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Molecular and Biochemical Characteristics of β-Propeller Phytase from Marine *Pseudomonas* sp. BS10-3 and Its Potential Application for Animal Feed Additives S

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Phytate is an antinutritional factor that impacts the bioavailability of essential minerals such as Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, and Fe^{2+} by forming insoluble mineral-phytate salts. These insoluble mineral-phytate salts are hydrolyzed rarely by monogastric animals, because they lack the hydrolyzing phytases and thus excrete the majority of them. The β-propeller phytases (BPPs) hydrolyze these insoluble mineral-phytate salts efficiently. In this study, we cloned a novel BPP gene from a marine *Pseudomonas* sp. This *Pseudomonas* BPP gene (PsBPP) had low sequence identity with other known phytases and contained an extra internal repeat domain (residues 24–279) and a typical BPP domain (residues 280–634) at the C-terminus. Structure-based sequence alignment suggested that the N-terminal repeat domain did not possess the active-site residues, whereas the C-terminal BPP domain contained multiple calcium-binding sites, which provide a favorable electrostatic environment for substrate binding and catalytic activity. Thus, we overexpressed the BPP domain from *Pseudomonas* sp. to potentially hydrolyze insoluble mineral-phytate salts. Purified recombinant PsBPP required Ca^{2+} or Fe^{2+} for phytase activity, indicating that PsBPP hydrolyzes insoluble Fe^{2+}-phytate or Ca^{2+}-phytate salts. The optimal temperature and pH for the hydrolysis of Ca^{2+}-phytate by PsBPP were 50°C and 6.0, respectively. Biochemical and kinetic studies clearly showed that PsBPP efficiently hydrolyzed Ca^{2+}-phytate salts and yielded *myo*-inositol 2,4,6-trisphosphate and three phosphate groups as final products. Finally, we showed that PsBPP was highly effective for hydrolyzing rice bran with high phytate content. Taken together, our results suggest that PsBPP has great potential in the animal feed industry for reducing phytates.

Keywords: β-Propeller phytase, *Pseudomonas* sp., Ca^{2+}-phytate salts

Introduction

Phytate is the principal storage form of phosphorus and inositol in plant seeds and tightly binds essential minerals such as Ca^{2+}, Mg^{2+}, Fe^{2+}, and Zn^{2+} to form indigestible mineral-phytate salts [6, 15]. Phytate represents up to 80% of the total phosphorus in plant seeds, including fruits, vegetables, nuts, and organic soils, with a content of 0.17–9.15% depending on the food source [16]. Monogastric animals, including poultry, rodents, and humans, have limited capability to hydrolyze mineral-phytate salts, thus excreting them undigested *via* the feces [8]. The loss of mineral-phytate salts contributes to the antinutritional impact of phytate and may result in mineral deficiencies in these animals [18]. Furthermore, long-term phytate diets inhibit intestinal calcium absorption, causing rickets in dogs [2, 4, 5, 10]. The bioavailability of phosphorus and Ca^{2+} in animal feed and food can be enhanced *via* the degradation of Ca^{2+}-phytate salts; thus, reducing phytate by enzymatic hydrolysis may be of considerable nutritional importance.

Phytases can be classified into four subclasses: the histidine acid phosphatases, the cysteine phytases, the β-propeller phytases (BPPs), and the purple acid phosphatases [9, 11, 13]. BPPs, which preferentially hydrolyze mineral-phytate salts, are potential candidates for the enzymatic reduction of phytate, which improves the bioavailability of phosphorus and minerals in foods with high phytate content [6, 17]. Isothermal titration calorimetric analysis of the final product, myo-inositol 2,4,6-triphosphate, revealed that BPPs efficiently eliminate the ability of phytate to strongly chelate several divalent cations, thereby providing free minerals and phosphorus as nutrients for bacterial growth [6]. Some BPPs from gram-positive bacteria, such as *Bacillus* sp. [12], *Shewanella oneidensis* MR-1 [3], *Pedobacter nyacknesis* MJ11CGMCC 2503 [5], and *Hahella chejuensis* [6], have been characterized, whereas those from gram-negative bacteria, such as *Pseudomonas* sp., have not.

In this study, we cloned the BPP gene from marine *Pseudomonas* sp. BS10-3 to gain insight into the biochemical and kinetic properties of BPPs from marine microorganisms. A sequence analysis clearly showed that *Pseudomonas* sp. BS10-3 β-propeller phytase (PsBPP) contains a unique N-terminal extra-repeat domain lacking active-site residues, whereas the C-terminal BPP domain contains multiple calcium-binding sites that provide a favorable electrostatic environment for substrate binding and catalytic activity. Furthermore, our results indicate that PsBPP efficiently abrogated the ability of phytate to chelate Ca^{2+} and other divalent cations by hydrolyzing mineral-phytate salts, thereby yielding *myo*-inositol 2,4,6-trisphosphate as a final product. The ability of PsBPP to hydrolyze mineral-phytate salts from rice bran as a natural substrate may be useful in biotechnological and nutritional applications.

Materials and Methods

Materials

Plasmid DNA was prepared using Miniprep kits (Qiagen Inc., Valencia, CA, USA). Restriction fragments and polymerase chain reaction (PCR) products were purified from agarose gels using the QIAquick gel extraction kit (Qiagen). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

DNA Cloning and Sequencing and Computer Analysis

We screened marine bacteria with BPP activity using Ca^{2+}-phytate as a substrate. Among them, we selected the marine bacteria *Pseudomonas* sp. We initially cloned the partial BPP gene from *Pseudomonas* sp. BS 10-3 using degenerative PCR primers (PN_BPP, 5′ GAY GAY CCN GCN RTN TGG 3′; PC_BPP, 5′ NGH NAB NCC YTC NAY RTC 3′) based on the highly conserved regions of BPPs (Fig. S1; black box [6]). After sequencing the amplified PCR products, we cloned the full-length BPP gene using an annealing control primer (ACP)-based PCR method [7] and GeneFishing DEG kits (SeeGene, Seoul, Korea). The primers used in this study are listed in Table S1. After PCR amplification, the amplified PCR products were separated on 1% agarose gels, extracted from the amplified PCR products using the GENCLEAN II Kit (Q-BIO Gene, Carlsbad, CA, USA), and cloned into the TOPO TA Cloning Vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After verifying the sequences of the cloned plasmids, we cloned the full-length BPP gene from *Pseudomonas* sp.

Cloning of the Phytase Gene from *Pseudomonas* sp.

The *Pseudomonas* sp. gene encoding BBP (amino acid residues 280–634) was subcloned into the pET 28a vector (Novagen, Gibbstown, NJ, USA) to generate a protein with a C-terminal (His) 6-tag. The following oligonucleotides were used as primers in the PCR: PsBPP_PN280 (*Nde*I), 5′-AAACATATGCCACAAGGGCTG GACGTGTGGGT-3′; PsBPP_PC634 (*Xho*I), 5′-AAACTCGAGTCA AGGCAAGTTCAGGGTTT-3′. Following PCR, the amplified DNA fragments were ligated using T4 DNA ligase. The ligation mixture was used to transform competent *E. coli* XL1 Blue cells (Invitrogen). Colonies were isolated, and plasmid DNA was extracted using Qiagen Miniprep kits. A plasmid harboring the PsBPP gene was introduced into competent *E. coli* BL21 (DE3) cells (Novagen).

Overexpression and Purification of Recombinant PsBPP

Cells were grown initially in 50 ml of LB-kanamycin (50 μg/ml) for 8 h at 37°C before inoculation into 2 L of LB-kanamycin (50 μg/ml), and then transferred immediately to a shaking incubator at 25°C. When the cultures reached an absorbance of 0.6–1.0 at 600 nm, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM. After 16 h, the cells were harvested by centrifugation (7,000 ×*g*; 30 min; 4°C) and resuspended in lysis buffer (50 mM Tris-HCl buffer (pH 7.0)). The cells were disrupted (10 min, 50% duty cycle), the supernatant was collected, and the recombinant enzyme was purified by nickel-nitrilotriacetic acid (Ni-NTA) affinity column chromatography (Qiagen) using 100 mM imidazole. The cell lysate and Ni-NTA mixture were loaded onto a column, which was washed twice with 4 ml of wash buffer (50 mM Tris-HCl (pH 7.0), 300 mM NaCl, and 10 mM imidazole). Then, 2.5 ml of elution buffer (50 mM Tris-HCl (pH 7.0), 300 mM NaCl, and 100 mM imidazole) was used to elute the target proteins twice. The molecular mass of the recombinant enzyme was estimated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Phytase Activity Assay

Phytase activity was assessed by measuring the production of inorganic orthophosphate (P_i) using a method described previously [12]. Experiments were performed in 100 mM Tris-HCl, pH 7.0, with various concentrations of Na-phytate (0.01–5.0 mM) and Ca^{2+} (0–5 mM). Enzymatic reactions were initiated by adding 50 µl of enzyme pre-incubated with increasing concentrations of Ca^{2+}, followed by 450 µl of 1 mM Na-phytate with 100 mM Tris-HCl of the appropriate pH and containing different Ca^{2+} concentrations. The reactions were quenched by adding 500 µl of coloring reagent solution containing 2.5% ammonium heptamolybdate, 0.175% ammonia, 0.1425% ammonium vanadate, and 22.75% nitric acid. Optical density (OD) was measured at 415 nm. One unit of phytase activity was defined as the amount of enzyme required to liberate 1 µM phosphate per minute under the assay conditions.

Effects of Metal Ions on Enzyme Activity

Mineral solutions (1 or 5 mM) of Ca^{2+}, Sr^{2+}, Fe^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Li^{2+}, and Zn^{2+} were prepared in Tris buffer (50 mM, pH 7.0). A 50-µl of aliquot of enzyme in Tris buffer (50 mM, pH 7.0) was pre-incubated with 500 µl of mineral solution at 37ºC for 30 min. After the incubation, 450 µl of sodium phytate solution (5 mM, pH 7.0) was added to the reaction mixture, which was incubated again at 37ºC for 30 min and then quenched by adding 500 µl of color quenching reagent. Residual enzyme activity was measured at an OD of 415 nm.

Determining Kinetic Parameters

The PsBPP activity for different concentrations of sodium phytate (0.1–6 mM) was determined. The reaction mixture was prepared and phytase activity determined. The K_m and V_{max} values were determined using a Lineweaver–Burk double-reciprocal plot.

High-Performance Ion Chromatography (HPIC) Analysis of the Reaction Products

The reaction products of the Ca^{2+}-phytate salt hydrolysis by PsBPP were analyzed using an HPIC system (ICS-3000; Dionex, Sunnyvale, CA, USA), as previously described [6]. In brief, an inositol phosphate analytical column (IonPac AS11; Dionex) and conductivity detector (Dionex) were used in conjunction with an anion suppressor. Filtered samples (20 µl) were eluted in a linear NaOH solution gradient (70–150 mM, 25 min). The separated inositol phosphates were detected using the conductivity detector.

Enzymatic Hydrolysis of Rice Bran

Rice bran was purchased from *Charm ssal* (Seoul, Korea). Phytate was hydrolyzed from rice bran in 10 mM Tris HCl, pH 7.0, at 37°C in a shaking incubator as previously described [17]. BPP from *Bacillus amyloliquefaciens* (BaBPP) and PsBPP were added to 500 U/kg rice bran. The enzymatic reaction was quenched at specific time points, and P_i was measured as described previously [12].

Results and Discussion

Cloning and Nucleotide Sequence Analysis of the PsBPP Gene

We cloned the BPP gene from *Pseudomonas* sp. BS 10-3 using an ACP-based PCR method with a set of degenerative primers based on highly conserved regions in BPPs (Fig. S1 and Table S1). The sequence analysis showed an open reading frame of 1,902 bp, which encoded a 633 amino acid residue with an estimated molecular mass of 68,852 Da (Fig. 1). Based on the BPP domain analysis for PsBPP using the SMART database (http://smart.embl-heidelberg.de/) and structure-based sequence alignments, it was clearly shown that the N-terminal repeat domain of PsBPP does not contain active-site residues, whereas the multiple calcium-binding residues of the C-terminal BPP domain provided a favorable electrostatic environment for Ca^{2+}-phytate and catalytic activity (Fig. S1 and Fig. 2A). Furthermore, the entire PsBPP sequence showed 64%, 65%, 75%, and 76% homology with *Pseudomonas fluorescens*, *Pseudomonas* sp. PH1b, *Pseudomonas* sp. CF149, and *Pseudomonas psychrophila* BPPs, respectively. PsBPP was 69%, 70%, 78%, and 80% homologous with the same *Pseudomonas* BPPs, respectively. However, PsBPP did not show significant sequence homology with other histidine acid phytases. Based on sequence homology, the results suggest that PsBPP can be classified into a phytase group separate from *Bacillus* BPPs.

Overexpression of the PsBPP BPP Domain and PsBPP Enzymatic Properties

The C-terminal PsBPP domain was overexpressed successfully in *E. coli* strain BL21 (DE3) at various temperatures. Maximum PsBPP expression levels were detected when the cells were cultured at 25°C for 16 h. After verifying the maximum PsBPP expression levels, we purified the PsBPP BPP domain by Ni-NTA and ion-exchange chromatography. The molecular mass of the purified enzyme was approximately 42 kDa, which differed from the deduced molecular mass (35 kDa) owing to the high content of negatively charged amino acids (11%). PsBPP enzymatic activity was assayed with Ca^{2+}-phytate salts, prepared by mixing 1 mM Na-phytate and 1 mM Ca^{2+} in 50 mM Tris-HCl, pH 7.0, at various temperatures. The optimal temperature for PsBPP was 50°C. The enzyme was stable up to 55°C in the presence of 1 mM Ca^{2+}, but its thermal stability decreased dramatically above 60°C. Nevertheless,

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ATGCGTTTTAACTGTAAACCGTGCCTGTTGCCGCTGTTGATCAGCCTGAGTGCAGGCCATGCGCAGGCCGCCACGCCCGTAACCGCGCCG   90
M  R  F  N  C  K  P  C  L  L  P  L  L  I  S  L  S  A  G  H  A  Q  A  A  T  P  V  T  A  P  30
ACGCTTAAGCCTTGGAGCACCACCAAAGCCCAGGCCTTGGGTTGGCTCGCCGGGGATCAGCGCCTGGCGGTGAGCAAGCGTGAAGGTGTG   180
T  L  K  P  W  S  T  T  K  A  Q  A  L  G  W  L  A  G  D  Q  R  L  A  V  S  K  R  E  G  V  60
TTGCTGCTCGACGCGCAAGGCAAAACCCTGAGCCATGTGCCGGGGGCATTCGCCTCGTTGGACAGTCGCGCCTTGGGCGATCAAGTGCTG   270
L  L  L  D  A  Q  G  K  T  L  S  H  V  P  G  A  F  A  S  L  D  S  R  A  L  G  D  Q  V  L  90
GTGGCCAGCCATGACGAGAAGAAACAGCAAGTGGCGCTGTTCAGCCTCAATCCCCAGAGCCACGAATGGCTGGCGCCGGTCTATTTACCG   360
V  A  S  H  D  E  K  K  Q  Q  V  A  L  F  S  L  N  P  Q  S  H  E  W  L  A  P  V  Y  L  P  120
AGGCGTGATTACGCGGTCAACGGTGTGTGTCTGTACCGCGACGAGGCAAGCAATATTTACCTGTTCACTGTCGGCGAGGAGGGCAAGGGC   450
R  R  D  Y  A  V  N  G  V  C  L  Y  R  D  E  A  S  N  I  Y  L  F  T  V  G  E  E  G  K  G  150
GAGCAATGGCTGGTGGCCGCAGACCGTAAACGGCTGAATCAGCCGCGTTTGGTGCGCAGCCTGCCGCTGCCGCCAGAGGCTGGCTTGTGT   540
E  Q  W  L  V  A  A  D  R  K  R  L  N  Q  P  R  L  V  R  S  L  P  L  P  P  E  A  G  L  C  180
CAGGTCGACGACGCGGCACATCAGTTGTTCGTCAATGAACAGAAGGTGGGCTGGTGGGCGTACCCGGCCCATGCCGAGGCGCAGGCTTCA   630
Q  V  D  D  A  A  H  Q  L  F  V  N  E  Q  K  V  G  W  W  A  Y  P  A  H  A  E  A  Q  A  S  210
CGGGTGCCGGTGGCGATGATCGAGCCGTTTGGCGAGGTGAAACAGGCGGCCGGGGCCATGGTGCCAGTACCGGGCGGGATGCTGGGCCTT   720
R  V  P  V  A  M  I  E  P  F  G  E  V  K  Q  A  A  G  A  M  V  P  V  P  G  G  M  L  G  L  240
GATCCAAAAGCTGGCGAACTGCACCTCTATCAGCAGCAAGGCAAGGGCTGGTCGCCCGTGGCCCGCTTCCCGTTGAAGCCGCTGGTTGAA   810
D  P  K  A  G  E  L  H  L  Y  Q  Q  Q  G  K  G  W  S  P  V  A  R  F  P  L  K  P  L  V  E  270
CCCGAGCACCTGGCGGTGCGCCAGACGCCACAAGGGCTGGACGTGTGGGTGCAGGACGCTGACAACAATCAGCTGTTTGAAGGCCGACTG   900
P  E  H  L  A  V  R  Q  T  P  Q  G  L  D  V  W  V  Q  D  A  D  N  N  Q  L  F  E  G  R  L  300
AGCTGGAATCCCGTCCCGGTGAGTGTGCCGCCGGTGTTGCCCGTGGTGAAACCTTCCGTACAAACCGACCCCGTCGTGAGCCAGGGAGGA   990
S  W  N  P  V  P  V  S  V  P  P  V  L  P  V  V  K  P  S  V  Q  T  D  P  V  V  S  Q  G  G  330
GGGGCGGATGACCCGGCGATTTGGCTTCACCCGCACGATCCGGCGTTGAGTCGGGTGCTGGGCACTAATAAAAAGAACGGCCTTGAGGTG   1080
G  A  D  D  P  A  I  W  L  H  P  H  D  P  A  L  S  R  V  L  G  T  N  K  K  N  G  L  E  V  360
TATGACCTGCAAGGGCGGCGCGTACAACATCTGGAAGTGGGGCGCTTGAACAACGTTGATGTACGCCCGGATTTTAAGCTGGGCACGCGC   1170
Y  D  L  Q  G  R  R  V  Q  H  L  E  V  G  R  L  N  N  V  D  V  R  P  D  F  K  L  G  T  R  390
ACTGTGGATCTGGCAGTGGCGACCAATCGGGATCACAACAGCCTGAGCGTGTTCAGCATCGACCGCGCCACAGGTGAGGTCCGTGCAGCA   1260
T  V  D  L  A  V  A  T  N  R  D  H  N  S  L  S  V  F  S  I  D  R  A  T  G  E  V  R  A  A  420
GGCGAGGTGCCGACACCGCTCAAGGATATTTACGGGTTGTGCCTGTTCAAGGCGCCCACGGGTGAGATCTACAGCTTTGCCAATGACAAG   1350
G  E  V  P  T  P  L  K  D  I  Y  G  L  C  L  F  K  A  P  T  G  E  I  Y  S  F  A  N  D  K  450
GACGGCACCTTTTTGCAACACCGCTTGAGCGCCAAGGGTGAGCAGGTGCAAGGCGAACTGGTGCGTCAGTTCAAGGTCGCGACCCAGCCT   1440
D  G  T  F  L  Q  H  R  L  S  A  K  G  E  Q  V  Q  G  E  L  V  R  Q  F  K  V  A  T  Q  P  480
GAAGGCTGTGTTGCCGATGACACGCATCAACGCCTGTTTATCGGTGAGGAAGACGTGGCGGTGTGGGCACTGGACGCGCGACCCGAGCAA   1530
E  G  C  V  A  D  D  T  H  Q  R  L  F  I  G  E  E  D  V  A  V  W  A  L  D  A  R  P  E  Q  510
CCGGCCGCCCTGAGCAGCGTGATCAACGTCGGTGGGCCAGTGCACGATGATATTGAAGGGCTGGCGCTGTATCAGGGCGAAAAAAACAGC   1620
P  A  A  L  S  S  V  I  N  V  G  G  P  V  H  D  D  I  E  G  L  A  L  Y  Q  G  E  K  N  S  540
TACCTGGTGATTTCCAGCCAGGGCAATGACAGCTACGTGGTGCTTGATGCGCAACCGCCGTATGCGTTACGTGGCGCCTTTCGGGTCGGC   1710
Y  L  V  I  S  S  Q  G  N  D  S  Y  V  V  L  D  A  Q  P  P  Y  A  L  R  G  A  F  R  V  G  570
GTGAACGCCGAGGCCGGGATTGACGGTGCGTCAGAGACCGATGGCCTGGAAGTGACCTCCGCCAACCTCGGCGGCCCGTTTACCCAAGGC   1800
V  N  A  E  A  G  I  D  G  A  S  E  T  D  G  L  E  V  T  S  A  N  L  G  G  P  F  T  Q  G  600
ATGCTGGTGGTGCAGGACGGGCGCAAGCGTATGCCCGAGCACAGTCAGAACTACAAATACATCCCGTGGGCCGATGTGGCCAAAACCCTG   1890
M  L  V  V  Q  D  G  R  K  R  M  P  E  H  S  Q  N  Y  K  Y  I  P  W  A  D  V  A  K  T  L  630
AACTTGCCTTGA                                                                               1902
N  L  P  *                                                                                 634
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Fig. 1. Nucleotide sequence of the β-propeller phytase (BBP) gene and its deduced amino acid sequence.

The first 23 amino acids correspond to the signal sequence. The blue-colored amino acid residues represent the internal repeat sequence, and the black-colored amino acids correspond to the BBP domain. The sequence was submitted to GenBank under the accession number KJ599466.

>20% PsBPP enzymatic activity was retained following incubation at 70°C for 30 min (Fig. 3A). These results suggest that PsBPP requires calcium ions for thermal stability, similar to the BPP from *Bacillus* sp. [12]. As shown in Fig. 3B, maximum PsBPP activity was observed at pH 6.0, and >65% of activity was retained in the pH range of 5.0–10.0. Moreover, the enzyme was stable over a broad pH range of 5.0–10.0. Its broad pH profile might be significant for potential PsBPP biotechnological applications, particularly for reducing the antinutritional effects of high phytate content foods in the gastrointestinal tract of monogastric animals [1].

Phytate exists as mineral-phytate salts in plant seeds, due to the strong negative charges on its six phosphate groups. Although Ca^{2+}-phytate is typically one of the most prevalent forms of mineral-phytate salts under physiological conditions [6], it is important to determine the types of mineral-phytate salts that can be used as PsBPP substrates. To address this question, we assessed the enzymatic activity of PsBPP to test the effects of divalent metal ions in the presence of various mineral ions. We found that PsBPP showed its highest enzymatic activity in the presence of 1 mM Ca^{2+}, followed by Fe^{2+}, Sr^{2+}, Mn^{2+}, and Cu^{2+} (Fig. 3C). Even in the presence of a high concentration of mineral ions (5 mM), PsBPP hydrolyzed Ca^{2+}-, Cu^{2+}-, Ni^{2+}-, and Fe^{2+}-phytate salts, suggesting an efficient hydrolysis of these salts by PsBPP (Fig. 3C). Together with other previously known BPPs [6], these results indicate that PsBPP efficiently eliminates phytate's chelation of various mineral ions, such as Ca^{2+}, Fe^{2+}, Sr^{2+}, Mn^{2+}, and Cu^{2+}, thus providing various minerals and phosphate groups as nutrients for marine bacteria such as *Pseudomonas* sp. BS 10-3.

Effect of Ca^{2+} on PsBPP Catalytic Activity

The biochemical characterization of PsBPP showed that the phytase requires Ca^{2+} for enzymatic activity as well as

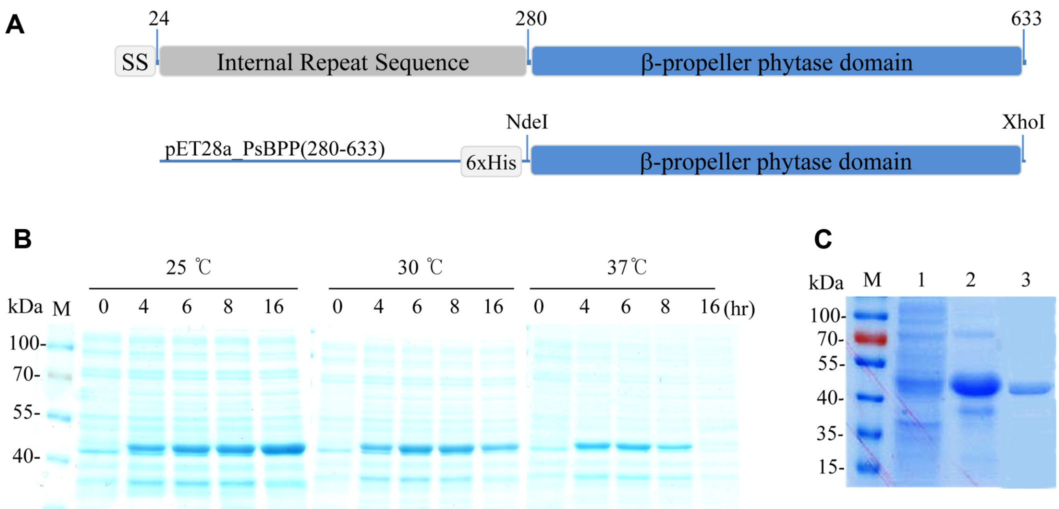

Fig. 2. Schematic illustration of the *Pseudomonas* sp. β-propeller phytase (PsBPP) structure and expression patterns at various temperatures.

(**A**) Schematic illustration of the PsBPP domain using the SMART database; amino acid residues 24–280 correspond to the internal repeat domain, and amino acid residues 280–633 correspond to the BPP domain. SS, signal sequence. The PsBPP domain was amplified by polymerase chain reaction and cloned into the pET 28a vector. (**B**) Time-course analysis of PsBPP expression at various temperatures (25°C, 30°C, and 37°C). Lane M, standard protein molecular weight markers. Lanes 1–5 correspond to different time points after IPTG induction. (**C**) Purification of PsBPP; M, protein standard marker; lane 1, cell lysates from 16 h of culture at 25°C after IPTG induction; lane 2, samples from Ni-NTA chromatography; lane 3, samples from Mono Q chromatography.

Ca^{2+}-phytate salts as a critical substrate component. To elucidate the effects of calcium ions on PsBPP catalytic activity, we measured the total amount of liberated phosphate at a fixed Na-phytate concentration (1 mM), while increasing the Ca^{2+} concentration from 0.1 to 3 mM. Increasing the concentration of Ca^{2+} enhanced PsBPP activity in a saturating manner to yield the Hill coefficient (h = 4.17 (0.16)), indicating that activation of the enzyme involved a minimum of four Ca^{2+}-binding sites and that enzyme activation was mediated by cooperative interactions between Ca^{2+} and PsBPP or phytate (Fig. 4A). These results suggest that the mode of Ca^{2+}-binding to the enzyme is quite similar to that of BaBPP. Moreover, PsBPP markedly differs from *Hahella chejuensis* BPP, which requires high levels of Ca^{2+} ions for catalytic activity [6]. To improve our understanding of the PsBPP kinetic mechanism, activities were measured using a single, fixed concentration of phytate (1 mM) and Ca^{2+} concentrations ranging from 0.1 to 6 mM. PsBPP enzymatic activities were maximal at a Ca^{2+} concentration of 1 mM. However, catalytic activity began to decline when the Ca^{2+} concentration exceeded 2 mM (Fig. 4B). Consistent with previous results [3, 6, 12], our data indicate that the rate of phytate hydrolysis depends on the relative Ca^{2+} and phytate concentrations and an equimolar ratio of Ca^{2+} to phytate. To further characterize the effects of Ca^{2+} ions on the kinetic properties of PsBPP, we determined the kinetic parameters of PsBPP at two fixed concentrations of Ca^{2+} (0.5 or 1 mM), while increasing the Na-phytate concentration from 0.1 to 3 mM. As shown in Fig. 4C, a double-reciprocal analysis of PsBPP showed that the apparent K_m increased as the Ca^{2+} concentration was increased, and the apparent V_{max} also increased. This result indicates that PsBPP requires Ca^{2+}-phytate salts, and not Ca^{2+}-free phytate, as a true substrate [12].

Final Product Identification and Time-Course Analysis of Natural Phytate

Previous results suggest that an equimolar concentration of Ca^{2+} to phytate is an optimal substrate condition for PsBPP enzymatic activity. Thus, we performed a time-course analysis of the total amount of phosphate liberated from Ca^{2+}-phytate salts, prepared by mixing 1 mM Na-phytate and 1 mM Ca^{2+} at 50°C in Tris-HCl at pH 7.0. The results showed that PsBPP efficiently hydrolyzed three phosphate groups per phytate molecule (Fig. 5A). This result was similar to the hydrolytic pattern and the number of phosphate groups hydrolyzed by other BPPs [6, 14]. To elucidate the final products of Ca^{2+}-phytate hydrolysis catalyzed by PsBPP, we further analyzed the reaction products using HPIC. Hydrolysis by PsBPP produced three

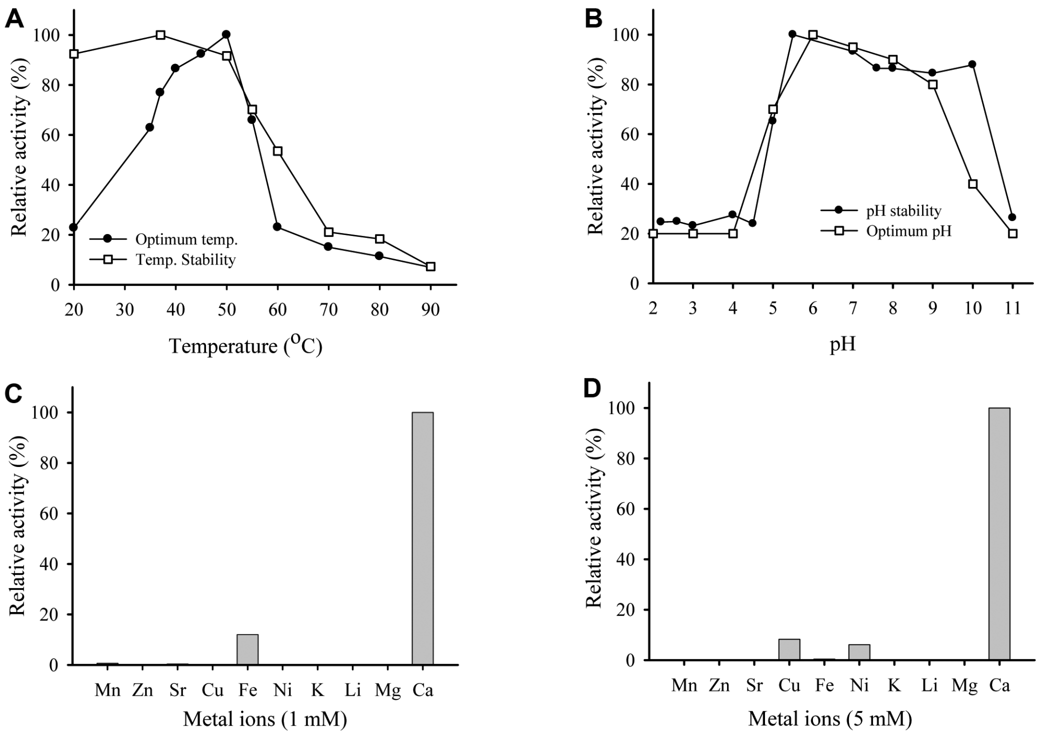

Fig. 3. Effects of temperature and pH on Ca^{2+}-phytate hydrolysis.

(**A**) *Pseudomonas* sp. β-propeller phytase (PsBPP) was pre-incubated at various temperatures for 30 min in the presence of 10 mM Ca^{2+}, and residual activity was measured at 50ºC in 50 mM Tris-HCl (pH 7.0). BPP activity in 50 mM Tris-HCl (pH 7.0) was assayed at various temperatures using a substrate prepared by mixing 1 mM phytate and 1 mM Ca^{2+}. (**B**) BPP activity at 50°C was measured in solutions of varying pH (●). In the pH stability test (■), PsBPP was pre-incubated with buffers of varying pH for 24 h at 4°C, and residual activity was measured at 50°C in 50 mM Tris-HCl (pH 7.0). The optimal temperature and pH for hydrolysis of Ca^{2+}-phytate by PsBPP were approximately 50°C and 6.0, respectively. (**C**, **D**) The effects of various minerals on different concentrations of PsBPP (1 and 5 mM). PsBPP was pre-incubated at 30°C for 30 min in the presence of various minerals (10 and 50 mM), and PsBPP activity was assayed at 50°C using a substrate prepared by mixing 1 mM phytate in 50 mM Tris-HCl (pH 7.0).

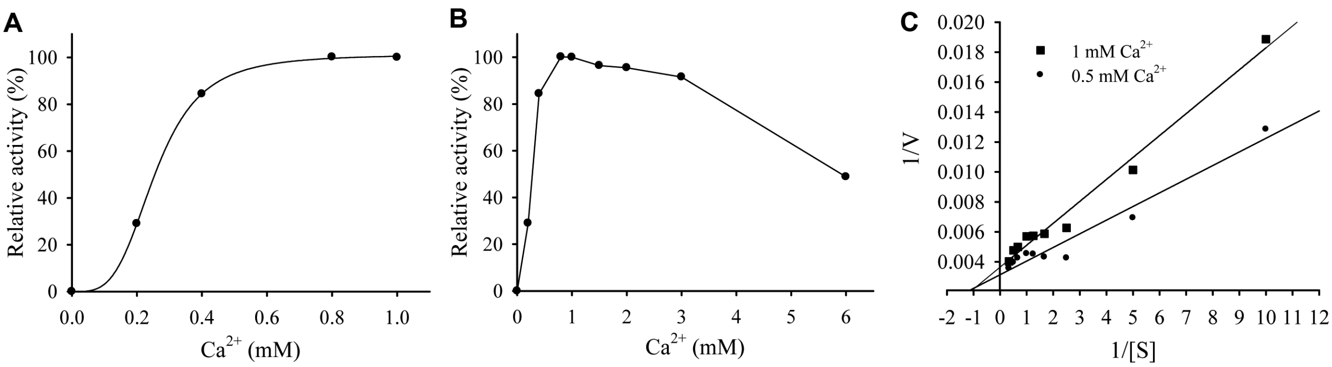

Fig. 4. Effects of Ca^{2+} concentrations on *Pseudomonas* sp. β-propeller phytase (PsBPP) activity.

(**A**) PsBPP was pre-incubated with increasing concentrations of Ca^{2+} and then mixed with 1.0 mM phytate in 50 mM Tris-HCl (pH 7.0). Curves were created by fitting the generated data to the Hill equation. The rate of phytate hydrolysis under each experimental condition is expressed as relative activity. (**B**) PsBPP activity was measured using a single, fixed concentration of phytate (1 mM) and Ca^{2+} concentrations ranging from 0 to 6 mM. (**C**) PsBPP activity was measured using a single, fixed Ca^{2+} concentration (0.5 or 1 mM) and phytate concentrations ranging from 0 to 4.0 mM. The K_m and V_{max} values were determined using a Lineweaver–Burk double reciprocal plot.

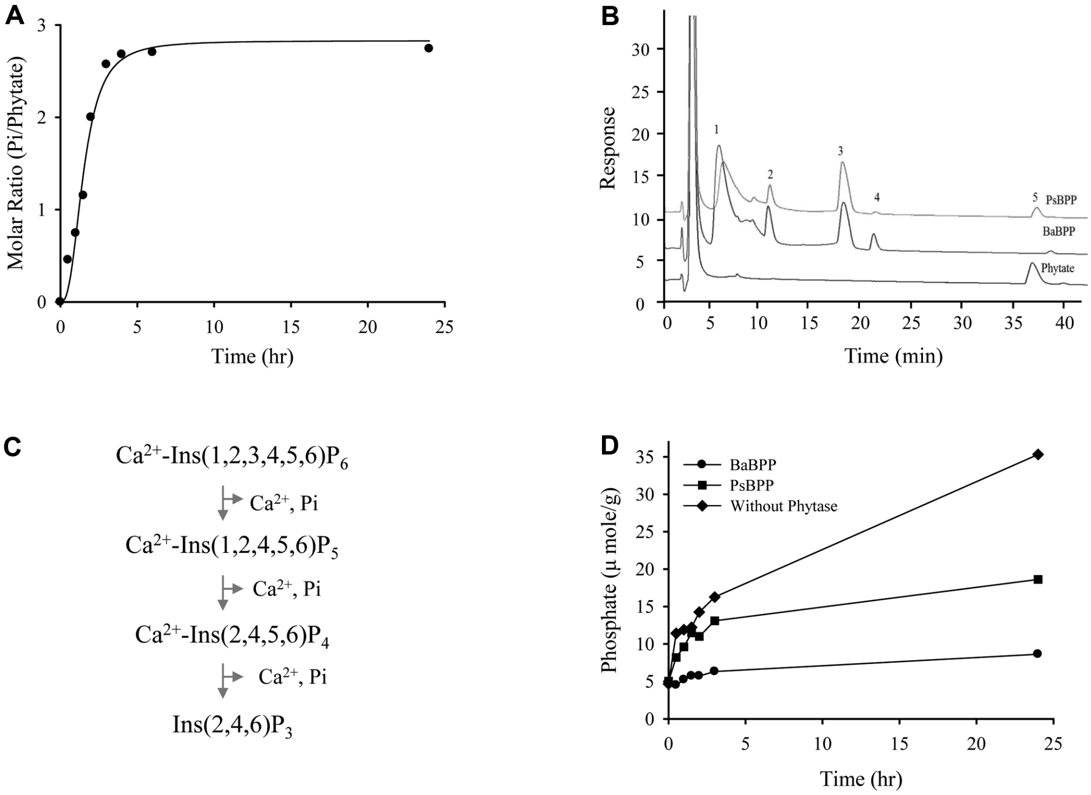

Fig. 5. Time-course analysis of inorganic phosphate liberation from Ca^{2+}-phytate by *Pseudomonas* sp. β-propeller phytase (PsBPP), high-performance ion chromatography (HPIC) analysis of the reaction products, schematic representation of the proposed hydrolytic pathway, and a time-course analysis for inorganic phosphate liberation from rice bran as a natural phytate source *via* PsBPP and *Bacillus amyloliquefaciens* BPP (BaBPP).

(**A**) Time-course analysis of the hydrolysis of Ca^{2+}-phytate using a substrate prepared by mixing 1 mM Ca^{2+} and 1 mM phytate. The phosphate concentration liberated as a result of hydrolysis of Ca^{2+}-phytate was very close to 3 mM, indicating that PsBPP hydrolyzed three phosphate groups per phytate molecule. (**B**) To determine their identities, the final reaction products of Ca^{2+}-phytate hydrolysis catalyzed by PsBPP were analyzed by HPIC. (**C**) Scheme for the hydrolytic pathway of Ca^{2+}-phytate salts catalyzed by PsBPP. PsBPP recognized insoluble Ca^{2+}-phytate salts and hydrolyzed Ca^{2+}-phytate at the D-3 position at initiation. The enzyme subsequently bound to the bidentate ligand inositol (1, 2, 4, 5, 6) P_5 and sequentially hydrolyzed the D-1 phosphate group, releasing inositol (2, 4, 5, 6) P_4, and finally the D-5 phosphate group, yielding inositol (2, 4, 6) P_3 as the final product. (**D**) PsBPP and BaBPP time course for inorganic phosphate formation using rice bran. The reaction was quenched at the indicated time points, and inorganic phosphate was measured.

phosphate groups and *myo*-inositol 2,4,6-trisphosphate (Ins(2,4,6)P_3, peak 3) (Fig. 5B), compared with the final products of BPP from *Bacillus* sp. DS11 [14]. We also identified Ins (2,4,5,6)P_4 (peak 4) as a reaction intermediate (Fig. 5B). Based on our kinetic data and the results of an HPIC analysis of the reaction intermediates and final products, we elucidated a schematic of the hydrolytic pathway of Ca^{2+}-phytate salts catalyzed by PsBPP (Fig. 5C).

To determine the capability of hydrolyzing natural phytate-rich foods, in which most phytates exist as Ca^{2+}-phytate salts, we hydrolyzed rice bran as a natural phytate source using two different enzymes. As shown in Fig. 5D, both BaBPP and PsBPP released similar amounts of phosphate from rice bran for the first 3 h. However, BaBPP more efficiently hydrolyzed natural phytate during long-term hydrolysis compared with PsBPP (Fig. 5C), indicating that the latter may be applicable for short-term hydrolysis of natural phytate. These results suggest that BPPs are strong candidates for reducing phytate in food and animal feed industries, such as soybean milk processing and starch hydrolysis. In conclusion, we showed that PsBPP efficiently hydrolyzed insoluble Ca^{2+}- or Fe^{2+}-phytate salts and completely abrogated the phytate chelation ability of various minerals such as Ca^{2+}, Mg^{2+}, Fe^{2+}, and Zn^{2+}. Most importantly, BPPs were capable of hydrolyzing natural phytates and eventually increasing the Ca^{2+} bioavailability from foods with high phytate contents.

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