yield of 15.9%. The purified r-APPA gave a single protein band of 45 kDa on SDS-PAGE gel, in accordance with its predicted molecular mass (Fig. 2).

Properties of the Purified Phytase

The purified r-APPA showed maximum activity at pH 5.0 and displayed more than 70% of peak activity at acidic pH values (4.0–5.5) (Fig. 3A). The r-APPA activity was stable over a wide range of pH; more than 80% of the total activity was retained after incubation at pH 3.0–10.0 at 37°C for 60 min.

The optimum temperature of the r-APPA activity was 50°C (Fig. 3B). The phytase displayed remarkable thermal

Table 2. Purification of r-APPA expressed in *E. coli*.

Purification steps	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)
Culture supernatant	500.0	1,430.0	57.4	24.9	100.0
$(NH_4)_2SO_4$ precipitation	15	835.1	3.56	234.4	58.4
Ion-exchange chromatography	5	227.4	0.212	1,072	15.9

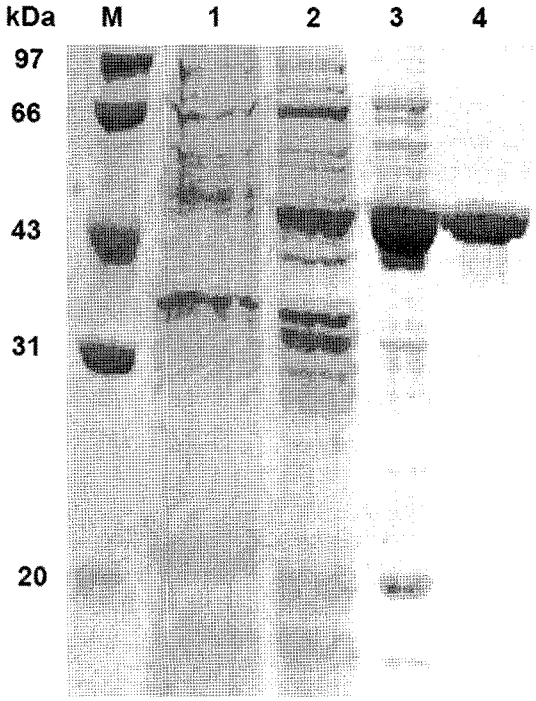

Fig. 2. SDS-PAGE analysis of the purified r-APPA expressed in *E. coli* BL21 (DE3).
Lane M, standard protein molecular mass markers; lane 1, culture supernatant of the uninduced transformant harboring pET-*appA*, as a control; lane 2, culture supernatant of the induced transformant harboring pET-*appA*; lane 3, precipitate of the culture supernatant after adding ammonium sulfate to 40% of saturation; lane 4, purified enzyme after performing anion-exchange chromatography.

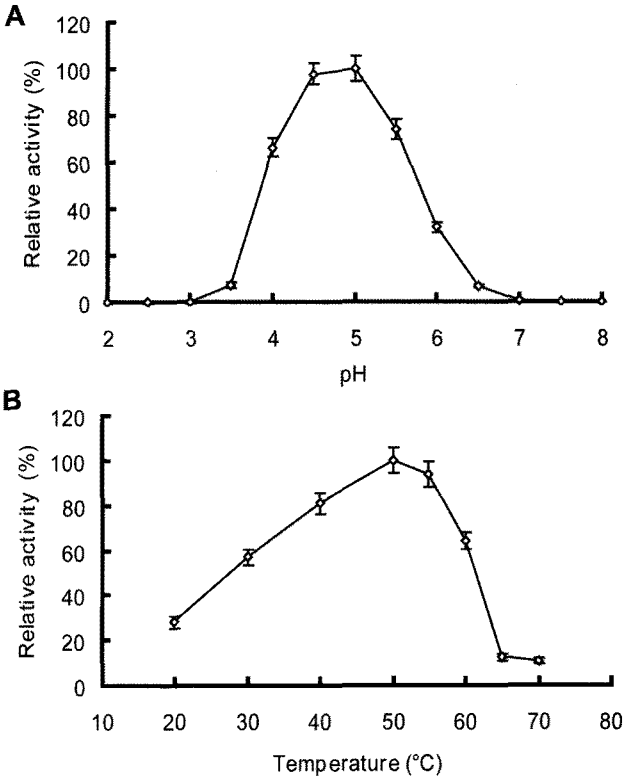

Fig. 3. Effects of pH and temperature on r-APPA activity.
A. pH profile of r-APPA activity. The enzyme assay was performed at 37°C in buffers of varying pH, and the relative activity was expressed as a percentage of the maximum activity, taken as 100% at pH 5.0. **B**. Optimal temperature for r-APPA. The enzyme assay was performed in 0.25 M acetate buffer (pH 5.0) containing 1.5 mM sodium phytate. The value obtained at 50°C was taken as 100%. The values in both panels are given as the mean±SD of three replicates.

stability when incubated at 50°C for 30 min, and over 70% activity retained. However, it lost activity rapidly at higher temperatures, with more than 90% activity lost after incubating at 70°C for 2 min.

The phytase activity of the r-APPA in the presence (1 mM or 5 mM each) of different metal ions or chemical reagents is shown in Table 3. The activity was absolutely inhibited by Hg^{2+} and SDS, even at 1 mM ion concentration (Table 3). Partial inhibition was observed in the presence of some metal ions at 1 mM or 5 mM concentration, and the enzyme inhibition was in the order of Fe^{3+}>Cu^{2}+Zn^{2}+Cr^{3}+>Ag^{+}. However, Ca^{2+}, Na^{+}, Mg^{2+}, and EDTA at 1 mM or 5 mM, respectively, appeared to slightly enhance the phytase activity (Table 3).

The enzyme had weak resistance to proteinase. After treatment with pepsin at 37°C for 10 min or at trypsin

Table 3. Effect of metal ions and chemical reagents on the activity of purified recombinant phytase.

Metal ions and chemical reagents	Relative activity (%)		Metal ions and chemical reagents	Relative activity (%)	
	1 mM	5 mM		1 mM	5 mM
Control	100.0	100.0	Mn^{2+}	84.6	72.7
EDTA	113.5	112.4	Pd^{2+}	67.4	18.4
Ca^{2+}	111.9	114.2	Ag^{+}	25.1	15.8
Mg^{2+}	106.9	98.7	Cr^{3+}	24.8	18.6
Na^{+}	106.6	122.4	Zn^{2+}	11.2	1.3
Li^{+}	101.6	115.8	Cu^{2+}	2.9	0.2
K^{+}	100.8	117.2	Fe^{3+}	2.4	1.7
Co^{2+}	94.8	80.6	Hg^{2+}	0.6	0.4
Ni^{2+}	92.2	78.8	SDS	0.4	0.3

at 37°C for 30 min, respectively, the enzyme lost over 90% activity.

The enzyme had a narrow substrate specificity, exhibiting 100% relative activity for phytic acid and almost no activity when the substrate was 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), *p*-nitrophenyl phosphate, or glucose 6-phosphate. For sodium phytate as substrate, the K_m and V_{max} were 0.17 mM and 1,714 μmol/min/mg, respectively.

DISCUSSION

The *P. wasabiae* phytase contains conserved active site residues (RHGXRXP and HDTN) that are typical of HAP family members, and it is most identical (48.5%) to PhyM from *P. syringae*, indicating its novelty among this family. Compared with PhyM, r-APPA has an acidic optimum pH of 5.0 (*vs.* pH 5.5 for PhyM) and higher specific activity (1,072 U/mg *vs.* 649 U/mg for PhyM [3]). Moreover, r-APPA activity is stable over a relatively wide range of pH (pH 3.0–10.0), suggesting its potential application in the feed industry.

It is well established that disulfide bonds can affect the thermal stability of proteins. Amino acid sequence analysis showed that r-APPA contains six cysteine residues, less than the eight or ten in phytases from *E. coli* [12] and *Aspergillus fumigatus* [23], which could form four and five disulfide bonds, respectively. Therefore, less disulfide bonds are assured in r-APPA and the structure of r-APPA is likely less rigid, leading to relatively poor thermal stability in comparison with the commercial recombinant phytases from *E. coli* [14] and *A. fumigatus* [23]. This characteristic could be improved by additional engineering and thus making it more adaptable to industrial requirements.

Xanthomonas oryzae pv. *oryzae* is an important rice bacterial pathogen that causes leaf blight. The *X. oryzae* pv. *oryzae* phytase gene (*phyA*) has been reported as a novel phytopathogenicity related gene [2]. *X. oryzae* pv. *oryzae* mutants lacking *phyA* are virulence-deficient owing to the inability to utilize host phytic acid, implying a correlation between phytase genes and phytopathogenicity. By searching bacterial genomes in the available databases, several genes encoding phytases belonging to the HAP family were also found in common to be phytopathogenic bacteria, including *Erwinia carotovora* subsp. *atroseptica*, *P. syringae* pv. *syringae*, and *Xanthomonas campestris* pv. *vesicatoria*, and several alkaline phytases were also found in *Pseudomonas* spp. and *Xanthomonas* spp. We also cloned the phytase gene from plant pathogens, *Erwinia* and *Dickeya* (data not shown). To some extent, the result proved the point of Chatterjee *et al.* [2] that the phytase gene was a novel phytopathogenicity related gene. Moreover, the r-APPA retained relative high activity at 20°C (Fig. 3B), which is the optimal temperature for *P. wasabiae* virulence, resulting in plant diseases [22]. Similar phenomena also exist in *P. syringae* and its phytase (PhyM) [3].

Collectively, these results raise the intriguing possibility that phytases from different bacteria might have a certain relationship with bacterial pathogenicity. It is possible that phytases exist in many other phytopathogenic bacteria. This study thus extends our knowledge of bacterial phytases. However, elucidation of the exact roles of phytases in bacterial pathogenicity will require further study.

Acknowledgments

This research was supported by the National Basic Research Program of China (973 Program, Grant No. 2004CB719606) and the National High Technology Research and Development Program of China (863 Program, Grant No. 2006AA020101).

REFERENCES

1. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
2. Chatterjee, S., R. Sankaranarayanan, and R. V. Sonti. 2003. PhyA, a secreted protein of *Xanthomonas oryzae* pv. *oryzae*, is required for optimum virulence and growth on phytic acid as a sole phosphate source. *Mol. Plant Microbe Interact.* **16:** 973–982.
3. Cho, J. S., C. W. Lee, S. H. Kang, and J. C. Lee. 2003. Purification and characterization of a phytase from *Pseudomonas syringae* MOK1. *Curr. Microbiol.* **47:** 290–294.
4. Cho, J. S., C. W. Lee, S. H. Kang, J. C. Lee, and H. G. Lee. 2005. Molecular cloning of a phytase gene (*phyM*) from *Pseudomonas syringae* MOK1. *Curr. Microbiol.* **51:** 11–15.
5. Choi, W. C., B. C. Oh, H. K. Kim, E. S. Lee, and T. K. Oh. 2002. Medium optimization for phytase production by recombinant *Escherichia coli* using statistical experimental design. *J. Microbiol. Biotechnol.* **12:** 490–496.
6. Chu, H. M., R. T. Guo, T. W. Lin, C. C. Chou, H. L. Shr, H. L. Lai, T. Y. Tang, K. J. Cheng, B. L. Selinger, and A. H. Wang. 2004. Structures of *Selenomonas ruminantium* phytase in complex with persulfated phytate: DSP phytase fold and mechanism for sequential substrate hydrolysis. *Structure* **12:** 2015–2024.
7. Duarte, V., S. H. de Boer, L. J. Ward, and A. M. R. de Oliveira. 2004. Characterization of atypical *Erwinia carotovora* strains causing blackleg of potato in Brazil. *J. Appl. Microbiol.* **96:** 535–545.
8. Holman, W. I. M. 1943. A new technique for the determination of phosphorus by the molybdenum blue method. *Biochem. J.* **37:** 256–259.
9. Huang, H., H. Luo, P. Yang, K. Meng, Y. Wang, T. Yuan, Y. Bai, and B. Yao. 2006. A novel phytase with preferable characteristics from *Yersinia intermedia*. *Biochem. Biophys. Res. Commun.* **350:** 884–889.

10. In, M. J., E. S. Jang, Y. J. Kim, and N. S. Oh. 2004. Purification and properties of an extracellular acid phytase from *Pseudomonas fragi* Y9451. *J. Microbiol. Biotechnol.* **14:** 1004–1008.
11. Kim, Y. H., M. N. Gwon, S. Y. Yang, T. K. Park, C. G. Kim, C. W. Kim, and M. D. Song. 2002. Isolation of phytase-producing *Pseudomonas* sp. and optimization of its phytase production. *J. Microbiol. Biotechnol.* **12:** 279–285.
12. Lim, D., S. Golovan, C. W. Forsberg, and Z. Jia. 2000. Crystal structures of *Escherichia coli* phytase and its complex with phytate. *Nat. Struct. Biol.* **7:** 108–113.
13. Liu, Y. G. and R. F. Whittier. 1995. Thermal asymmetric interlaced PCR: Automatable amplification and sequencing of insert end fragments from P1 and YAC clones for chromosome walking. *Genomics* **25:** 674–681.
14. Luo, H., B. Yao, T. Yuan, Y. Wang, X. Shi, N. Wu, and Y. Fan. 2004. Overexpression of *Escherichia coli* phytase with high specific activity. *Chinese J. Biotechnol.* **20:** 78–84.
15. Luo, H., H. Huang, P. Yang, Y. Wang, T. Yuan, N. Wu, B. Yao, and L. Fan. 2007. A novel phytase *appA* from *Citrobacter amalonaticus* CGMCC 1696: Gene cloning and overexpression in *Pichia pastoris*. *Curr. Microbiol.* **55:** 185–192.
16. Mullaney, E. J. and A. H. J. Ullah. 2003. The term phytase comprises several different classes of enzymes. *Biochem. Biophys. Res. Commun.* **312:** 179–184.
17. Mullaney, E. J., C. Daly, and A. H. J. Ullah. 2000. Advances in phytase research. *Adv. Appl. Microbiol.* **47:** 157–199.
18. Pandey, A., G. Szakacs, C. R. Soccol, J. A. Rodriguez-Leon, and V. T. Soccol. 2001. Production, purification and properties of microbial phytases. *Bioresour. Technol.* **77:** 203–214.
19. Perombelon, M. C. M. and A. Kelman. 1980. Ecology of the soft rot erwinias. *Annu. Rev. Phytopathol.* **18:** 361–397.
20. Rodriquez, E., Y. Han, and X. G. Lei. 1999. Cloning, sequencing and expression of an *Escherichia coli* acid phosphatase/phytase gene (*appA2*) isolated from pig colon. *Biochem. Biophys. Res. Commun.* **257:** 117–123.
21. Sajidan, A., A. Farouk, R. Greiner, P. Jungblut, E. C. Muller, and R. Borris. 2004. Molecular and physiological characterization of a 3-phytase from soil bacterium *Klebsiella* sp. ASR1. *Appl. Microbiol. Biotechnol.* **65:** 110–118.
22. Smadja, B., X. Latour, S. Trigui, J. F. Burini, S. Chevalier, and N. Orange. 2004. Thermodependence of growth and enzymatic activities implicated in pathogenicity of two *Erwinia carotovora* subspecies (*Pectobacterium* spp.). *Can. J. Microbiol.* **50:** 19–27.
23. Wang, Y., X. Gao, Q. Su, W. Wu, and L. An. 2007. Cloning, expression, and enzyme characterization of an acid heat-stable phytase from *Aspergillus fumigatus* WY-2. *Curr. Microbiol.* **55:** 65–70.
24. Wodzinski, R. J. and A. H. J. Ullah. 1996. Phytase. *Adv. Appl. Microbiol.* **42:** 263–302.
25. Wyss, M., R. Brugger, A. Kronenberger, R. Remy, R. Fimbel, G. Oesterhelt, M. Lehmann, and A. P. van Loon. 1999. Biochemical characterization of fungal phytases (*myo*-inositol hexakisphosphate phosphohydrolases): Catalytic properties. *Appl. Environ. Microbiol.* **65:** 367–373.
26. Yin, C., Z. Z. Zhu, X. A. Lin, Y. Z. Yi, Z. F. Zhang, and G. F. Shen. 2005. Overexpression and characterization of *appA* phytase expressed by recombinant *Baculovirus*-infected silkworm. *J. Microbiol. Biotechnol.* **15:** 466–471.
27. Zinin, N. V., A. V. Serkina, M. S. Gelfand, A. B. Shevelev, and S. P. Sineoky. 2004. Gene cloning, expression and characterization of novel phytase from *Obesumbacterium proteus*. *FEMS Microbiol. Lett.* **236:** 283–290.