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Gene Cloning, Expression, and Characterization of a Novel Phytase from *Dickeya paradisiaca*

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Abstract A novel phytase gene, *appA*, was isolated by degenerate polymerase chain reaction (PCR) and thermal asymmetric interlaced PCR from *Dickeya paradisiaca.* The full-length *appA* comprises 1278 bp and encodes 425 amino acid residues, including a 23-residue putative N-terminal signal peptide. The deduced amino acid sequence of *appA* reveals the conserved motifs RHGXRXP and HD, which are typical of histidine acid phosphatases; significantly, APPA shows maximum identity (49%) to a phytase from *Klebsiella pneumoniae*. To characterize the properties of APPA, *appA* was expressed in *Escherichia coli* and purified. The purified recombinant APPA has two pH optima at pH 4.5 and 5.5, optimum temperature at 55 °C, specific activity of 769 U/mg, and good pH stability. The K_m value for the substrate sodium phytate is 0.399 mM with a V_{max} of 666 U/mg. To our knowledge, this is the first report of a phytase or phytase gene isolated from *Dickeya*.

Keywords Degenerate PCR · *Dickeya paradisiaca* · Histidine acid phosphatase · Phytase · TAIL-PCR

Abbreviation

HAP	histidine acid phosphatase
IPTG	isopropyl β-D-1-thiogalactopyranoside
r-APPA	recombinant APPA
TAIL-PCR	thermal asymmetric interlaced PCR

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Introduction

Phytase (myoinositol hexakisphosphate phosphohydrolase, EC 3.1.3.8 and EC 3.1.3.26) catalyzes the hydrolysis of phytate, the major phosphate storage compound in seeds of higher plants, such as cereals and legumes [1], to various lower phosphate myoinositol derivatives and inorganic phosphate [2]. Monogastric animals lack phytase in their digestive tracts and, thus, are incapable of utilizing the phosphorus bound in phytate; therefore, inorganic phosphate or a phytase of microbial origin has to be supplemented to their feed. Supplementation with inorganic phosphate increases the total phosphate burden and intensifies environmental pollution from animal waste; thus, phytase supplementation is preferable because it improves the utilization of phosphate and the nutritional quality of animal diets [2–5].

Microorganisms are important sources of phytases. To date, many phytase genes have been cloned from bacteria such as *Escherichia coli* [6, 7], *Bacillus* sp. [8–10], *Citrobacter braakii* [11], *Klebsiella* sp. [12], *Yersinia intermedia* [13], *Obesumbacterium proteus* [14] and *Pseudomonas syringae* [15]. Most of them belong to *Enterobacteriaceae*. The main purpose of this work was to identify additional phytase genes from other genera of *Enterobacteriaceae*, with the specific goal of obtaining phytases having properties superior to those of known phytases.

Materials and Methods

Strains, Plasmids, and Chemicals

Dickeya paradisiaca (DSMZ 18069) was obtained from DSMZ. *E. coli* JM109 (TaKaRa, Japan) was the host for recombinant plasmid vector pGEM-T Easy (Promega, USA). *E. coli* BL21 and plasmid pET-22b(+) were purchased from Novagen (Germany) for protein expression and recombinant expression plasmid construction. All strains were grown at 37 °C in Luria–Bertani medium. All chemicals were of analytical grade and commercially available.

Cloning of the Phytase Gene

The phytase gene was cloned according to Huang et al. [13]. Briefly, based on the conserved amino acid sequences (RHGXRXP and HDTN) of histidine acid phosphatases (HAP) from *Enterobacteriaceae*, two degenerated primers (F1, R1; see Table 1) were designed and used to amplify the core region between these two conserved sequences. The amplified fragment was analyzed by basic local alignment search tool (BLAST). The 5′ and 3′ flanking regions of the core region were amplified using thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR). The reaction parameters for TAIL-PCR were as in Liu et al. [16]. Up special primer (usp1 3), down special primer (dsp1 3), and arbitrary degenerate (AD) primers are listed in Table 1. The full-length gene, thus, obtained was named *appA*.

Sequence Analysis

The *appA* promoter was predicted using BDGP (http://www.fruitfly.org/seq_tools/promoter.html). The signal peptide was predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP/). The molecular mass of the mature peptide was determined using the software

Table 1 Primers used in this study.

Primer name	Primer sequence	Size (bases)
F1	5′-GTKSTKAWWKTGAGYCGCCA-3′	20
R1	5′-TWKGCMAKRTTRGTATCATG-3′	20
usp1	5′-GTGTCCTGTTCTTCGCTTGCCGCGCCGC-3′	28
usp2	5′- GCAGCCGGGGAAGACACCGTCCATCAGC-3′	28
usp3	5′-CTGCCTTCATACTGGCCTTTCAGCAC-3′	26
dsp1	5′-CGGTGCTGAATGGCCCGGCGGCGTTG-3′	26
dsp2	5′-GGCGGCGTTGGCCAACATGGCGGAAACC-3′	28
dsp3	5′-CACCAACGATCTGCCCTATGTCGC-3′	24
AD5	5′-AGWGNAGWANCAWAGG-3′	16
AD9	5′-WCAGNTGWTNGTNCTG-3′	16
AD10	5′-TCTTICGNACITNGGA-3′	16
D1F	5′-GCAGGATCCAGCCTCCACATCATGGAG-3′	27
D1R	5′-GCAAAGCTTTTAGCGACTATAGCTTACCGGCAG-3′	33

The *Bam*HI (D1F) and *Hin*dIII (D1R) restriction sites are boxed.

DNAStar. Homology searches in GenBank were performed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Homology modeling was performed using Swiss Model (http://swissmodel.expasy.org/workspace).

Expression of *appA* in *E. coli*

The gene *appA* without the signal peptide was amplified from genomic DNA of *D. paradisiaca* by PCR using primers D1F and D1R (Table 1). PCR was performed for 30 cycles consisting of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 2 min and 20 s. The amplified product was digested by *Bam*HI and *Hin*dIII and cloned into the corresponding sites of the expression vector pET-22b(+). *E. coli* BL21(DE3) cells harboring the plasmid pET-22b(+)-*appA* were incubated in Luria–Bertani medium containing 100 μg/mL ampicillin and grown at 37 °C to OD_{600} of 0.6. The induction of phytase production was performed by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The cultures were grown for an additional 4 h at 30 °C, and protein expression in the cells and culture supernatant were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described in Sambrook et al. [17].

Purification of the Phytase

The induced culture was centrifuged at 4,000×*g* for 10 min, and the cell-free supernatant was collected for phytase purification. The phytase in the supernatant was precipitated with ammonium sulfate (50–80% saturation) in an ice bath. The resulting precipitate was dissolved in buffer A (20 mM Tris-HCl, pH 7.5) and dialyzed against the same buffer. The dialyzed solution was concentrated using PEG8000. The concentrated solution was loaded onto a HiTrap Q Sepharose XL fast protein liquid chromatography column (Amersham Pharmacia Biotech, Sweden) pre-equilibrated with buffer A. Elution was done by buffer B (20 mM Tris-HCl and 0.6 M NaCl, pH 7.5) with a linear NaCl concentration gradient (0–0.6 M) at a flow rate of 2 mL/min. Fractions with phytase activity were pooled as the purified recombinant APPA enzyme, r-APPA. The protein concentration of the purified enzyme was determined by the Bradford assay, using bovine serum albumin as a standard.

Enzyme Activity and Properties of r-APPA

Phytase activity was determined by the ferrous sulfate-molybdenum blue method [18]. One unit of phytase activity was defined as the amount of enzyme required to release 1 μmol phosphate per min at 37 °C.

The optimum pH of the purified r-APPA was determined by measuring the enzyme activity at pH 2.0–9.0 for 30 min in the following buffers: 0.1 M glycine-HCl (pH 2.0–3.5), 0.1 M sodium acetate (pH 4.0–5.5), 0.1 M Tris-acetate (pH 6.0–6.5), and 0.1 M Tris-HCl (pH 7.0–9.0). The detailed procedures are as follows: 50 μl of enzyme solution and 950 μl of substrate solution were incubated in the buffers with different pH values at 37 °C for 30 min. The reaction stopped by adding 1 ml of 10% (*w*/*v*) trichloroacetic acid. After adding 2 ml of a coloring reagent C [1% (*w*/*v*) ammonium molybdate, 3.2% (*v*/*v*) sulfuric acid solution, and 7.2% (*w*/*v*) ferrous sulfate solution], the released inorganic phosphate was analyzed by measuring the absorption at 700 nm. The pH value at which r-APPA has the highest activity is the optimum pH. As to test the pH stability of r-APPA, the phytase samples were pretreated for 60 min in different pH systems (ranging from 2.0 to 9.0). The residual enzyme activity of all the treated samples were measured under the same condition (at 37 °C for 30 min) at the optimum pH obtained. A control sample with no pretreatment was assayed at same time, and its enzyme activity was taken as 100%.

The optimum temperature of the purified r-APPA was determined at optimum pH and at temperatures ranging from 30 to 65 at 5 °C intervals. The temperature stability was estimated by assessing the residual enzyme activity after preincubation of the enzyme at 50 and 60 °C for 5, 10, 20, or 30 min, respectively.

The effects of metal ions and chemical reagents on r-APPA activity were determined by assaying the enzyme activity at pH 4.5, 37 °C for 30 min in the presence of various reagents at two concentrations (1 and 5 mM).

The K_m and V_{max} values were determined by Lineweaver–Burk analysis at optimum pH and at 37 °C.

To study the substrate affinity of the purified r-APPA, the enzyme activity was measured with different phosphate-containing compounds as substrate at a concentration of 1.5 mM. The substrates used were: phytic acid sodium, sodium phenyl phosphate dibasic dehydrate, ATP disodium, ADP, P-NPP disodium, and D-glucose-6-phosphate sodium salt.

Results

Molecular Cloning and Sequence Analysis of the Phytase Gene *appA*

The core region of *appA* was obtained by degenerate PCR using primers F1 and R1. Sequencing showed that the core region comprised 844 bp. The 5′ and 3′ flanking regions of the core region were amplified by TAIL-PCR and cloned into pGEM-T Easy vector and sequenced. Assembly of the three PCR products yielded a full-length gene (GenBank accession No. EU086595) containing an open reading frame of 1,278 bp encoding 425 amino acid residues. The most likely position of the cleavage site at the N-terminus, Ala23-Ala24, was determined using the SignalP program (Fig. 1). The mature protein without the signal peptide has a calculated molecular weight of 43.9 kDa and a theoretical p*I* of 5.8. Within the 5′-noncoding region of the fragment, a possible –10 promoter sequence (ATGCTT) and the –35 sequence (TTGACG) were determined. BLAST search showed that

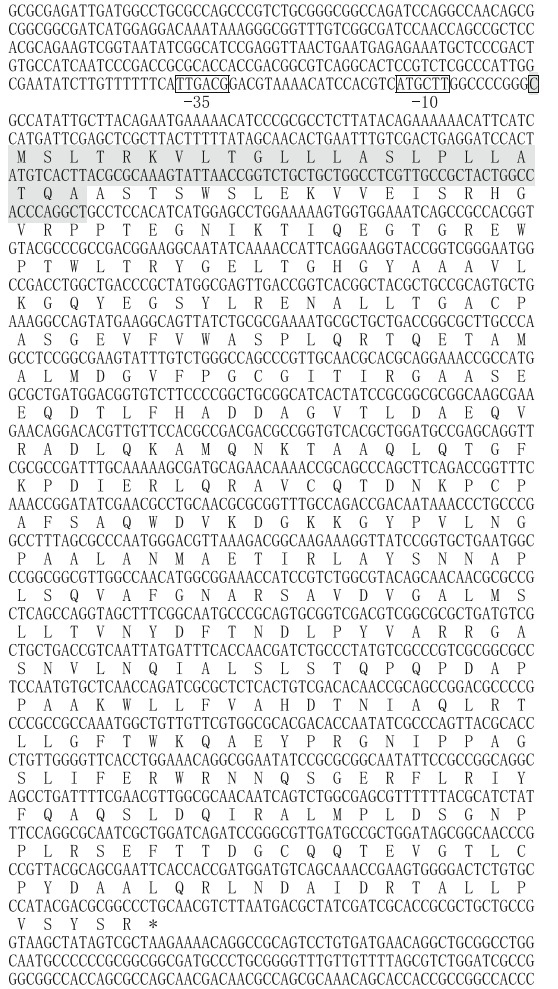

Fig. 1 Nucleotide and deduced amino acid sequences of the phytase gene, *appA*, from *D. paradisiaca*. The putative –35 and –10 regions are *boxed*. The putative transcription initiation site nucleotide (C) is *boxed and shaded*. The stop codon is shown by an *asterisk*. The putative signal peptide sequence is shaded in *gray*

the deduced amino acid sequence of *appA* had 49% and 46% identity to the phytases from *Klebsiella pneumoniae* and *P. syringae*, respectively, implying that *appA* under study encodes a novel phytase. Homology modeling indicated that the structure of APPA is highly similar to those of other HAP family phytases.

Enzyme Expression and Purification

Expression vector pET-22b(+)-*appA* was constructed and transformed into *E. coli*. Phytase activity of r-APPA was determined in the culture supernatant and cell lysate after IPTG induction (Fig. 2). The uninduced transformant and the transformant harboring the empty pET-22b(+) vector showed no phytase activity. SDS-PAGE analysis indicated that the molecular weight of r-APPA was approximately 43 kDa, corresponding to the calculated value.

The phytase in the supernatant was concentrated with gradient ammonium sulfate (50–80%). The concentrated enzyme was purified on a HiTrap Q Sepharose XL column. The

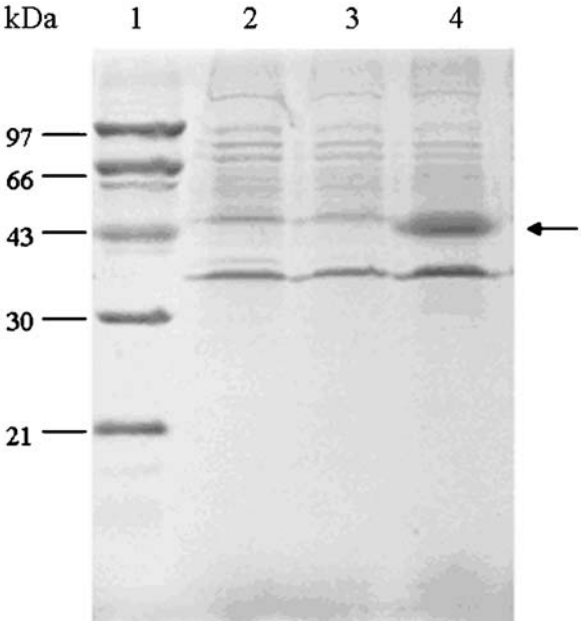

Fig. 2 SDS-PAGE analysis of the r-APPA expression in *E. coli* BL21. *Lane 1*, protein molecular weight standards; *lane 2*, culture supernatant of *E. coli* BL21 harboring the empty pET-22b(+) vector; *lane 3*, culture supernatant of *E. coli* BL21 harboring the recombinant plasmid pET-22b (+)-*appA* without IPTG induction; *lane 4*, culture supernatant of *E. coli* BL21 harboring the recombinant plasmid pET-22b (+)-*appA* induced by 1 mM IPTG for 4 h

phytase activity of peak marked by an arrow was detected (Fig. 3). The purified r-APPA appeared as a single protein band on an SDS-PAGE gel stained with Coomassie Brilliant Blue (Fig. 4). As a result, the specific activity increased from 196.5 U/mg in the crude enzyme preparation to 768.8 U/mg for the purified r-APPA (Table 2). A 3.9-fold purification of the enzyme was achieved with a recovery of 57%.

Properties of r-APPA

The phytase activity of r-APPA was measured at various pH values. Optimal activity was apparent over a pH range of 4.0–5.5, and >50% activity was retained up to pH 6.5 (Fig. 5A). No activity was detected at values lower than pH 3.5 or above pH 7.0. A pH stability assay indicated that r-APPA was stable over a wide range of pH and that the

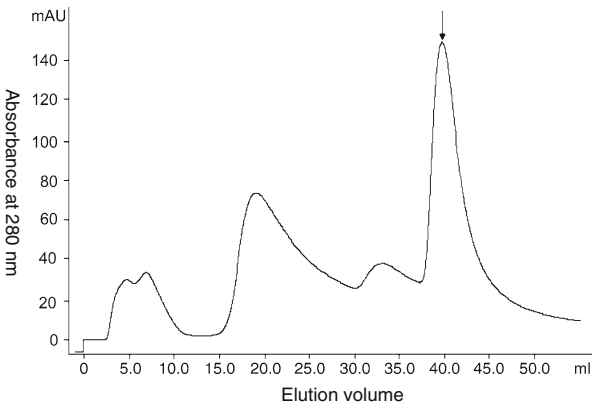

Fig. 3 Purification of r-APPA by anion exchange chromatography. The peak of r-APPA fraction was marked by an *arrow*

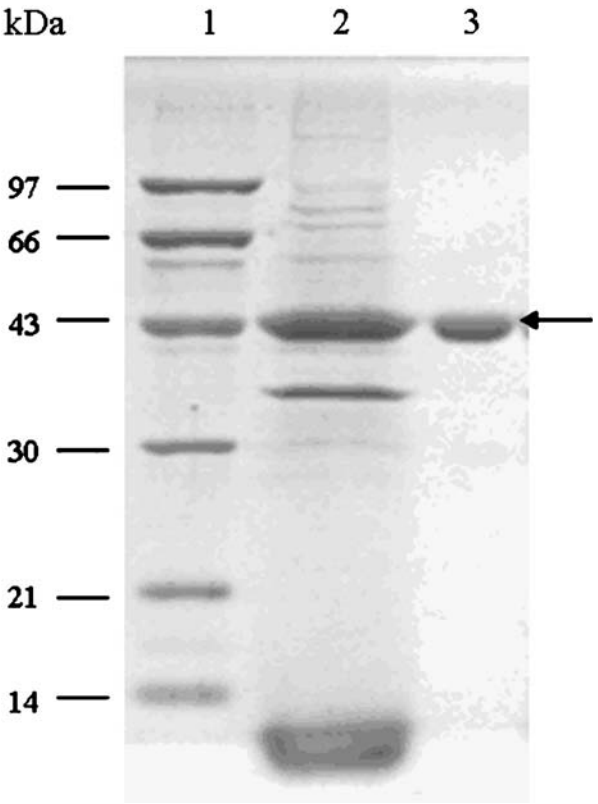

Fig. 4 SDS-PAGE analysis of the purified r-APPA. *Lane 1*, protein molecular weight standards; *lane 2*, the supernatant of *E. coli* with recombinant plasmid pET-22b(+)-*appA*; *lane 3*, the purified r-APPA after anion exchange chromatography. The gel was stained with Coomassie Brilliant Blue

enzyme retained maximal activity over the pH 4.5–7.5 (Fig. 5B). The optimum temperature for r-APPA activity was 55 °C (Fig. 5C). A thermal stability assay showed that r-APPA retained 43% of its activity when incubated at 50 °C for 30 min (data not shown). No activity was detected by incubating the enzyme at 60 °C for 5 min.

The effects of various metal ions and chemical reagents on the activity of purified r-APPA are presented in Table 3. The r-APPA activity was strongly inhibited by Cr^{3+}, Cu^{2+}, Fe^{3+}, and Zn^{2+}, and slightly inhibited by Ni^{2+} and Mn^{2+} at 1 mM concentration. Ca^{2+}, Mg^{2+} and ethylene diamine tetraacetic acid at 1 mM slightly enhanced the enzyme activity. Raising the concentration of the various reagents to 5 mM had very little effect on r-APPA activity relative to activity measured at 1 mM.

The specific activity of the purified r-APPA was 768.8 U/mg. The K_m and V_{max} values were 0.399 mM and 666 U/mg, respectively.

Table 2 Purification of r-APPA expressed in *E. coli* BL21.

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	3537.7	18.0	196.5	1.0	100
$(NH_4)_2SO_4$ precipitation	3103.3	11.9	260.7	1.3	88
HiTrap Q XL	1998.9	2.6	768.8	3.9	57

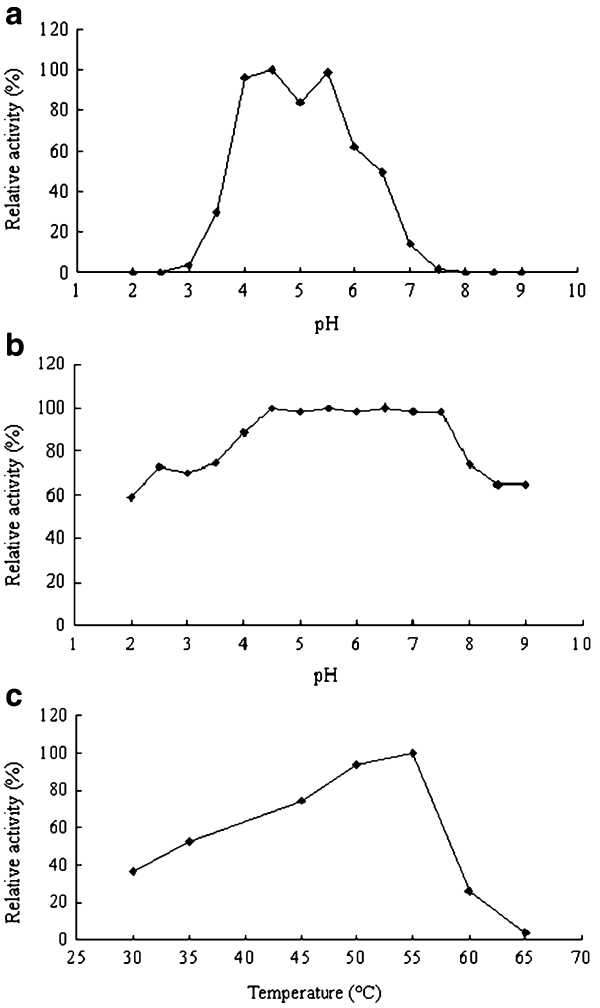

Fig. 5 Effects of pH and temperature on the activity of purified r-APPA with sodium phytate as substrate. The 100% relative activity was 768.8 U/mg. (**A**) Effect of pH on r-APPA activity. The effect of pH on the activity of r-APPA was tested in 0.1 M glycine-HCl (pH 2.0–3.5), 0.1 M sodium acetate (pH 4.0–5.5), 0.1 M Tris-acetate (pH 6.0–6.5), and 0.1 M Tris-HCl (pH 7.0–9.0) at 37 °C. (**B**) Effect of pH on stability of r-APPA. pH stability of the enzyme was determined by measuring the residual activity after incubating the enzyme at 37 °C for 60 min in buffers described above at pH 2.0–9.0. (**C**) Profile of r-APPA activity at pH 5.5 over the temperature range of 30 to 65 °C

The hydrolysis of various phosphorylated compounds by purified r-APPA was measured relative to a natural substrate, sodium phytate (Table 4). Sodium phenyl phosphate dibasic dehydrate, ATP disodium, and P-NPP disodium were hydrolyzed to some extent (4–12%), although the enzyme exhibited little activity for ADP and D-glucose-6-phosphate sodium salt. These results suggested that r-APPA has narrow substrate specificity and high specificity for phytic acid.

Discussion

To date, five genera of *Enterobacteriaceae* have been reported to produce phytase, and the relevant genes have been cloned [6, 11–14, 19]. The cloning of *D. paradisiaca appA* here demonstrates that the conserved amino acid sequences (RHGXRXP and HDTN) of the HAP family are very helpful for identifying phytase genes. The deduced amino acid

Table 3 Effects of metal ions and chemical reagents on purified r-APPA activity.

Chemical	Relative activity (%)	
	1 mM	5 mM
CK	100	100
Ca^{2+}	119	109
Mg^{2+}	111	100
K^{+}	98	113
Na^{+}	96	115
Li^{2+}	96	113
Co^{2+}	87	73
Ni^{2+}	77	53
Mn^{2+}	68	53
Zn^{2+}	10	0
Cr^{3+}	8	0
Cu^{2+}	3	0
Fe^{3+}	0	0
EDTA	120	130

Relative activity of 100% was 768.8 U/mg when assayed with sodium phytate as substrate.

sequence of *appA* from *D. paradisiaca* revealed low identity to phytases from *K. pneumoniae* and *P. syringae*. To our knowledge, our current work is the first to report a phytase gene from *Dickeya*. It might be possible that phytases exist in most genera of *Enterobacteriaceae*, but additional phytases need to be identified to support this hypothesis.

The gene *appA* was expressed in *E. coli*. The purified r-APPA displayed optimum activity at 55 °C (Fig. 5C), consistent with the activity of certain commercialized phytases [20, 21]. At the porcine physiological temperature of 39 °C, the enzyme displayed about 60% of its maximum activity. Interestingly, like the phytase from *O. proteus*, r-APPA has two pH optima at pH 4.5 and 5.5 (Fig. 5A). More than 50% of the maximal activity was observed between pH 4.0 and 6.5, the same as the characteristic pH range from the stomach to small intestine. These characteristics of APPA suggest that it would be a good candidate phytase for use as an additive in animal feed.

Phytases from *Enterobacteriaceae* exhibit remarkable differences in specific activity. For example, the specific activity of purified r-APPA was 768.8 U/mg, similar to that of the *P. syringae* phytase (649 U/mg), but it is much higher than that of *Klebsiella* sp. ASR1 phytase (224 U/mg) [12, 22]. Wyss et al. [23] attempted to classify phytases into two groups based on substrate specificity. One group includes phytases having wide substrate specificity but relatively low specificity for phytate, whereas the other group includes phytases having narrow substrate specificity but high specificity for phytate. According to

Table 4 Substrate specificity of r-APPA.

Substrate (1.5 mM)	Relative activity (%)
Sodium phytate[a]	100.00
Sodium phenyl phosphate dibasic dehydrate	4.62
ATP disodium	4.62
ADP	1.13
P-NPP disodium	12.09
D-glucose-6-phosphate sodium salt	0.44

Relative activity of 100% was 768.8 U/mg when assayed with sodium phytate as substrate.

the specific activity of r-APPA and the property that r-APPA was very specific for phytic acid, our r-APPA might be belong to the latter group.

D. paradisiaca is one of the phytopathogenic *Enterobacteriaceae* that can cause soft rot diseases in many important crops. Phytases have been found in many plant pathogens, such as *Y. intermedia* [13], *Erwinia carotovora* (data not shown), *Xanthomonas oryzae* pv. *Oryzae* [24], and others. Generally, the host plants for these pathogens contain phytic acid. Because phytase activity results in the release of phosphate, which is essential for pathogen physiology, the expression of phytase by the pathogen upon infection of phytic acid-producing plants may be critical for pathogen survival. Chatterjee et al. [24] reported that the phytase gene, *phyA*, from *X. oryzae* pv. *Oryza* is involved in virulence because a mutant of *phyA* was deficient for virulence, at least in part due to the mutant's inability to utilize host phytic acid as a source of phosphate. Thus, *appA* from *D. paradisiaca* may be involved in the pathogenicity of this bacterium.

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