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Purification and Properties of an Extracellular Acid Phytase from *Pseudomonas fragi* Y9451

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Abstract An extracellular acid phytase from *Pseudomonas fragi* Y9451 was purified to homogeneity from the culture supernatant by salting-out, DEAE-Sepharose column chromatography, CM-Sepharose column chromatography, and Sephacryl S-300 gel filtration. The molecular weight of the purified enzyme was estimated to be 74 kDa on gel filtration and 54 kDa and 25 kDa on SDS-PAGE, suggesting that the native enzyme was a heterodimeric protein. The purified enzyme was most active at pH 4.5 and 70°C and fairly stable from pH 4.0–6.0. It was specific for phytate and exhibited a K_m value of 27 mM (sodium phytate, pH 4.5, 50°C). The phytase activity was strongly inhibited (at maximum by 87%) by Fe^{3+}, Cu^{2+}, Fe^{2+}, and Zn^{2+} at 5 mM concentration, and greatly inhibited by Ca^{2+} at 10 mM concentration. However, EDTA notably stimulated the phytase activity at 10 mM concentration. With optimum pH and stability, *Pseudomonas fragi* phytase could be a potential candidate for animal feed applications.

Key words: *Pseudomonas fragi*, extracellular acid phytase, heterodimer

Phytases (EC 3.1.3.8) catalyze the hydrolysis of phytate (*myo*-inositol hexaphosphate), the major storage form of phosphorus in plant seed and seed-based animal feed, to the inorganic phosphate and less-phosphorylated *myo*-inositol derivatives [14]. Monogastric animals, such as pigs and poultry, virtually lack phytase activity in their digestive tracts. Because phytate phosphorous is metabolically unavailable to these animals, the supplementation of the feed with inorganic phosphate becomes necessary, even though this method increases the phosphorus burden in the manure, causing environmental problems by the eutrophication of surface water. Phytate also acts as an anti-nutrient by forming complexes with proteins and various metal ions that decrease the dietary availability of these nutrients. In order to decrease environmental pollution by high-phosphorus manure and improve the nutritional value of feed, the hydrolysis of phytate in animal feeds is necessary.

Phytases are present in plants, certain animal tissues, and microorganisms. They have been studied most intensively in the seeds of plants [3, 4]. Phytase activity in microorganisms has been found most frequently in fungi [16, 21] and bacteria [2, 6, 11, 13, 25]. Among the bacterial phytases, the optimum pHs for extracellular and intracellular phytases are 6.0–7.0 and 4.5–6.0, respectively. For industrial feed applications, a phytase with a pH activity profile ideally suited for maximal activity in the digestive tract of monogastric animals is desirable. Because of its great environmental and industrial importance, there is an ongoing interest in isolating new microbial strains capable of producing novel and efficient phytases.

A bacterial strain producing phytase was isolated from a streamlet near a livestock shed in Korea and identified as a *Pseudomonas fragi* [12]. In this study, an extracellular phytase produced by a bacterial strain, *P. fragi* Y9451, which showed maximum activity at acidic pH range, was purified and its enzymatic properties were characterized.

Materials and Methods

Chemicals

DEAE-Sepharose, CM-Sepharose, and Sephacryl S-300 were purchased from Amersham Biosciences AB (Uppsala, Sweden). Molecular weight marker protein for gel filtration, sodium phytate, sodium triphosphate, disodium *p*-nitrophenyl phosphate, disodium pyrophosphate, disodium α-naphtyl phosphate, disodium β-glycerophosphate, and disodium glycerophosphate were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Molecular weight markers

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for SDS-PAGE were supplied by Invitrogen Corp. (Carlsbad, CA, U.S.A.). All other chemicals were of analytical grade.

Bacterial Cultivation

P. fragi Y9451, one of the isolates capable of producing phytase, was grown aerobically in a suspension culture. The preculture was conducted in nutrient broth (Difco Laboratories, Detroit, MI, U.S.A.) at 30°C for 12 h on a rotary shaker at 150 rpm. The medium of main cultivation for enzyme production contained 16 g of nutrient broth and 50 g of fructose per liter and adjusted to pH 7.0 prior to autoclaving. The main culture was carried out for 120 h under the same conditions as the preculture.

Enzyme Purification

After removing cells from fermented broth by centrifugation (3,000 ×*g*, 15 min), extracellular protein fractions were precipitated by ammonium sulfate at 40–80% saturation. The pellets were dissolved in 50 mM Tris-HCl buffer (pH 7.0) containing 2 mM 2-mercaptoethanol and dialyzed against the same buffer for 24 h.

The dialyzed enzyme solution was loaded onto a DEAE-Sepharose column (3×24 cm) equilibrated with the same buffer, and washed with 6 column volumes of 50 mM acetate buffer (pH 4.5) containing 2 mM 2-mercaptoethanol. This buffer is hereafter referred to as the standard buffer. The fractions showing phytase activity were collected in the washing step without elution.

The collected solution was applied onto a CM-Sepharose column (3×34 cm) equilibrated with the standard buffer and washed with 4 column volumes of the standard buffer. Elution was done with a linear gradient of KCl (0–0.5 M) in the standard buffer at a flow rate of 36 ml/h. The active fractions were pooled and dialyzed against the standard buffer.

The concentrated protein solution was loaded for gel permeation chromatography using a Sephacryl S-300 column (2×140 cm) equilibrated with the standard buffer. The column was developed at 22 ml/h. The fractions containing phytase were pooled and concentrated using cold sugar.

Enzyme Activity Assay

Phytase assays were carried out using the Shimizu method [17] with some modification. The enzymatic reactions were initiated by adding 100 µl of enzyme to the assay mixture. The assay mixture consisted of 900 µl of 0.1 M sodium acetate buffer, pH 4.5, containing 2 µmol of sodium phytate. After incubation at 50°C for 10 min, the liberated inorganic phosphate was measured according to the ammonium molybdate method [8]. One milliunit (mU) of phytase was defined as one nanomole of phosphate liberated per minute.

Determination of Molecular Mass

The molecular mass of the purified enzyme was estimated by gel permeation chromatography, using a Sephacryl S-300 column equilibrated with the standard buffer containing 50 mM KCl. Subunit analysis was done by SDS-PAGE, using commercially available 4–20% Tris-glycine pre-cast gel (Tefco, Tokyo, Japan) calibrated with standard proteins [9, 15]. The gel was stained with Coomassie blue R-250 dye.

Other Analytical Methods

Total protein concentration was determined by Coomassie blue G-250 dye binding assay using bovine serum albumin as a standard [1]. Protein in the column eluents was monitored by measuring the absorbance at 280 nm. The K_m and V_{max} values were determined from a Lineweaver-Burk double reciprocal plot.

Results and Discussion

Production of the Phytase

In previous work, a *Pseudomonas* sp. producing phytase was isolated from a streamlet near a livestock shed in Korea and the 16S rDNA sequence of isolated strain was 99% homologous to that of *Pseudomonas fragi* [12]. Therefore, the isolated strain was considered to be a variant of *Pseudomonas fragi* and named *P. fragi* Y9451. When *P. fragi* Y9451 was cultured in phytase-producing medium containing nutrient broth and 5% fructose at 30°C, extracellular phytase activity reached up to 839 mU/ml at 120 h. After centrifugation of the culture broth, the clear supernatant was used for phytase purification.

Purification and Properties of the Phytase

Phytase from the culture supernatant of *P. fragi* Y9451 was purified using ammonium sulfate fractionation, anion-exchange chromatography, cation-exchange chromatography, and gel

Table 1. Purification of phytase from *Pseudomonas fragi* Y9451.

Step	Activity (U)	Protein (mg)	Specific activity (U/mg protein)	Purification (fold)	Yield (%)
Crude enzyme	275	14,765	0.019	1	100
$(NH_4)_2SO_4$ fractionation	267	12,966	0.021	1.10	97
DEAE-Sepharose	194	3,937	0.049	2.63	70
CM-Sepharose	112	214	0.362	28.13	41
Sephacryl S-300	46	16	0.912	49.79	17

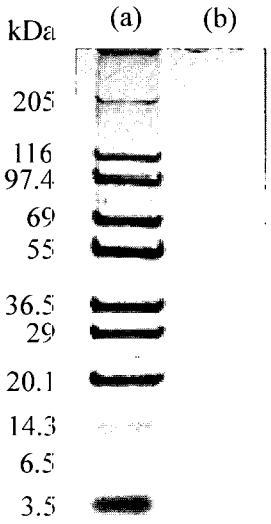

Fig. 1. SDS-PAGE of phytase from *Pseudomonas fragi* Y9541.
Lane (a), molecular weight markers in kDa; Lane (b), phytase.

filtration. The overall scheme of purification is shown in Table 1. The phytase was purified about 50-fold with a yield of 17%. The purified enzyme exhibited a specific activity of 0.91 U/mg protein.

The enzyme gave a single peak with the estimated molecular mass of 74 kDa on gel filtration, and two bands on SDS-PAGE corresponding to molecular masses of 54 kDa and 25 kDa (Fig. 1). These results suggest that this enzyme is a heterodimeric enzyme. The subunit composition and molecular mass of this enzyme differ from the reported phytases of the following bacterial strains: *Bacillus* sp. DS11 (monomeric enzyme of 44 kDa [13]), *E. coli* (monomeric enzyme of 42 kDa [6]), *B. subtilis* (monomeric enzyme of 43 kDa [10]), and *Klebsiella terrigena* (monomeric enzyme of 40 kDa [5]). On the other hand, fungal phytases from the *Aspergillus* species origin have been reported to possess a large molecular weight: 214 kDa (a homehexamer) for *A. terrus* [23] and 85–100 kDa (a monomer) for *A. ficuum* [21]. Dimeric phytases, such as those from rat intestine [24] and soybean [7], have also been described; however, they have much larger subunits and markedly different properties. Since the extracellular phytase from *P. fragi* Y9451 is different in many aspects from the already known phytases of other sources, it is considered to be a new phytase.

Effect of pH and Temperature

The effect of pH on the enzyme activity was examined in three different buffer systems, 0.1 M glycine-HCl (pH 2.0–3.0), 0.1 M acetate (pH 4.0–6.0), and 0.1 M Tris-HCl (pH 7.0–9.0). The optimum pH was determined to be 4.5 (Fig. 2). However, the enzyme remained highly active in the pH range of 4.0 to 6.0, retaining more than 80% of the maximum activity. To measure pH stability, the remaining

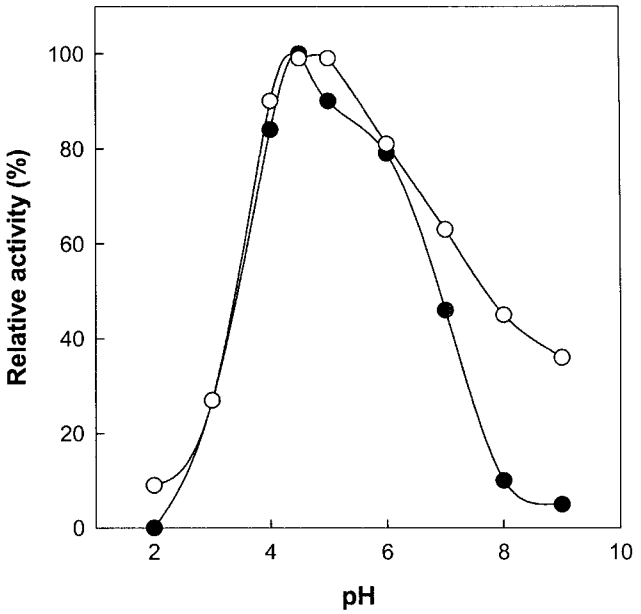

Fig. 2. Effects of pH on phytase activity (●) and stability (○).
The enzyme was assayed at various pHs. For pH stability, the enzyme was incubated at various pH buffers for 20 h at 25°C and the remaining activity was measured at pH 4.5.

activity of Y9451 phytase was determined after incubation at various pHs for 20 h at 25°C. The enzyme was stable at pH ranging from 4.0–6.0 (Fig. 2). The pH profiles of

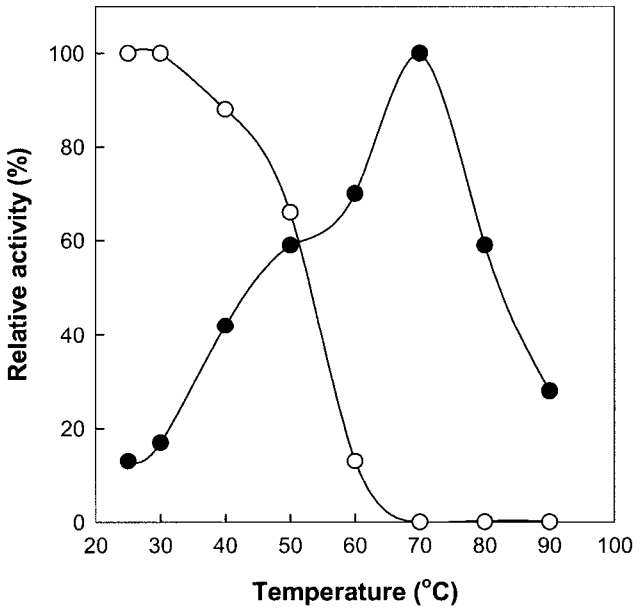

Fig. 3. Effects of temperature on phytase activity (●) and stability (○).
The enzyme was assayed at various temperatures. For thermal stability, the enzyme was incubated in 0.1 M acetate buffer (pH 4.5) at various temperatures for 20 h and the remaining activity was measured at pH 4.5.

Y9451 phytase for activity and stability showed greatly similar patterns. The phytase activity was measured at various temperatures ranging from 30 to 90°C at pH 4.5, and maximal activity was exhibited at 70°C under the assay condition (Fig. 3), which is higher than the optimum temperatures of other bacterial phytases. After incubation of the enzyme in 0.1 M acetate buffer (pH 4.5) at 50°C for 20 h, the remaining enzyme activity was 66% of the initial activity.

By comparing the optimal pH and temperature of the phytases, it could be concluded that the extracellular phytase of *P. fragi* Y9451 was different from already known phytases from other bacteria in optimal pH and temperature [12]. *Aspergillus ficuum* phytase (extracellular, pH 5.0-5.5 and 58°C) [21], *E. coli* phytase (intracellular, pH 4.5 and 55°C) [6], and *Lactobacillus plantarum* phytase (extracellular, pH 5.5 and 65°C) [25] were active in acidic pH. These acidic phytases were applied as feed additives, as they could release inorganic phosphate from feed under acidic conditions during the digestion process of animals [18]. To shift the pH activity profile to more acidic pH regions, Tomschy *et al.* [19] attempted to modify the pH activity profiles of fungal phytases by chemical modification and site-directed mutagenesis. Commercially available fungal phytases, originated from *Aspergillus ficuum* (Natuphose®, optimum pH 5.5) and *Peniophora lycii* (Ronozyme®, optimum pH 4.0-5.0), have been used in the animal feed industry. Therefore, acid phytases have more potential for industrial applications.

Substrate Specificity and Kinetic Analysis

The dephosphorylation of various phosphorylated compounds by purified phytase in 0.1 M acetate buffer (pH 4.5) was examined by measuring free phosphates. The relative rate of phosphate release is summarized in Table 2. The enzyme was very specific for phytate, but slightly specific for other phosphate esters including *p*-nitrophenyl phosphate, a general substrate for acid phosphatase. Among the reported fungal and bacterial phytases, the *Bacillus* extracellular phytases had shown high specificity for phytate [10, 13]. The phytase from *L. plantarum*, one of the bacterial extracellular acid phytases, had a very low specificity for phytate and was a nonspecific acid phosphatase [25]. The substrate specificity of many fungal phytases has proven to be very broad [22]. In addition, as a practical application, the substrate specificity of phytases has also become important in transgenic plant studies, in the introduction of microbial phytase gene into plants, because a nonspecific phytase might disturb the other metabolic pathways of plant cells [20]. Consequently, the *P. fragi* Y9451 phytase characterized in the current study might also be a suitable gene source for plant transformation.

The kinetic parameter of hydrolysis of sodium phytate was determined at 50°C (pH 4.5). The *P. fragi* Y9451 phytase followed Michaelis-Menten kinetics with the Michaelis constant (K_m) of 27 mM. However, the K_m values of the phytase studied ranged from 10 to 650 μM [14]. Most of the phytases showed higher affinity to phytate than *P. fragi* Y9451 phytase. Therefore, further work is needed to improve the affinity for phytate.

Table 2. Substrate specificity of phytase from *Pseudomonas fragi* Y9451.

Substrate	Relative activity (%)
Sodium phytate	100
Sodium triphosphate	17
Disodium *p*-nitrophenyl phosphate	9
Disodium pyrophosphate	4
Disodium α-naphtyl phosphate	3
Disodium β-glycerophosphate	1
Disodium glycerophosphate	0

Effect of Metal Ions

By incubating the enzyme with various metal ions, the enzyme activity was measured with sodium phytate as a substrate (Table 3). The enzyme activity was greatly inhibited by Fe^{3+}, Cu^{2+}, Fe^{2+}, and Zn^{2+}, and moderately inhibited by Co^{2+} and Ni^{2+} at a 5 mM concentration. According to Greiner *et al.* [5], the reduction of phytase activity in the presence of Fe^{2+} and Fe^{3+} was based on a lower substrate concentration because of the formation of a poorly stable complex between metal ions and phytate. Ca^{2+} ion was slightly inhibited at a 5 mM concentration and strongly inhibited (84%) at a 10 mM concentration. It was reported that Ca^{2+} had no significant effect on *Bacillus* sp. DS11 phytase [13], was slightly inhibitory on phytases from *E. coli* [6] and *K. terrigena* [5], and that Ca^{2+} was required for the activity of *B. subtilis* phytase [17]. In contrast, 5 and 10 mM EDTA stimulated the enzyme activity by up to 70%. It was known that EDTA was greatly inhibitory on phytase from *B. subtilis* [17], whereas 1 and

Table 3. Effects of reagent and metal ions on the activity of *Pseudomonas fragi* Y9451 phytase.

Reagents	Relative enzyme activity (%)	
	5 mM	10 mM
None	100	100
LiCl	91	65
$CaCl_2$	83	16
$FeCl_2$	29	22
$NiCl_2$	78	37
$MgCl_2$	98	59
$CoCl_2$	65	33
$CuCl_2$	20	11
$ZnCl_2$	20	13
$FeCl_3$	17	12
EDTA	130	171

10 mM EDTA stimulated the activity of fungal phytase from *A. fumigatus* [22]. The effects of the metal ions mentioned above, along with the fact that EDTA stimulated phytase activity, showed that the phytase from *P. fragi* Y9451 clearly differed from the other bacterial phytases and that the metals were not required for the activity.

In summary, *P. fragi* Y9451 phytase, with its desirable activity profile under acidic pH, thermal stability, and substrate specificity, has potential in animal nutritional and environmental applications.

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