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Full Length Research Paper

The phytase from antarctic bacterial isolate, *Pseudomonas* sp. JPK1 as a potential tool for animal agriculture to reduce manure phosphorus excretion

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Intracellular phytase activity was found in the Antarctic bacterial isolate, JPK1. Based on 16S rRNA gene sequence analysis, the strain was related to *Pseudomonas* sp. The optimal activity of JPK1 phytase occurred at 50°C and pH 5.0 to 5.5. The enzyme was highly specific for phytate with little or no other phosphate conjugates. Enzyme activity was strongly inhibited by Cu^{2+} and Zn^{2+}, and completely inactivated by sodium dodecyl sulfate. The enzyme effectively liberated inorganic phosphate from wheat bran, a main feedstuff with high phytate content. The enzyme may be a good candidate for use as an environmental-friendly feed additive to enhance the nutritive value of phytate and reduce phosphorus pollution.

Key words: Antarctic, phytase, *Pseudomonas* sp., phytate, feed additive.

INTRODUCTION

Phytate or phytic acid (*myo*-inositol hexakisphosphate; $InsP_6$) is the primary storage form of phosphorus in higher plants such as cereals, oilseeds, and legumes, which are the major constituents of animal feed (Haefner et al., 2005; Lei and Porres, 2003). Monogastric animals such as poultry and pigs, and fish cannot metabolize phytate phosphorus, owing to very limited levels of phytate-degrading enzyme activity in their gastrointestinal tracts (Cao et al., 2007; Maenz and Classen, 1998). Thus, inorganic phosphate is frequently added to poultry and swine diets to meet the nutritional requirements (Oh et al., 2004). Excess amounts of undigested phytate plus inorganic phosphate are discharged in the feces of these animals and can contribute to the eutrophication of water adjacent to the runoff from farmland (Cho et al., 2006). The absorption of nutritionally important cations including Ca^{2+}, Zn^{2+} and Fe^{2+}, and some proteins by phytate can contribute to malnutrition (Haefner et al., 2005; Stevenson-Paulik et al., 2005).

Phytate-degrading enzymes, which are termed phytases (*myo*-inositol hexakisphosphate phosphohydrolase), largely fall into three different groups: histidine acid phosphatase, β-propeller phytase and purple acid phosphatase. Phytases catalyze the stepwise hydrolysis of phytate to inorganic phosphate, a series of lower phosphate esters of *myo*-inositol and eventually *myo*-inositol (Brinch-Pedersen et al., 2002; Lei and Porres, 2003; Mullaney and Ullah, 2003). Phytases can be roughly classified as 3-phytases (EC 3.1.3.8) or 6-phytases (EC 3.1.3.26), based on the position-specificity of the initial hydrolysis from phytate (Brinch-Pedersen et al., 2002 ; Oh et al., 2004). In general, plant-derived phytases are considered to be 6-phytases, while the ones from microorganisms tend to be 3-phytases (Brinch-Pedersen et al., 2002). Ultimately, phytase supplementation of feed can improve phosphorus and mineral bioavailability in monogastric animals, and reduce the phosphorus pollution in the environment (Lei et al., 1993; Maenz, 2001). Although phytases are widely distributed in nature, occurring in plants, microorganisms, as well as in some animal tissues (Konietzny and Greiner, 2002), so far most have been extensively screened and characterized from microorganisms, which are the best sources for commercially biotechnological production of the enzymes because of their favorable catalytic

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properties and easy production (Haefner et al., 2005). However, only a few reports have been published concerning phytases from *Pseudomonas* sp., one of ubiquitous soil bacterial strains, which can survive on phytate as a sole source of carbon and phosphorus and seems to play a pivotal role in utilizing inositol phosphates which are rich in soils and waterbodies (Cho et al., 2003; Cho et al., 2005; Richardson and Hadobas, 1997). In addition, the genus *Pseudomonas* has been known to produce the phytase which belongs to the class of histidine acid phosphatase and shows a strict substrate specificity for phytate in comparison with one from well-known *Aspergillus* sp. (Cho et al., 2003; Cho et al., 2005). Recently, *Pseudomonas* is also reported to be an important source of another phytase type, β-propeller phytase in the fish intestine and it may be a promising target for studying the main source of phytases elsewhere in nature (Huang et al., 2009a; Patel et al., 2010).

Therefore, the present study partially characterizes an intracellular phytase produced by an Antarctic *Pseudomonas* sp. designated JPK1, by comparing the general catalytic properties of the enzyme with those of two different counterparts including a fungal *Aspergillus* phytase that is commercially-available as a feed additive (Haefner et al., 2005) and a wheat phytase.

MATERIALS AND METHODS

Reagents and source of other phytases

Unless otherwise stated, general chemicals were purchased from Sigma (St.Louis, USA). Two phytate substrates were used. Phytate dodecasodium salt (sodium phytate; Na-InsP_6., P0109) was purchased from Sigma and phytate pentamagnesium salt (Mg-InsP_6) was prepared as previously described (Torres et al., 2005). Crude phytases from *Aspergillus ficuum* (P9792) and wheat (P1259) were both purchased from Sigma.

Organism

A bacterial isolate JPK1 that was obtained from the Antarctic soil samples was supplied by Korea Polar Research Institute (KOPRI) operating the King Sejong Station (South Korea) in Antarctica.

Taxonomic identification of JPK1

Genomic DNA was extracted from isolate JPK1 using the FastDNA kit (6540-400., Qbiogene) according to the manufacturer's protocol. The 16S rRNA gene was amplified from genomic DNA by polymerase chain reaction (PCR) using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (William et al., 1991). The sequences of the amplified 1,435 bp fragments were determined using an automated ABI PRISM 3730 XL DNA analyzer (Applied Biosystems). The resulting sequences were compared with the GenBank database (NCBI) using BLAST (Altschul et al., 1990). Sequences showing a relevant degree of similarity were imported into the CLUSTAL W program (Thompson et al., 1994) and aligned. The evolutionary distances with other strains of *Pseudomonas* were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and the phylogenetic relationships were determined using the software MEGA version 4.0 (Tamura et al., 2007).

Partial purification of JPK1 phytase

Isolate JPK1 was cultivated in 100 ml of phytase production medium [0.5% $(NH_4)_2SO_4$ (A4418., Sigma) 0.01% NaCl (S9888), 0.01% $CaCl_2 2H_2O$ (223506), 0.01% $MgSO_4 7H_2O$ (230391), 0.001% $MnSO_4 4H_2O$ (M8179), and 0.001% $FeSO_4 7H_2O$ (F8048); pH 6.5] with 0.5% sodium phytate (phytic acid dodecasodium salt; Sigma) as a sole source of carbon and phosphorus for 96 h at 28°C. The cells were recovered by centrifugation at 10,000 x g, 20 min, 4°C and resuspended in 20 mL of 50 mM Tris-HCl (T1503) (pH 7.4). To extract the cytoplasmic proteins, 3 g of chilled glass beads (425 to 600 microns; G8772, Sigma) was added to the cell suspensions and vortexed at maximum speed for total of 30 min with 2 min chilling on ice for every 2 min of vortexing. The homogenate was centrifuged at 10,000 x g for 30 min at 4°C. After filtering the supernatant with a syringe filter (0.45 µm; 25CS045AS., ADVANTEC), the crude extract was loaded onto a cation exchange column (2.4 ml bed volume; 731-1550., BioRad) packed with SP sepharose high performance resin (17-1087-01., GE healthcare) that had been previously equilibrated with 50 mM Bis-Tris (B4429) (pH 6.0). The column was washed with the equilibration buffer, and bound proteins were eluted by sodium chloride gradient (0.25-1 M). Fractions showing highest phytase activity were pooled and dialyzed overnight against 50 mM Tris-HCl (pH 7.4) at 4°C. The dialyzed solution was used as the JPK1 phytase source throughout this work to examine its catalytic properties.

Zymogram analysis of JPK1 phytase

The enzyme was subjected to non-denaturing 6% polyacrylamide gel electrophoresis (PAGE) using a Modular Mini-Protein II Electrophoresis System (165-8001., Bio-Rad) according to the manufacturer's instructions. After gel electrophoresis, the gel was overlaid with 1.5% (w/v) molten agar (214010, BD) in 200 mM sodium acetate (S8625) (pH 5.5) containing 0.2% (w/v) Na-InsP_6. After overlay solidification, the gel was incubated at 50°C for 24 h, followed by flooding with 10% (w/v) $CaCl_2$. The band of phytase activity was detected as a transluscent zone against the opaque background (Casey and Walsh, 2003).

Measurement of phytase activity and substrate specificity

Unless otherwise stated, the JPK1 and wheat phytase assays were performed in 1 ml of 50 mM acetate buffer (pH 5.0) containing 1 mM Mg-InsP_6 at 50°C, and the *Aspergillus* phytase assay was done in 1 ml of 50 mM acetate buffer (pH 6.0) containing 1 mM Mg-InsP_6 at 50°C. The released inorganic phosphates were measured by a modified method of Heinonen and Lahti (1980), with a freshly prepared acetone ammonium molybdate (AAM) reagent consisting of acetone (154598), 5N sulfuric acid (320501), and 10 mM ammonium molybdate (A7302) (2:1:1, v/v). Two milliliters of the AAM solution and, thereafter 0.2 ml of 1 M citric acid (C2404) were added to the phytase assay mixture. Absorbance was read at 355 nm after blanking the spectrophotometer with an appropriate control. The assay for enzyme activity with other phosphorylated compounds such as ATP (A3377, Sigma), AMP (A1752), ribose-5-phosphate (83875) and glucose-6-phosphate (G7879) was carried out as described above by using 1 mM of each substrate. Acid phosphatase activity was assayed by using *p*-nitrophenyl

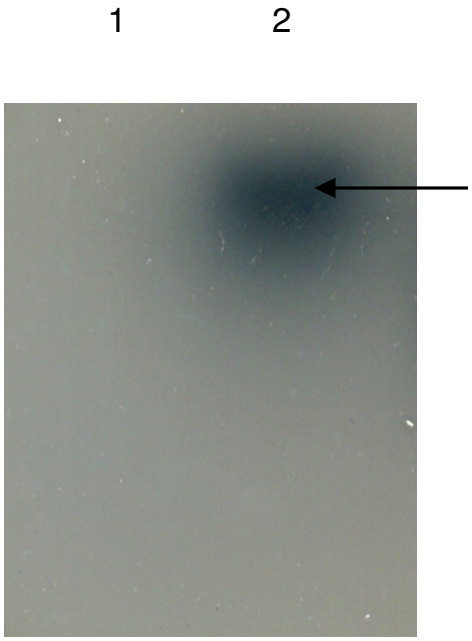

Figure 1. Zymogram analysis of phytase from *Pseudomonas* sp. JPK1 on a non-denaturing electrophoretic gel. Lane 1: BSA (bovine serum albumin) as a negative control ; lane 2: partially purified JPK1 phytase.

phosphate (71768, Sigma) as previously described (Greiner et al., 1993). The release of *p*-nitrophenol was measured at the absorbance of 405 nm. One unit (U) of enzyme activity was defined as the amount of enzyme to produce 1 µmol of inorganic phosphate or *p*-nitrophenol per minute under the defined assay conditions.

Effect of pH and temperature on enzyme activity

Phytase activities were investigated in the pH range of 2 to 8.5 [50 mM glycine-HCl (G8898) (pH 2-3); 50 mM sodium acetate (pH 4 to 5.5); 50 mM Bis-Tris-HCl (pH 6-7); 50 mM Tris-HCl (pH 7.4-8.5)] at 30°C and temperatures between 0 and 80°C at each optimum pH.

Effect of metal ions and chemicals on enzyme activity

The effects of different metal ions and chemicals on phytase activities were determined under standard enzyme assay conditions in the presence of 1 mM of Ba^{2+} (B0750.,Sigma), Mg^{2+}(M8266), Ca^{2+}, Co^{2+}(60820), Zn^{2+}(96468), Ni^{2+}(N6136), Cu^{2+}(212946), Mn^{2+}(221279), ethylenediaminetetraacetic acid (EDTA) (E5134), sodium dodecyl sulfate (SDS) (L4390), or phenylmethylsulfonyl fluoride (PMSF) (P7626).

Phosphate liberation from feed ingredient

Finely ground wheat bran was autoclaved for 15 min at 121°C to inactivate the endogenous phytase, and aliquots (5 g) of the autoclaved wheat bran were resuspended in 40 ml of 0.2 M acetate buffer (pH 5.0). The suspensions were incubated as such or with 200 U of partially purified JPK1 phytase per kg of the wheat bran for 300 min at 37°C in a shaking water bath. From the incubation mixtures, 1 ml aliquots were removed at different time points and centrifuged at 10,000 x g for 2 min at 4°C. The supernatant was collected and the liberated inorganic phosphates were quantified as described above.

Statistical analysis

The data were expressed as mean and standard errors from three experiments, and their significance was analyzed using Student's *t*-test.

RESULTS AND DISCUSSION

Nucleotide sequence accession numbers

The nucleotide sequence of the 16S rRNA gene of the bacterial isolate JPK1 has been deposited in the GenBank database under Accession No. GU393025.

Identification of the bacterial isolate JPK1

To identify the organism displaying phytase activity (Figure 1), the 16S rRNA gene of isolate JPK1 was cloned and its sequence was compared with those available in the public GenBank database. A phylogenic tree based on the 16S rRNA gene sequences from nine members of bacterial *Pseudomonas* strains showed that strain JPK1 was closely related to the type strain, *Pseudomonas collierea* PR212, with 99.8% identity (Figure 2). Therefore, it was designated *Pseudomonas* sp. JPK1.

Partial catalytic properties of JPK1 phytase, compared with those of *Aspergillus ficuum* and wheat phytase

Until now, conventional phytase research has routinely used the chemically synthesized dodecasodium salt of phytate (Na-InsP_6) as a substrate for the quantitative enzyme assays (Cho et al., 2006; Wyss et al., 1999). However, a recent and comprehensive re-evaluation of the nature of soluble InsP_6 in biological surroundings has revealed that the pentamagnesium salt (Mg-InsP_6) is the most physiologically-relevant form of InsP_6 (Cho et al., 2006; Torres et al., 2005). Therefore, Mg-InsP_6 was used as a main substrate in the present differential phytase assay.

Optimal JPK1 phytase activity occurred at pH 5.0 to 5.5, which matches with the low-temperature active phytase recently isolated from a bacterial strain, *Erwinia carotovora* var. *carotovota* (Huang et al., 2009b) and over 40% of the activity was retained at pH 4 to 5.5, which is relatively close to the pH range at potential sites of degradation of feed derived phytate such as salivary glands (pH 5.0) and upper part of small instestine (pH 4.0

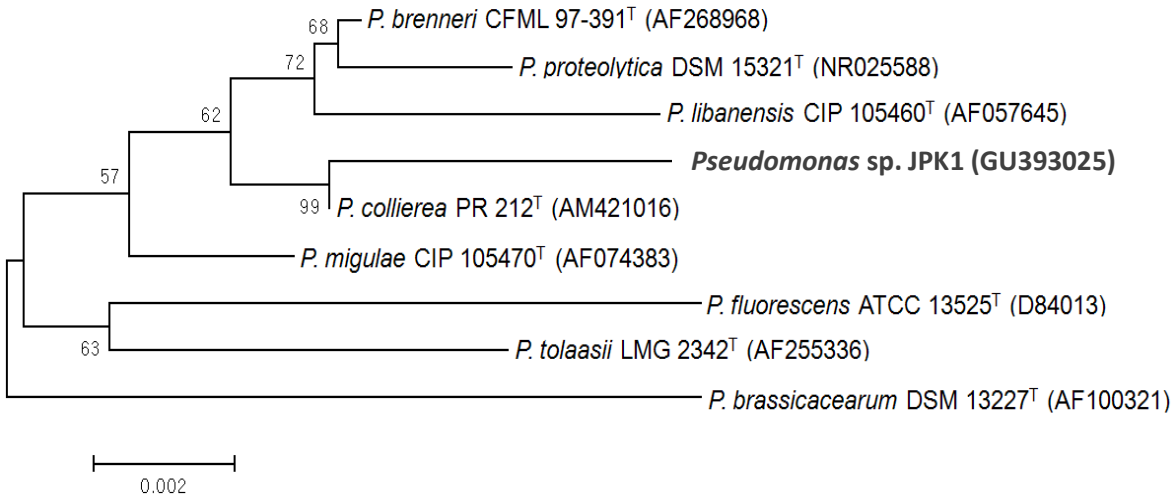

Figure 2. Phylogenic relationship of the 16S rRNA sequences of *P.* sp. JPK1 with other type strains of *Pseudomonas.* Bootstrap values (based on 1,000 trials and only values > 60%) are shown at the nodes. The GenBank accession numbers are indicated in parentheses. *Bar,* 2-base substitutions per 1,000 nucleotide positions. *P.* represents the abbreviation of *Pseudomonas*.

to 6.0) (Casey and Walsh, 2003; Haefner et al., 2005) and pH 8 (Figure 3A). Most acidic phytases present optimal activities at pH range of 4.5 to 6 (Konietzny and Greiner, 2002), but phytases from *Bacillus* sp. have neutral or alkaline pH optima (Lei and Porres, 2003). Until now, most effective feeding trials have been done with the acidic phytase (Haefner et al., 2005). Nevertheless, it still seems difficult to define an ideal pH profile concerning the phytase activity, because phytase with neutral pH optimum occasionally displays significant biological activity (Haefner et al., 2005). Surprisingly, three pH optima, at pH 3.0, 6.0 and 7.4, were observed for *A. ficuum* phytase (Figure 3B). This is different from the previous result that *A. niger* NRRL 3135 phytase exhibits optimal activity at pH 2.5 and 5.5 for Na-InsP_6 (Casey and Walsh, 2003). Presumably, at the additional pH 7.4, the local electrostatic field attraction between the Mg-InsP_6 and specific amino acid residues constituting catalytic active center in the *A. ficcum* phytase molecule may be increased, which may help to enhance the activity (Mullaney et al., 2002; Wyss et al., 1999). On the other hand, wheat phytase showed optimal activity at pH 5.0 (Figure 3C), as previously reported (Nakano et al., 1999).

The optimal activity for JPK1 and *A. ficuum* phytase was shown at a temperature of 50°C. JPK1 phytase retained more than 90% of the activity at 30 to 40°C (Figure 4A), whereas more than 70% of *A. ficuum* phytase activity was retained at the same temperature range (Figure 4B). JPK1 phytase may be a more favorable target for its application as a feed additive because its temperature range for the maximal activity is very close to the gastrointestinal temperature of pig or poultry (37 to 40°C) (Lei and Porres, 2003). Both enzymes displayed a marked loss of activity at higher temperatures (60 to 80°C). In contrast, wheat phytase maintained more than 50% of its highest activity relatively at 30 to 80°C with an optimum of 50°C (Figure 4C). Although enzymes obtained from microorganisms inhabiting cold environments such as polar regions and deep sea show higher catalytic efficiency at low temperatures than their mesophilic counterparts (Gerday et al., 1997), some phytases isolated from psychrophilic microorganisms display optimal activity at a temperature typical of enzymes from mesophilic producers. For instance, the phytase from the Antarctic yeast strain, *Cryptococcus laurentii* exhibits maximal activity at 40°C (Pavlova et al., 2008), whereas *Pedobacter nyackensis* derived from glacier soil produces a phytase with a temperature optimum of 45°C (Huang et al., 2009c). JPK1 phytase was close to a mesophilic enzyme, which is similar to a previous report that the optimal activity for MOK1 phytase from *P. syringae* is maximal at 40°C (Cho et al., 2003).

JPK1 phytase was highly specific for phytate and showed little or no activity on other phosphate esters (Figure 5). This is in good agreement with the results obtained with *P. syringae* MOK1 phytase (Cho et al., 2003). On the other hand, *A. ficuum* and wheat phytase showed broader specificity for phytate and various other phosphate compounds (Figure 5). Futhermore, *A. ficuum* phytase was 4-fold less active against Na-InsP_6 compared to Mg-InsP_6, while JPK1 and wheat phytase equally hydrolyzed these two substrates (Figure 5). Interestingly, the wheat enzyme hydrolyzed ATP 5.4-fold higher than phytate substrates, which supports the fact that phytate-degrading enzymes from plants generally yield the highest relative rates of hydrolysis with

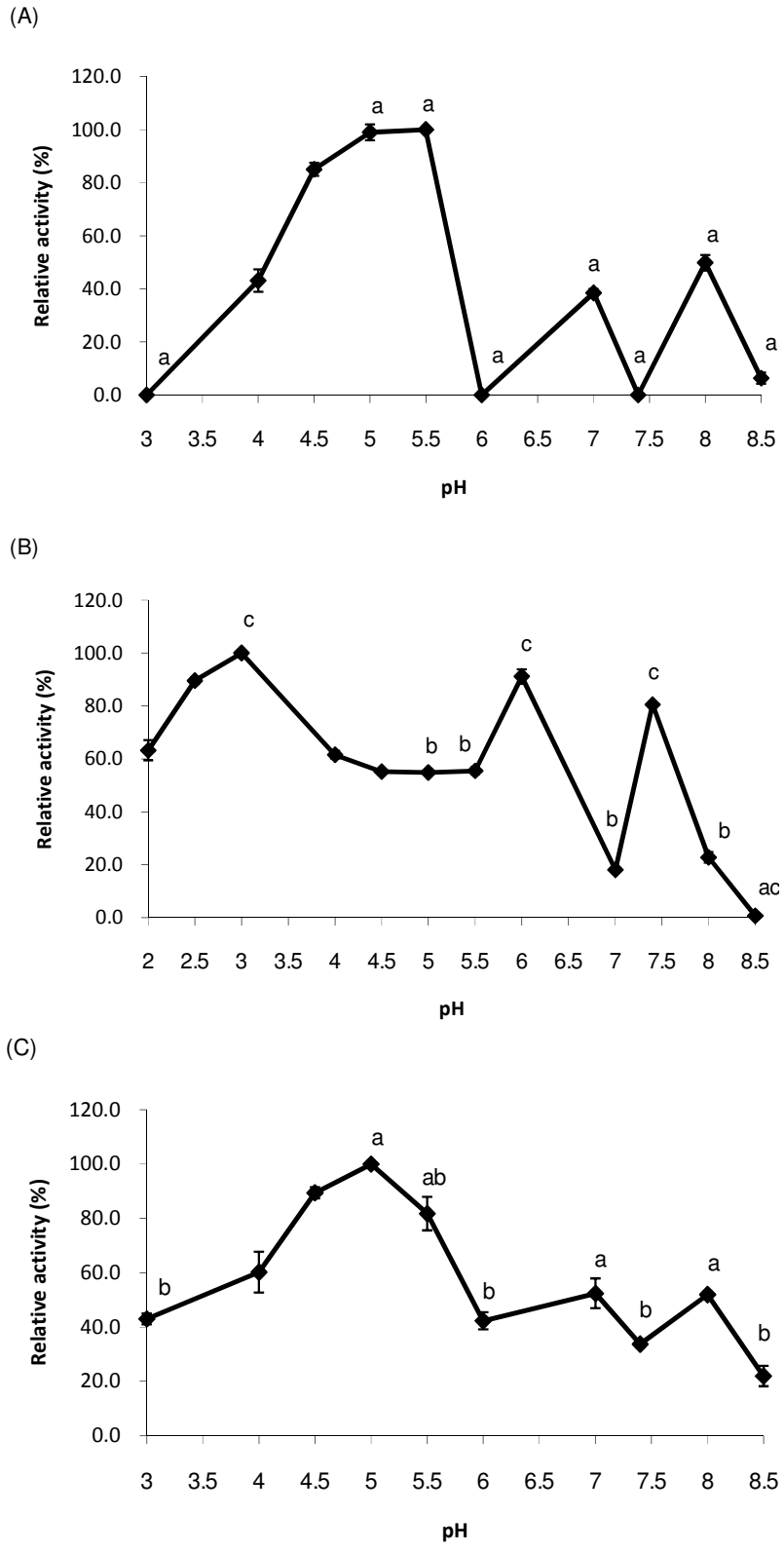

Figure 3. pH activity profiles. Relative activity is expressed as a percentage of the maximum (100% of relative activity in JPK1, *Aspergillus*, and wheat phytase equates to 0.37, 0.71, and 0.13 U/ml, respectively). Data represent means and standard errors from three experiments. Values in the same pH without a common letter differ significantly ($P<0.05$) among the phytases. (A) *Pseudomonas* sp. JPK1 phytase, (B) *A. ficuum* phytase, (C) Wheat phytase.

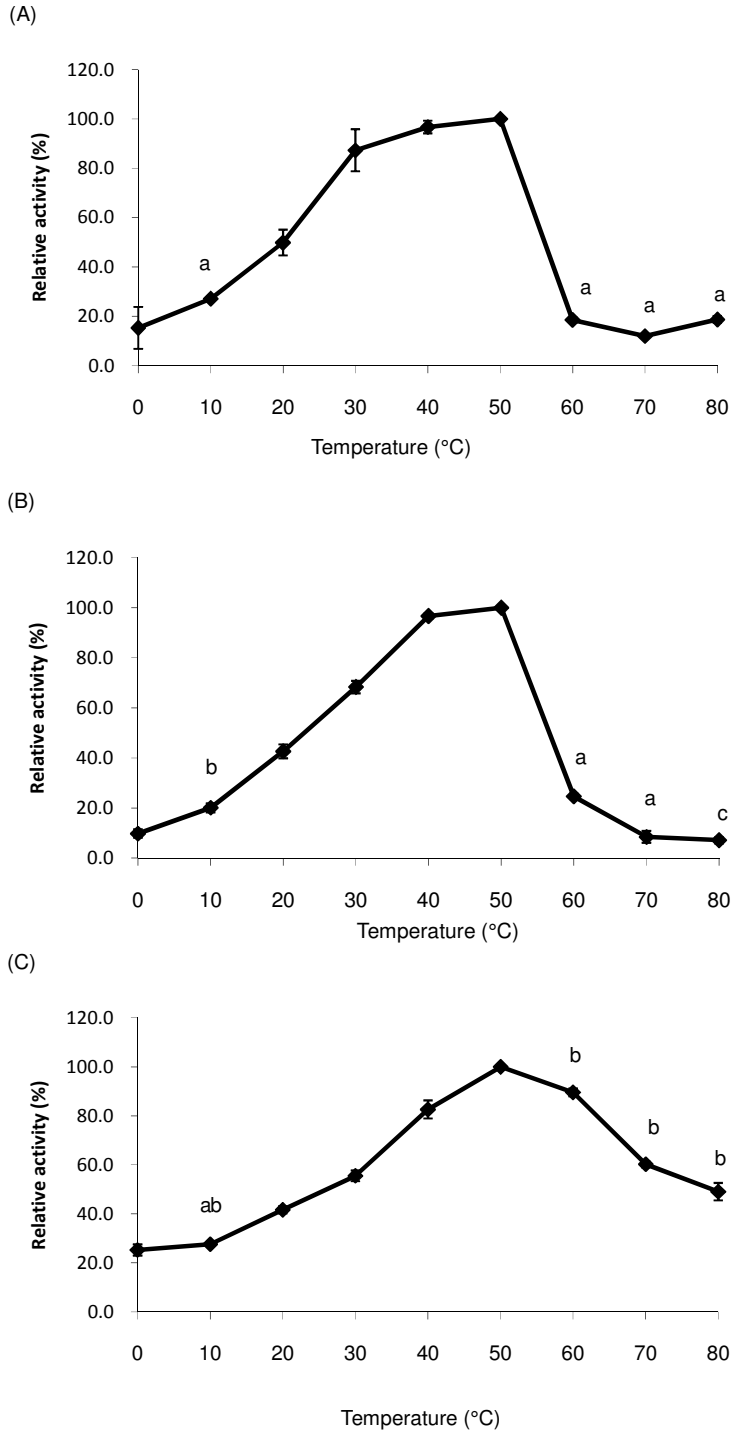

Figure 4. Temperature activity profiles. Relative activity is expressed as a percentage of the maximum (100% of relative activity in JPK1, *Aspergillus*, and wheat phytase equates to 0.38, 1.09, and 0.32 U/ml, respectively). Data represent means and standard errors from three experiments. Values in the same temperature without a common letter differ significantly ($P<0.05$) among the phytases:(A) *Pseudomonas* sp. JPK1 phytase, (B) *A. ficuum* phytase, (C) Wheat phytase.

pyrophosphate and ATP (Konietzny and Greiner, 2002). Although many phytases in a subfamily of histidine acid phosphatases typically characterized by the conserved active site motif, RHGXRXP, do not tend to display strict

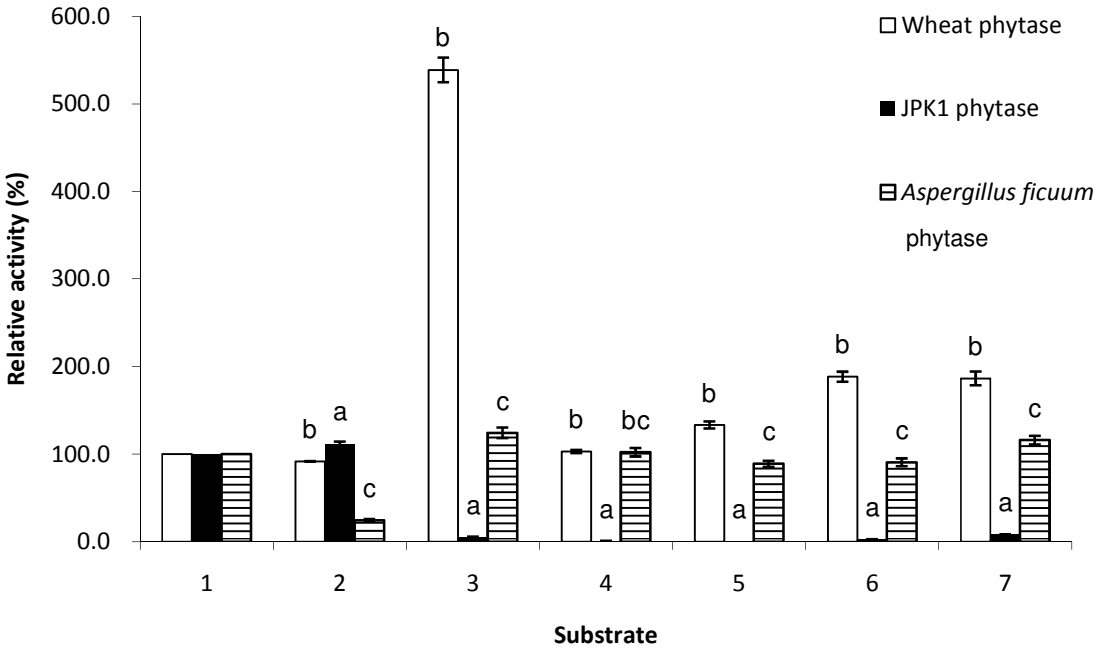

Figure 5. Substrate specificities of *P.* JPK1, *A. ficuum*, and wheat phytase. Relative activity is expressed as a percentage of the maximum (100% of relative activity in JPK1, *Aspergillus*, and wheat phytase equates to 0.54, 1.12, and 0.22 U/ml, respectively). The values below 0.5% in the relative activity were unseen. Data represent means and standard errors from three experiments. Values in the same substrate without a common letter differ significantly ($P<0.05$) among the phytases.1. Mg-InsP_6, 2. Na-InsP_6, 3. ATP, 4. AMP, 5. ribose-5-phosphate, 6. glucose-6-phosphate, 7. *p*-nitrophenyl phosphate.

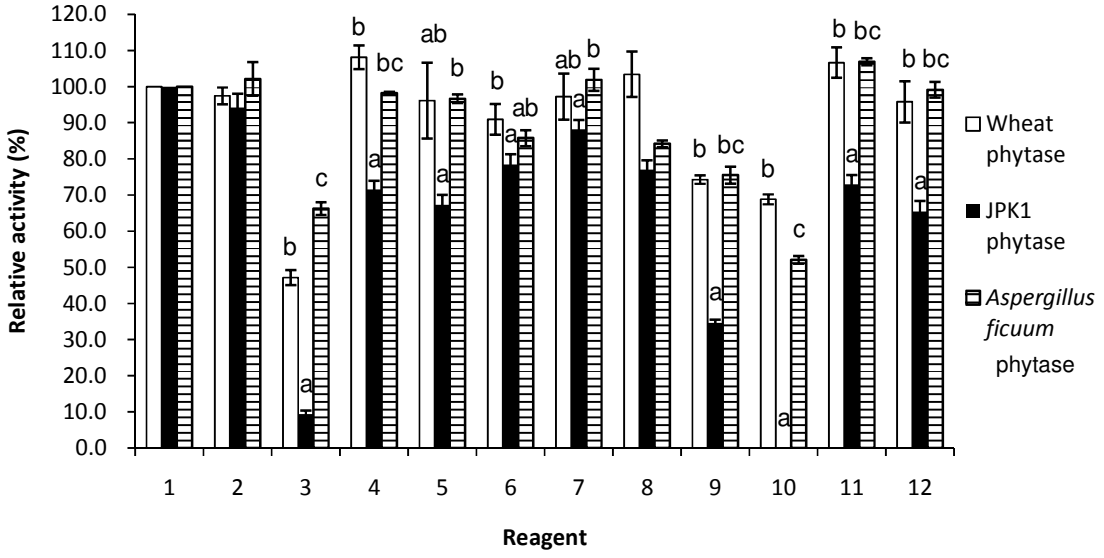

Figure 6. Effect of metal ions and chemicals on phytase activity. Relative activity is expressed as a percentage of the maximum (100% of relative activity in JPK1, *Aspergillus*, and wheat phytase equates to 0.77, 0.94, and 0.29 U/ml, respectively). The value (0%) in the relative activity was unseen. Data represent means and standard errors from three experiments. Values in the same reagent without a common letter differ significantly ($P<0.05$) among the phytases. 1. control (no addition) ; 2. Mg^{2+}, 3. Cu^{2+}, 4. Mn^{2+}, 5. Ca^{2+}, 6. Co^{2+}, 7. Ba^{2+}, 8. Ni^{2+} , 9. Zn^{2+}, 10. SDS ,11. EDTA and 12. PMSF.

substrate specificity for phytate (Oh et al., 2004), MOK1 phytase from *P. syringae* (Cho et al., 2003) and avian multiple inositol polyphosphate phosphatase (Cho et al., 2006), which belong to the subfamily, show exceptionally

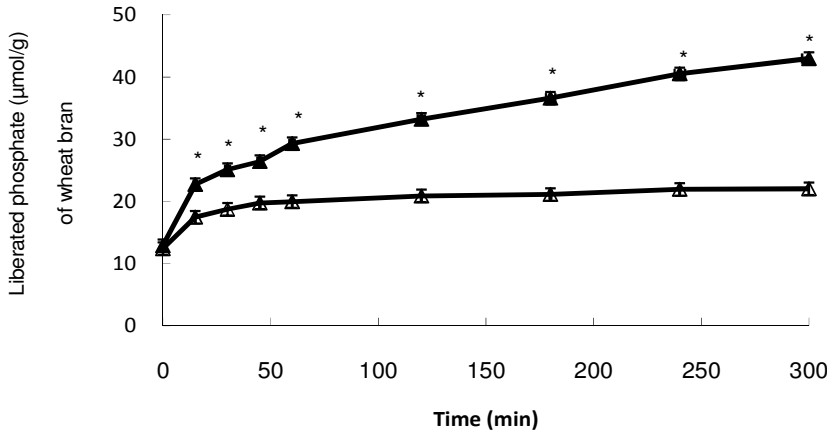

Figure 7. *In vitro* phosphate liberation from wheat bran by JPK1 phytase. Symbols represent no enzyme supplementation (open triangle) and enzyme supplementation (200 U) (filled triangle). Data represent means and standard errors from three experiments. Asterisks indicate significantly differences ($P<0.05$) between no enzyme supplementation and enzyme supplementation.

strict substrate specificity for phytate. As a comparison, alkaline β-propeller phytases, which do not contain the conserved RHGXRXP motif of histidine acid phosphatases and exist in some *Bacillus* strains, are also reported to be highly specific for phytate (Oh et al., 2004). Some metal ions and chemicals modulate phytase activity (Konietzny and Greiner, 2002). As shown in Figure 6, the activity of JPK1 phytase was by far more strongly inhibited by Cu^{2+} and Zn^{2+}, which are regarded as representative inhibitors in most phytases characterized so far (Konietzny and Greiner, 2002), than *A. ficuum* and wheat phytase. However, no major effects on the activities of JPK1, *A. ficuum*, and wheat phytase were observed in the presence of Mg^{2+}, Co^{2+}, Ba^{2+}, Ni^{2+}, or the chelating agent, EDTA. In contrast, the metal ion-dependent phytase of *Bacillus subtilis* is readily inhibited by EDTA (Kerovuo et al., 2000), but the activities of *A. fumigatus* and *A. ficuum* NTG-23 phytase are increased up to 50% and about 10% in the presence of 1 mM and 1.25 mM EDTA, respectively (Wyss et al., 1999; Zhang et al., 2010). Unlike two other phytases, the common anionic surfactant SDS completely inactivated JPK1 phytase, indicating hydrogen bonds may play a crucial role in maintaining the enzyme activity (Wang et al., 2005). Additionally, Mn^{2+}, Ca^{2+}, and the well-known serine protease inhibitor PMSF moderately inhibited JPK1 phytase. The activities of *B. subtilis*, *Bacillus amyloliquefaciens*, lily pollen, and cattail pollen phytate-degrading enzymes are Ca^{2+}-dependent (Konietzny and Greiner, 2002).

In vitro phosphate liberation from feed ingredient by JPK1 phytase

As shown in Figure 7, the net amount of inorganic phosphate released from wheat bran, which is a representative feed ingredient with high phytate content (Haefner et al., 2005), was 21 ± 3.1 μmol/g of wheat bran at pH 5.0 for 300 min. The ability of JPK1 phytase to degrade natural phytate in feeds as well as chemically pure soluble phytate is practically important, because phytate in cereals largely exists as an insoluble salt form (Cho et al., 2003).

Surprisingly, the growth of animal feed enzymes in the global market for industrial enzymes is remarkable, with an expected average annual growth rate of 4%, supported in large part by the increased use of phytase to fight phosphorus pollution (Hasan et al., 2006). Advanced feed production relies on biotechnological approaches. JPK1 phytase may be potentially useful as an environmental-friendly microbial feed additive because of its strict substrate specificity and its favorable activity levels at physiologically relevant pH and temperature. In the future, more detailed research on protein engineering and recombinant DNA work will be considered to improve the catalytic efficiency and productive yield of the enzyme.

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