J. Microbiol. Biotechnol. (2005), 15(4), 745–748

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Purification and Characterization of a Novel Extracellular Alkaline Phytase from *Aeromonas* sp.

SEO, MYUNG-JI[1], JEONG-NYEO KIM[2], EUN-AH CHO[2], HOON PARK[3], HAK-JONG CHOI[4], AND YU-RYANG PYUN[2]*

[1]*Bioproducts Research Center and* [2]*Department of Biotechnology, Yonsei University, Seoul 120-749, Korea*
[3]*Division of Applied Biological Sciences, Sunmoon University, Asan, Chung Nam 336-708, Korea*
[4]*Gwen Knapp Center for Lupus and Immunology Research, Committee on Immunology and Department of Pathology, University of Chicago, IL 60637-5420, U.S.A.*

Received: September 16, 2004
Accepted: December 31, 2004

Abstract A phytase from *Aeromonas* sp. LIK 1-5 was partially purified by ammonium sulfate precipitation and DEAE-Sephacel column chromatography. Its molecular weight was 44 kDa according to SDS-PAGE gel. Enzyme activity was optimal at pH 7 and at 50°C. The purified enzyme was strongly inhibited by 2 mM EDTA, Zn^{2+}, Co^{2+}, or Mn^{2+}, and activated by 2 mM Ca^{2+}. The K_m value for sodium phytate was 0.23 mM, and the enzyme was resistant to trypsin. The N-terminal amino acid sequence of the phytase was similar to that of other known alkaline phytases. The phytase was specific for ATP and sodium phytate, which is different from other known alkaline phytases. Based on the substrate specificity, the phytase may therefore be a novel alkaline phytase.

Key words: *Aeromonas* sp., alkaline phytase, ATP hydrolysis, sodium phytate

Phytic acid (myo-inositol hexaphosphate; phytate) is abundant in plant materials used as animal feedstuffs. However, the phytic acid has a low bioavailability for monogastric animals due to the lack of phytase and contributes to environmental pollution. The addition of phytases to monogastric animal feeds may ameliorate the antinutritive properties of phytic acid and decrease phosphate pollution [9, 10].

Recently, based on their biochemical properties and amino acid sequence, phytases have been classified into HAPs (histidine acid phytases) and alkaline phytases. Most phytases, such as *Aspergillus niger* phytase, belong to HAPs, which share a conserved RHGXRXP motif, are stimulated by EDTA, and exhibit a broad substrate specificity. However, alkaline phytases, such as that from *Bacillus amyloliquefaciens*, do not have the conserved motif, are inhibited by EDTA, and have a high substrate specificity for sodium phytate. Despite these considerable differences between HAPs and alkaline phytases, most enzymatic characterization of phytases has been focused on HAPs, and only limited knowledge on alkaline phytases is currently available [3, 4, 12, 14].

In this study, the extracellular alkaline phytase-producing *Aeromonas* sp. was isolated from Indonesia soil and identified. The molecular and biochemical properties of the phytase were investigated to expand the knowledge on alkaline phytases.

*Corresponding author
Phone: 82-2-2123-2883; Fax: 82-2-312-6821;
E-mail: yrpyun@yonsei.ac.kr

Materials and Methods

Isolation, Cultivation, and Identification of Phytase-Producing Bacteria

Soil samples from Likupang area in Indonesia were incubated on BHI (Brain Heart Infusion, Difco) agar plates at 37°C for 24 h, and then overlaid with a soft agar medium (100 mM sodium acetate, pH 5, 10 mM sodium phytate, 50 mM $CaCl_2$, and agar 0.8%). Colonies producing a clear zone were selected and cultivated on MPB medium (maltose 1%, peptone 1%, beef extract 0.5%, $CaCl_2$ 0.1%, and $MgSO_4 \cdot 7H_2O$ 0.1%, pH 7). Identification of the bacteria was performed by 16S rDNA sequence analysis. To determine the 16S rDNA sequence, the genomic DNA of the isolated LIK 1-5 was isolated by the method of Zhou *et al.* [18]. The PCR (Polymerase Chain Reaction) was performed to amplify a partial 16S rDNA fragment of LIK 1-5 using the universal primers (FP: 5'-AGAGTTTGATCCTGGCTCAG-3' and RP: 5'-GGTTACCTTGTTACGACTT-3'). The amplified PCR product was then purified from the agarose gels using a gel elution kit (Qiagen),

Table 1. Purification scheme of *Aeromonas* sp. LIK 1-5 phytase.

Step	Total activity (Unit)	Total protein (mg)	Specific activity (Unit/mg)	Purification (fold)	Yield (%)
Crude enzyme solution	974.6	1,292.0	0.8	1.0	100.0
Ammonium sulfate[a]	376.1	58.6	6.4	8.6	38.6
DEAE-Sephacel[b]	38.4	2.2	17.3	23.0	3.9

[a]Culture supernatans were precipitated with ammonium sulfate (80% saturation) and resuspended in 10 mM Tris-HCl buffer (pH 8).
[b]The dialyzed fractions were loaded onto a DEAE-Sephacel column and eluted with 200 mM NaCl.

ligated into a pGEM-T vector (Promega), and sequenced according to the manufacturer's instructions. The 16S rDNA sequence of strain LIK 1-5 was then aligned with reference sequences obtained from the GenBank databases (NCBI).

Phytase Activity Assay

The modified method of Engelen and Heeft [4] was used to measure the phytase activity. The reaction was performed in 250 μl of 0.1 M Tris-HCl buffer (pH 7) containing 2 mM sodium phytate at 37°C for 30 min. The reaction was stopped by adding 250 μl of 10% (w/v) trichloroacetic acid. One unit of phytase activity was defined as the amount of enzyme required to liberate 1 μmol of phosphate per min under the standard assay condition.

Purification of Phytase

In order to produce the enzyme, *Aeromonas* sp. LIK 1-5 was cultivated at 37°C, 200 rpm, and pH 7 in 2 l of MPB medium for 48 h. The culture broth was collected by centrifugation and used as a crude enzyme solution. The phytase was purified through ammonium sulfate precipitation and ion-exchange chromatography.

Protein Determination and Analysis of N-Terminal Amino Acid

Protein concentrations were measured by the Bradford method [1]. The purified enzyme was electrophoretically transferred to a sheet of polyvinylidene difluoride (PVDF) membrane (Bio-Rad) after SDS-PAGE using 15% (v/v) gel. The phytase band was cut out and its N-terminal amino acid sequence was analyzed at the Analytical Core Facility of Tufts University (Boston, MA, U.S.A.).

Results and Discussion

Isolation and Identification of Phytase-Producing Bacteria

The isolated strains showing the clear zone were tested for extracellular phytase production. Among the strains isolated, LIK 1-5 strain had the highest phytase activity. Based on the result of 16S rDNA sequence analysis and phylogenetic tree, this strain was identified as *Aeromonas* sp. and it was, therefore, designated as *Aeromonas* sp. LIK 1-5. This is the first report that the strain of *Aeromonas* sp. produces the phytase.

Purification of *Aeromonas* sp. LIK 1-5 Phytase

The purification scheme of the phytase is summarized in Table 1. The enzyme was purified 23-fold with 4% overall yield. The specific activity of the purified enzyme was 17 U mg^{-1}.

Molecular Properties of Phytase

The molecular mass of the purified enzyme was approximately 44 kDa, as determined by SDS-PAGE (Fig. 1). The fungal phytases have larger molecular weights; for example, 85–100 kDa for *A. fumigatus* [17] and 120–140 kDa for *A. oryzae* [15]. On the other hand, most bacterial phytases have molecular weights of 38–47 kDa. The phytase from *E. coli* [7] has molecular mass of 42 kDa and that of *B. amyloliquefaciens* [13] phytase is 44 kDa, similar to that of *Aeromonas* sp. LIK 1-5 phytase.

The N-terminal amino acid sequence analysis of the phytase is indicated in Table 2. Among the 14 amino acids analyzed, 9 amino acids (YHFTVNAAA) were the same as those of *Bacillus* sp. phytase, but the first, second, and third amino acid residues of the phytase were replaced with glutamine, proline, and tyrosine, respectively. On the other hand, the N-terminal amino acid sequences of the

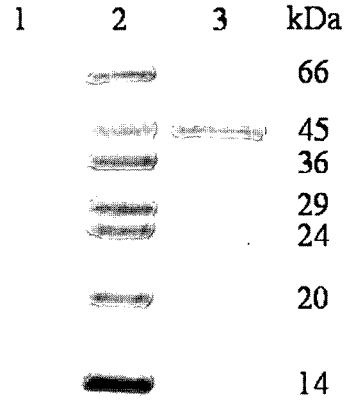

Fig. 1. SDS-PAGE analysis of the phytase from *Aeromonas* sp. LIK 1-5.
Electrophoresis was performed on a 15% polyacrylamide gel, and proteins were stained with Coomassie Brilliant Blue R-250. Lanes: 1, crude enzyme solution; 2, molecular size standards from Sigma; 3, the purified *Aeromonas* sp. LIK 1-5 phytase.

Table 2. N-terminal amino acid sequences of *Aeromonas* sp. LIK 1-5 phytase and comparison with sequences of other phytases.

Phytase-producing origin	N-terminal amino acid sequences	Reference
Aeromonas sp.	Gln-Pro-Tyr-Tyr-His-Phe-Thr-Val-Asn-Ala-Ala-Ala-Glu-Thr[a]	This study
Bacillus sp.	Ser-Asp-Pro-Tyr-His-Phe-Thr-Val-Asn-Ala-Ala-Ala	Kim *et al.* [10]
E. coli	Ser-Glu-Pro-Glu-Leu-Lys-Leu-Glu-Ala-Val-Val	Greiner *et al.* [6]
Aspergillus niger	Ala-Ser-Arg-Asn-Gln-Ser-Ser-Cys-Asp-Thr-Val	Hartingsveldt *et al.* [8]
Pseudomonas syringae	Ala-Ser-Ala-Asp-Gly-Tyr-Val-Leu-Asp-Lys-Val-Val-Gln	Cho *et al.* [2]
Citrobacter braakii	Glu-Glu-Gln-Asn-Gly-Met-Lys-Leu-Glu-Arg	Kim *et al.* [11]

[a]The 9 amino acid sequences from 4[th] to 12[th] amino acid sequence were the same, compared with *Bacillus* sp. reported by Kim *et al.* [10].

Aeromonas sp. LIK 1-5 phytase were quite different from the other phytases such as *E. coli* and *Pseudomonas syringae* phytases, because the other phytases do not have the sequence YHFTVNAAA.

Biochemical Properties of Phytase

The optimum temperature of the phytase was approximately 50°C, and decreased sharply at 60°C. The phytase was stable up to 50°C for 60 min, and the residual activity was about 60%, after preincubation of the enzyme at 60°C for 60 min. The phytase showed high activity at pH 7 and was virtually inactive at pH below 5 and above 9. The phytase was strongly inhibited by 2 mM EDTA, Zn^{2+}, Co^{2+}, or Mn^{2+}, but activated by Ca^{2+} (data not shown). These biochemical properties were similar to those of alkaline phytases [14]. The Michaelis constant (K_m) of the phytase for sodium phytate was 0.23 mM. This K_m value is much higher than that of *A. ficuum* phytase (0.04 mM) [16], but lower than that of *Bacillus* sp. DS11 phytase (0.55 mM) [13] and *E. coli* phytase (0.78 mM) [6]. The substrate specificities of the phytase on several phosphate esters are summarized in Table 3. The phytase was very specific for sodium phytate, ATP, and ADP. This result is of interest since the substrate specificity of the HAPs is broad, and that of alkaline phytases is very specific for only sodium phytate [14]. To determine the protease resistance, the phytase was incubated with 0.1 mg ml^{-1} trypsin at 37°C for 2 h, and the phytase activity was not significantly changed with trypsin.

Aeromonas sp. LIK 1-5 phytase, which has not been reported before, was similar to alkaline phytases produced by microorganisms such as *B. amyloliquefaciens*, but different from HAPs in their molecular and biochemical properties, especially amino acid sequence, metal effect, and substrate specificity. Additionally, the three amino acid residues among the fourteen N-terminal amino acid sequences of the phytase were different from those of other known alkaline phyases, and the phytase was specific for ATP as well as sodium phytate. The results indicate that the phytase from *Aeromonas* sp. LIK 1-5 is a novel alkaline phytase.

Table 3. Substrate specificity of *Aeromonas* sp. LIK 1-5 phytase.

Substrates (2 mM)	Relative activity[a] (%)
α-D-glucose-1-phosphate	0
Tetrasodium pyrophosphate	0
Sodium phytate	100
β-Glycerophosphate	0
Sodium hexametaphosphate	0
ATP	104
ADP	47
p-Nitrophenylphosphate	0

[a]Hydrolysis rate of sodium phytate was taken as 100%.

REFERENCES

1. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
2. Cho, J.-S., C.-W. Lee, S.-H. Kang, J.-C. Lee, J.-D. Bok, Y.-S. Moon, H.-G. Lee, S.-C. Kim, and Y.-J. Choi. 2003. Purification and characterization of a phytase from *Pseudomonas syringae* MOK1. *Curr. Microbiol.* **47:** 290–294.
3. Choi, W.-C., B.-C. Oh, H.-K. Kim, S.-C. Kang, and T.-K. Oh. 2002. Characterization and cloning of a phytase from *Escherichia coli* WC7. *Kor. J. Microbiol. Biotechnol.* **30:** 1–7.
4. Choi, Y. M., D. O. Noh, S. H. Cho, H. K. Lee, H.-J. Suh, and S.-H. Chung. 1999. Isolation of a phytase-producing *Bacillus* sp. KHU-10 and its phytase production. *J. Microbiol. Biotechnol.* **9:** 223–226.
5. Engelen, A. J. and F. C. Heeft. 1993. Simple and rapid determination of phytase activity. *J. AOAC. Int.* **77:** 761–764.
6. Golovan, S., G. Wang, J. Zhang, and C. W. Forsberg. 2000. Characterization and overproduction of the *Escherichia coli* appA encoded bifunctional enzyme that exhibits both phytase and acid phosphatase activities. *Can. J. Microbiol.* **46:** 59–71.
7. Greiner, R., U. Konietzny, and K. D. Jany. 1993. Purification and characterization of two phytases from *Escherichia coli*. *Arch. Biochem. Biophys.* **303:** 107–113.
8. Hartingsveldt, W., C. M. Zeijl, G. M. Harteveld, R. J. Gouka, M. E. Suykerbuyk, R. G. Luiten, P. A. Paridon, G. C. Selten, A. E. Veenstra, and R. F. Gorcom. 1993. Cloning, characterization and overexpression of the phytase-enccding gene (phyA) of *Aspergillus niger*. *Gene* **127:** 87–94.

9. 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.
10. Kim, D. H., B. C. Oh, W. C. Choi, J. K. Lee, and T. K. Oh. 1999. Enzymatic evaluation of *Bacillus amyloliquefaciens* phytase as a feed additive. *Biotechnol. Lett.* **21:** 925–927.
11. Kim, H. W., Y. O. Kim, J. H. Lee, K. K. Kim, and Y. J. Kim. 2003. Isolation and characterizatio of a phytase with improved properties from citrobacter braakii. *Biotechnol. Lett.* **25:** 1231–1234.
12. 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.
13. Kim, Y. O., H. K. Kim, K. S. Bae, J. H. Yu, and T. K. Oh. 1998. Purification and properties of a thermostable phytase from *Bacillus* sp. DS11. *Enz. Microb. Technol.* **22:** 2–7.
14. Oh, B. C., W. C. Choi, S. Park, Y. O. Kim, and T. K. Oh. 2004. Biochemical properties and substrate specificities of alkaline and histidine acid phytases. *Appl. Microbiol. Biotechnol.* **63:** 362–372.
15. Shimizu, M. 1993. Purification and characterization of phytase and acid phosphatase produced by *Aspergillus oryzae* K1. *Biosci. Biotechnol. Biochem.* **57:** 1364–1365.
16. Ullah, A. H. and D. M. Gibson. 1987. Extracellular phytase (E.C. 3.1.3.8) from *Aspergillus ficuum* NRRL 3135: Purification and characterization. *Prep. Biochem.* **17:** 63–91.
17. Ullah, A. H., K. Sethumadhavan, X. G. Lei, and E. J. Mullaney. 2000. Biochemical characterization of cloned *Aspergillus fumigatus* phytase (phyA). *Biochem. Biophys. Res. Commun.* **275:** 279–285.
18. Zhou, J., M. A. Bruns, and J. M. Tiedje. 1996. DNA recovery from soils of diverse composition. *Appl. Environ. Microb.* **62:** 316–322.