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Novel Low-Temperature-Active Phytase from *Erwinia carotovora* var. *carotovota* ACCC 10276

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A phytase with high activity at low temperatures has great potential for feed applications, especially in aquaculture. Therefore, this study used a degenerate PCR and TAIL PCR to clone a phytase gene from *Erwinia carotovora* var. *carotovota*, the cause of soft rot of vegetables in the ground or during cold storage. The full-length 2.5-kb fragment included an open reading frame of 1,302 bp and encoded a putative phytase of 45.3 kDa with a 50% amino acid identity to the *Klebsiella pneumoniae* phytase. The phytase contained the active site RHGXRXP and HD sequence motifs that are typical of histidine acid phosphatases. The enzyme was expressed in *Escherichia coli*, purified, and displayed the following characteristics: a high catalytic activity at low temperatures (retaining over 24% activity at 5°C) and remarkably thermal lability (losing >96% activity after incubation at 60°C for 2 min). The optimal phytase activity occurred at pH 5.5 and ~40°C, and the enzyme activity rapidly decreased above 40°C. When compared with mesophilic counterparts, the phytase not only exhibited a high activity at a low temperature, but also had a low K_m and high k_{cat}. These temperature characteristics and kinetic parameters are consistent with low-temperature-active enzymes. To our knowledge, this would appear to be the first report of a low-temperature-active phytase and its heterogeneous expression.

Keywords: Aquaculture, low-temperature-active enzyme, *Erwinia carotovora*, phytase

Phytate, the major storage form of phosphorus in plants [22], is an antinutrient in animal feed, reducing the quality of the feed and leading to environmental pollution [11]. However, phytases initiate the stepwise removal of phosphate from phytate, and are widely used as an additive to animal feed to improve the uptake of phosphorus and other nutrients, and to reduce the phosphorus pollution of animal waste [11, 32].

Many phytases have already been cloned and characterized from fungi and bacteria, including from *Aspergillus niger* [17], *A. fumigatus* [20], *Escherichia coli* [4], *Bacillus subtilis* [29], *Klebsiella pneumoniae* [24], *Pseudomonas fragi* [9], *Obesumbacterium proteus* [18], *Aeromonas* sp. [25], and *Yersinia intermedia* [8]. All these phytases are typical mesophilic enzymes, with a maximum activity at 50–60°C and lower activity at low temperatures [19, 27, 31].

The effect of temperature on phytase activity is important as regards functional application. Thus, phytases that can function at low temperatures would be valuable for use in certain applications. For example, the application of phytases in aquaculture, a current active field of phytase research, requires enzymes with a high activity at temperatures generally lower than 28°C [3, 16]. Moreover, although the physiological temperature of poultry and swine is 39°C, the initial stages of digestion of cold feed occur at a lower temperature when the enzyme is ingested with cold feed [2]. Therefore, a phytase with a high activity at low temperatures, particularly at 20–39°C, would have many potential industrial applications.

Accordingly, this paper reports on a phytase from *Erwinia carotovora* var. *carotovota* ACCC 10276, a bacterium that causes soft rot of vegetables in the ground or during cold storage [21]. The enzyme was expressed in *E. coli* and displayed the characteristics of low-temperature-active enzymes. A comparison of the temperature properties with typical mesophilic phytases also suggested that the phytase has properties in common with other low-temperature-active enzymes. Therefore, this low-temperature-active phytase, with a high activity at 20–40°C, may be useful for many low-temperature applications.

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Materials and Methods

Bacterial Strains, Plasmids, and Chemicals

The *Erwinia carotovora* var. *carotovota* (ACCC 10276) was obtained from the Agricultural Culture Collection of China, Beijing, China. *E. coli* JM109 and BL21 (DE3) (TaKaRa, Kyotanabe, Japan) were used as the hosts for the recombinant plasmid amplification and protein expression, respectively. The plasmids pGEM-T Easy (Promega, Madison, WI, U.S.A.) and pET22-b(+) (Novagen, Madison, WI, U.S.A.) were utilized to clone the PCR fragments for sequencing and to construct the recombinant expression plasmids, respectively. The phytic acid (from rice, P0109) was purchased from Sigma (St. Louis, MO, U.S.A.). All the other chemicals were of analytical grade and commercially available.

Phytase Production in *E. carotovora*

The *E. carotovora* (ACCC 10276) was grown at 25°C in either a Luria–Bertani (LB) medium, a low-phosphate medium [14], or a high-phosphate medium consisting of the low-phosphate medium and 10 mM phosphate for 16 h, or a phytate-specific medium [30] that utilizes phytic acid as the only source of carbon and phosphate at pH 5.5 for 150 h, respectively. The phytase activities in the supernatants and cell pellets from the four cultures were measured using the ferrous sulfate–molybdenum blue method, as described below [8].

Cloning of Phytase Gene

The phytase gene, *appA*, was cloned using a two-step PCR approach, which included a degenerate PCR step and a thermal asymmetric interlaced PCR (TAIL–PCR) step [8]. Briefly, a set of degenerate primers (E-FI and E-RI, Table 1) was designed based on two regions (RHGXRXP and HDTN) in the conserved sequence of histidine acid phosphatases (HAPs) from *Enterobacteriaceae* using the preferred codons of *Erwinia*. To obtain the core region between the two conserved blocks, a degenerate PCR was performed with an annealing temperature of 50°C and *E. carotovora* ACCC 10276 genomic DNA as the template. The amplified fragment with the appropriate size was ligated into the pGEM-T Easy vector for sequencing. The 5' and 3' flanking regions of the core region were then obtained using a TAIL–PCR. The nested insertion-specific primers (Table 1) for the TAIL–PCR were named usp1-3 (up special primers) and dsp1-3 (down special primers), respectively. The TAIL–PCR was performed using a Genome Walking kit (TaKaRa) and following the manufacturer's instructions.

Sequence and Phylogenetic Analysis

The sequence assembly was performed using Vector NTI advance 10.0 software (Invitrogen, Carlsbad, CA, U.S.A.), and the nucleotide sequence analyzed using the NCBI ORF Finder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The signal peptide and hydrolytic domain were predicted using SMART (http://smart.embl-heidelberg.de). The DNA and protein sequence similarity tests were carried out using the BLASTn and BLASTp programs (http://www.ncbi.nlm.nih.gov/BLAST/), respectively. Multiple alignments of the protein sequences were conducted using the CLUSTALW program (http://www.ebi.ac.uk/clustalW/). A phylogenetic tree of the multiple microbial phytases was constructed using the neighbor-joining (NJ) algorithm in MEGA version 4.0 [28], and the reliability assessed based on 1,000 bootstrap repetitions.

Expression of *appA* in *E. coli*

appA without the signal peptide coding sequence was amplified from the genomic DNA of *E. carotovora* by a PCR using primers E2-mF and E2-mR (Table 1). The fragment was cloned into the NcoI–NotI site of pET-22b(+), and then the recombinant plasmid, pET-*appA*, was transformed into *E. coli* BL21 (DE3) competent cells. Several positive transformants were sequenced and cultured in 25 ml of the LB medium (pH 5.5, 100 μmol/ml ampicillin) at 37°C to an OD_{600} of 0.5–0.7. Phytase expression was then induced by the addition of 1 mM IPTG for an additional 8 h at 25°C. Protein expression in both the pellet and the culture supernatant was checked using an activity assay and SDS–polyacrylamide gel electrophoresis (SDS–PAGE).

Purification of Recombinant APPA (r-APPA)

The induced culture (500 ml) was centrifuged at 12,000 ×*g* at 4°C for 10 min to remove any cell debris. The phytase activity of the culture supernatant was fractionated by ammonium sulfate (40–60% saturation), and the resulting precipitate dissolved in 5 ml of a 50 mM potassium phosphate buffer (pH 5.5) with 20% (w/v) ammonium sulfate. The enzyme was then applied to a Phenyl Sepharose HP column (1 ml) (Amersham Pharmacia Biotech, Uppsala, Sweden), and the proteins were eluted with an ammonium sulfate linear gradient of 20–0% in a 50 mM potassium phosphate buffer. Fractions with enzyme activity were collected based on the protein concentration as detected at A_{280nm}. To concentrate the fractions and change the buffer, the fractions with phytase activity were further eluted with 0.25 M sodium acetate (pH 5.5) using a Nanosep Centrifugal 10K

Table 1. Primers used in this study.

Primer name	Primer sequence[a]	Size (bases)
E-FI	5'-GTGGTKATWKTKAGYCGYCATGGYGT-3'	26
E-RI	5'-AKRTWKGCAAKRTTGGTRCATG-3'	23
usp1	5'-CGTGCGTTGCTACGCCACAGCCGG-3'	24
usp2	5'-CATGTAGGCCTGTGCGGTCGCACGG-3'	25
usp3	5'-CGGTGGCAGCATAGCCGTGTCCG-3'	23
dsp1	5'-CGGTTCGATTTCTATCAGCGGTCTGAATACGC-3'	32
dsp2	5'-GCTGGAGTACACTGAGAACCTACCACAGG-3'	29
dsp3	5'-CAGCGCGGCGGCTCACTACTCATG-3'	24
E2-mF	5'-GCA***CCATGG***CCCAGGATCGCTACCAGTTGG-3'	30
E2-mR	5'-GCA***GCGGCCGC***TTACAGCGCGTAATGCAC-3'	29

[a]The restriction enzyme sites incorporated into the primers are shown in boldface and italic font.

Device (Pall, Ann Arbor, MI, U.S.A.). The protein concentration of the purified enzyme was determined by a Bradford assay, using bovine serum albumin as the standard [1].

Phytase Activity Assay

The phytase activity was determined using a modified ferrous sulfate–molybdenum blue method [8]. Briefly, 50 μl of the enzyme solution was incubated with 950 μl of a 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 then stopped by the addition of 1 ml of 10% (w/v) trichloroacetic acid. Thereafter, the released inorganic phosphate was analyzed by adding 2 ml of color reagent C, containing 1% (w/v) ammonium molybdate, 3.2% (v/v) sulfuric acid, and 7.2% (w/v) ferrous sulfate, and the optical density measured at 700 nm. All the phytase activity determinations were performed in three independent experiments, and are shown as the means ± standard deviation (SD). One unit (U) of phytase activity was defined as the amount of enzyme that released 1 μmole of phosphate per minute at 37°C.

Properties and Substrate Affinity of r-APPA

The pH versus activity profile was determined by measuring the phytase activity at 37°C and pH 1.0–10.0 using the following buffers: glycine-HCl (0.25 M, pH 1.0–3.5), sodium acetate (0.25 M, pH 3.5–6.0), Tris-HCl (0.25 M, pH 6.0–8.5), and glycine-NaOH (0.25 M, pH 8.5–10). For the pH stability assays, the enzyme was incubated in the same buffers over a range of pHs from 1.0 to 10.0 at 37°C for 1 h, and the residual enzyme activity measured under standard conditions (pH 5.5, 37°C, 15 min).

The optimum temperature was determined at the optimum pH by varying the temperature from 0 to 80°C at 5°C intervals. The thermal stability was measured by assessing the residual enzyme activity after incubating the enzyme in 0.25 M sodium acetate (pH 5.5) at 50 or 60°C for 2, 4, 6, 8, and 10 min, respectively.

The K_m, V_{max}, and k_{cat} values for the r-APPA were determined using a Lineweaver–Burk analysis. The enzyme activity was measured at 37°C in 0.25 M sodium acetate containing 0.125 to 4.0 mM phytic acid as the substrate.

The substrate affinity of the purified enzyme was determined by replacing the phytic acid in the standard assay mixture with an equal concentration (1.5 mM) of the following phosphorylated compounds: adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), *p*-nitrophenyl phosphate, and glucose 6-phosphate.

RESULTS

Phytase Production in *E. carotovora*

To measure the phytase activity of *E. carotovora*, strain ACCC 10276 was grown in four different media with varying phosphate contents, and the phytase activity measured in both the pellet and the culture supernatant. Phytase activity was only detected in the cell lysate of the phytate-specific medium culture with a phytase activity of 0.53 U/ml, indicating that *E. carotovora* ACCC 10276 can produce phytase, and that the phytase production may depend on the source of carbon and phosphate and/or medium pH [10].

Cloning of Phytase Gene *appA* and Sequence Analysis

The core region of the phytase gene was obtained by a PCR using degenerate primers for the two regions conserved in histidine acid phosphatases, and the length of the PCR product was 872 bp. Fragments of 999 bp and 786 bp were obtained at the 5' and 3' ends of the core region, respectively, by a TAIL–PCR. The assembly of these three fragments yielded a 2,462-bp fragment (GenBank Accession No. EU234501), containing a complete open reading frame of 1,302 bp from nucleotides 801–2,102 and encoding 433 amino acid residues with a typical signal peptide (residues 1–27) (Fig. 1). The mature protein had a theoretical molecular mass of 45.3 kDa. The encoded phytase also contained the RHGXRXP and HD sequence motifs, the typical active sites of HAPs. Therefore, like the *E. coli* phytase gene *appA* in the HAP family, the *E. carotovora* gene was also named *appA*. A BLAST search using the DNA sequence and deduced amino acid sequence also identified the protein as a member of the acid phosphatase A family. An alignment of the *E. carotovora* phytase and six known phytases from the GenBank was performed using the CLUSTALW program (Fig. 1), and a phylogenetic tree then constructed based on the alignment using the NJ method (Fig. 2). The *E. carotovora* phytase was found to be genetically close to the known phytases from *K. pneumoniae* (50% identity) (GenBank Accession No. AAM23271) and *E. coli* (30%) (GenBank Accession No. ABF60232), indicating the novelty of the *E. carotovora* phytase.

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

To produce a recombinant phytase, *appA* was expressed in *E. coli* BL21 (DE3). The medium supernatant showed a phytase activity of 1.73 U/ml after induction at 25°C for 8 h. Meanwhile, no phytase activity was detected in the transformant harboring an empty pET-22b(+) vector under the same induction conditions. The recombinant phytase, r-APPA, was purified to electrophoretic homogeneity by ammonium sulfate precipitation and hydrophobic chromatography. As a result, the specific activity of the purified r-APPA was 792 U/mg after 57-fold purification, with a final activity yield of 6.3%. The purified enzyme yielded a single band with a molecular mass of 45 kDa, as determined by SDS–PAGE.

Properties of Purified r-APPA

The purified r-APPA displayed activity in a narrow pH range (Fig. 3), retaining more than 30% of its maximal activity at pH 4.0–6.0 with a pH optimum at 5.5, and losing all activity below pH 3.5 or above pH 6.5. The purified phytase exhibited a weak pH stability, retaining <40% activity after incubation at pH 3.0–4.0 and 7.0–10.0 for 1 h (Fig. 3).

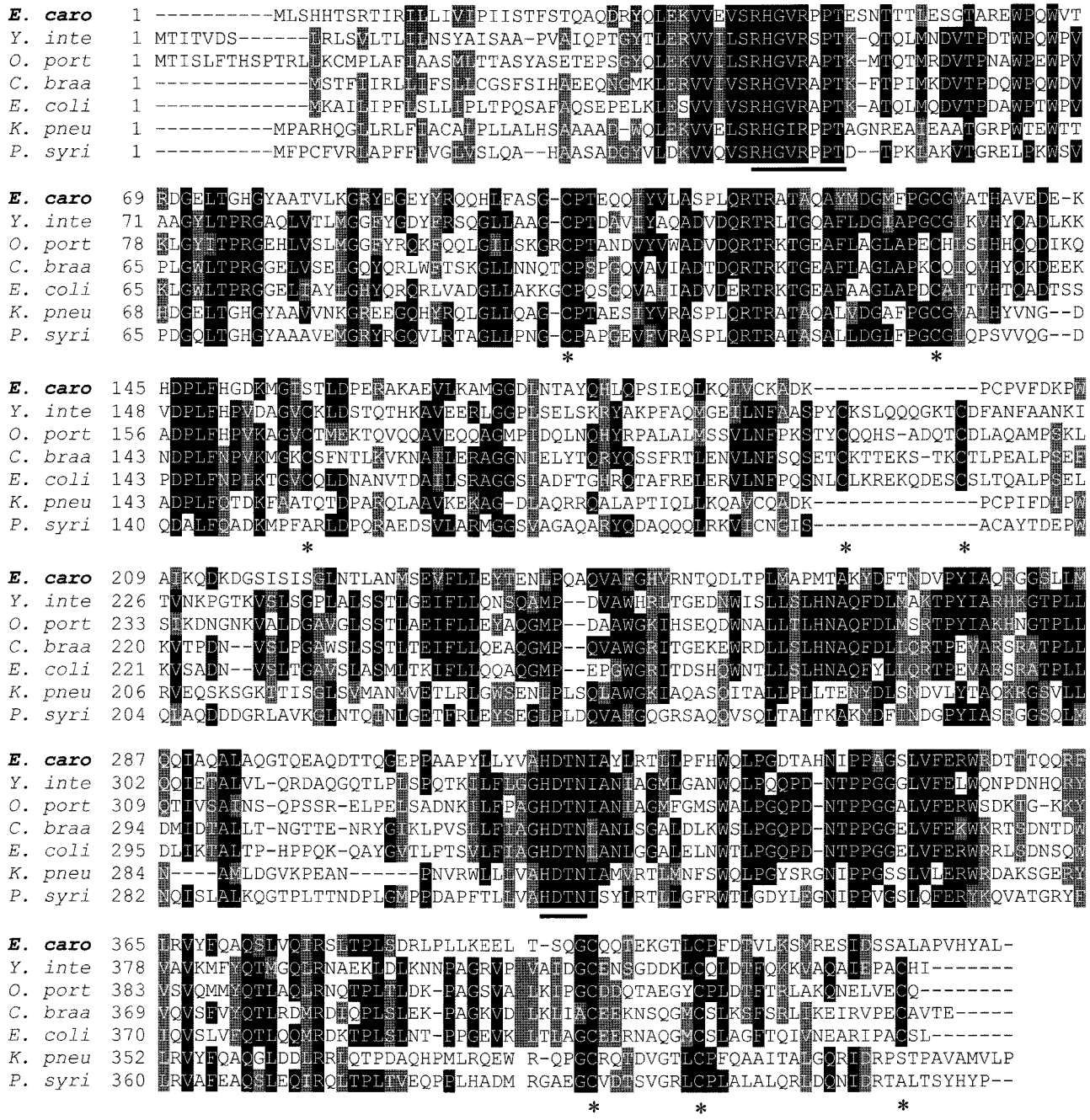

Fig. 1. Multiple alignment of amino acid sequences of *E. carotovora* phytase and six other known phytases.
The conserved motifs of the HAP family are underlined and gray; cysteine residues are marked using an asterisk. The phytase abbreviations, microbial source, and GenBank accession numbers are as follows: *E. caro*, *Erwinia carotovora* EU234501; *C. braa*, *Citrobacter braakii* AAS45884; *E. coli*, *Escherichia coli* AAN28334; *K.pneu*, *Klebsiella pneumoniae* AAM23271; *P. syri*, *Pseudomonas syringae* AAN77879; *O. prot*, *Obesumbacterium proteus* AAQ90419; *Y. inte*, *Yersinia intermedia* AAQ90419.

The optimum temperature for the purified r-APPA as regards phytase activity was ~40°C, with a rapid decrease in activity above 40°C (Fig. 4A). The enzyme exhibited >90% of its maximum activity at 30°C, and retained >24% activity even at 5°C. In addition, the r-APPA displayed remarkable thermal lability, losing over 96% activity after incubation at 50°C for 4 min or 60°C for 2 min (Fig. 4B).

The kinetic parameters for the hydrolysis of phytic acid gave a K_m of 0.25 mM, V_{max} of 1,250 μmol/min/mg, and k_{cat} of 950 s^{-1}, which were determined from a Lineweaver–Burk plot (Table 2).

To determine the substrate affinity of the *E. carotovora* phytase, the hydrolysis of various phosphorylated compounds by the purified r-APPA was studied. The enzyme had a

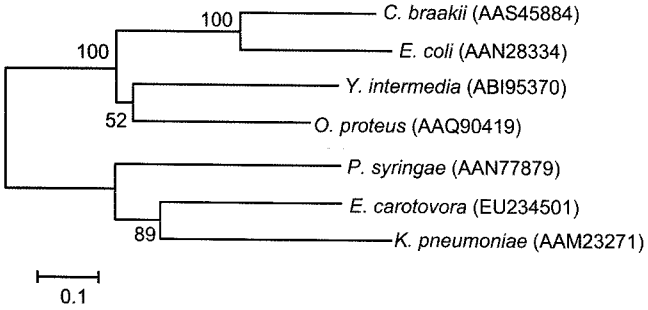

Fig. 2. Phylogenetic dendrogram obtained from neighbor-joining method of amino acid sequences, showing the position of *E. carotovora* phytase with respect to six other known phytases.
Bootstrap values are expressed as percentages of 1,000 replications. Bar, 0.1 sequence divergence.

narrow substrate affinity (Table 3), exhibiting 100% relative activity with phytic acid, yet almost no activity when the substrate was adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), *p*-nitrophenyl phosphate, or glucose 6-phosphate.

DISCUSSION

This study cloned a phytase gene from *E. carotovora*, a bacterium that causes soft rot of vegetables in the ground or during cold storage [21]. The cloned phytase contained the conserved active site residues (RHGXRXP and HDTN) that are typical among HAP family members. The cloned enzyme also exhibited the highest identity (86%) with a putative exported phosphatase from the genomic sequence (GenBank Accession No. YP_052285) of *E. carotovora* subsp. *atroseptica* SCRI1043; however, there was no more information to indicate that the putative sequence was a phytase with a function. When compared with other phytases reported previously, the phytase identified in this study was most similar to the phytase from *K. pneumoniae*, with an amino acid sequence identity of only 50%, and a 30% identity with the phytase from *E. coli*, confirming that the phytase from *E. carotovora* was a phytase with a low similarity to other known phytases.

The phylogenetic analysis (Fig. 2) also showed that the deduced amino acid sequence of the *E. carotovora* phytase was most similar to the *K. pneumoniae* phytase, which is a typical mesophilic enzyme that has an optimum temperature at 50°C (Fig. 4A) and retains over 75% activity after incubation at 60°C for 10 min [5, 24]. In addition, the phytase from *E. coli* is also a typical mesophilic enzyme that has an optimum temperature at 65°C (Fig. 4A) and good thermal stability (Fig. 4B) [13]. The phytases from *K. pneumoniae* and *E. coli* have already been well studied

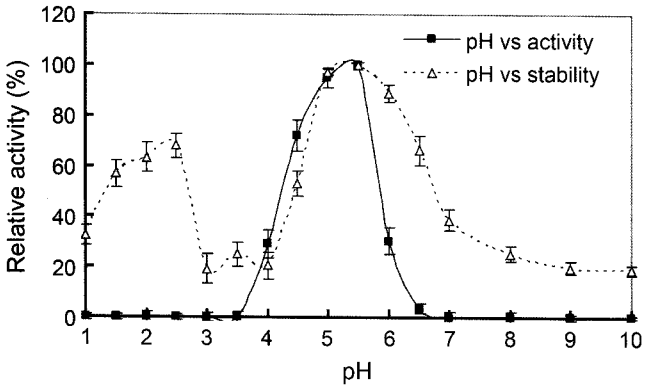

Fig. 3. pH versus activity profiles and pH stability of purified r-APPA from *E. carotovora*.
The activity and stability of the purified phytase were determined in the following buffers: glycine-HCl (0.25 M, pH 1.0–3.5), sodium acetate (0.25 M, pH 3.5–6.0), Tris-HCl (0.25 M, pH 6.0–8.5), and glycine-NaOH (0.25 M, pH 8.5–10.0). Each value in the panel represents mean±SD (n=3).

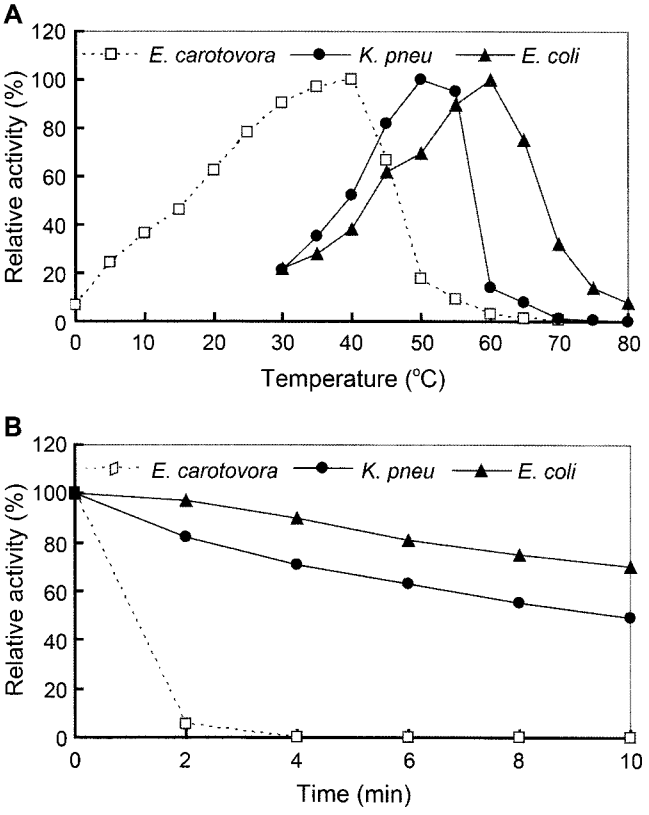

Fig. 4. Temperature versus activity profiles and thermal stability of purified r-APPA from *E. carotovora*, and controls *K. pneumoniae* and *E. coli* phytases.
A. The temperature profiles of the three enzymes were determined by measuring the enzymatic activity after incubating the enzymes in a sodium acetate buffer (0.25 M) at the pH optimum for each enzyme, pH 4.5 for *E. coli* phytase, pH 5.0 for *K. pneumoniae* phytase, and pH 5.5 for r-APPA, respectively, and at 0 to 80°C with 5°C intervals in a water bath for 30 min. **B.** The thermal stability was determined after incubating the phytase in 0.25 mM sodium acetate at pH 5.5 and 60°C for 2, 4, 6, 8, or 10 min, and then measuring the phytase activity under standard conditions. Each value in the panels represents the mean of triplicate tests.

Table 2. Specific activity and kinetics of three phytases.

Source of phytase	Specific activity (U/mg)	V_{max} (μmol/mg/min)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)
E. carotovora	792	1,250	252	950	3.77
K. pneumoniae	99	94	280	66	0.24
E. coli	3,191	3,934	550	2,950	5.36

[5, 13, 24], along with the crystal structures of *E. coli* phytase and its complex with phytate [12]. Therefore, these two phytases were selected as mesophilic counterparts to analyze the *E. carotovora* phytase. When compared with its mesophilic counterparts (Fig. 4), the *E. carotovora* phytase had a relatively lower optimum temperature (40°C), retained a high activity at low temperatures (>24% activity even at 5°C), and lost most of its activity at 50°C after 4 min. The temperature profile for the *E. carotovora* phytase was consistent with those for other low-temperature-active enzymes.

It has been proposed that low-temperature-active enzymes have a relatively high k_{cat}/K_m value and highly flexible structures [6, 26]. When comparing the kinetic parameters of the three phytases in Table 2, the *E. carotovora* phytase exhibited a lower K_m value and high k_{cat} value based on the specific activity. However, the *E. coli* phytase exhibited a higher k_{cat}/K_m value than the other two phytases, probably due to the high specific activity of the *E. coli* phytase. Nonetheless, the *E. carotovora* phytase had a relatively high k_{cat}/K_m value. Disulfide bridges are generally known to increase both stability and heat tolerance in proteins [15]. Thus, eliminating disulfide bridges may improve the domain flexibility and catalytic efficiency, while reducing the thermal stability [23]. The *E. coli* phytase has four disulfide bonds involving all eight Cys residues [12], yet only six Cys residues were detected in the *E. carotovora* phytase, suggesting fewer disulfide bonds and more flexibility (Fig. 1). As such, these characteristics of r-APPA indicate that the *E. carotovora* phytase is a low-temperature-active enzyme with remarkable thermal lability, probably derived from the cold environment of the source (cold storage).

The potential applications of the *E. carotovora* phytase with a relatively high activity at low temperatures, especially at 20–30°C, could be extended to aquaculture [3, 16] and food processing [7]. No phytase with appropriate temperature properties is currently used in aquaculture. Generally, the water temperature for aquaculture is 20–30°C, and most available phytases are mesophilic and cannot degrade phytic acid in fish feed efficiently under such conditions. The low-temperature-active *E. carotovora* phytase is also attractive for food processing by avoiding damage to the food ingredients at low temperatures [7]. Moreover, owing to its typical properties as a low-temperature-active enzyme, the *E. carotovora* phytase could be a good model protein to study the relationship between structure and function. Therefore, future studies will focus on clarifying the low-temperature properties of enzymes based on their protein structure using site-directed mutagenesis and three-dimensional structure analyses.

Table 3. Substrate affinity of purified *E. carotovora* r-APPA.

Substrate (4 mM)	Relative activity (%)
Phytic acid	100.00
Adenosine 5'-diphosphate (ADP)	0.02
Adenosine 5'-triphosphate (ATP)	0.26
p-Nitrophenyl phosphate	0.65
Glucose 6-phosphate	0.39

Acknowledgments

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