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Purification and Characterization of Phytase from *Bacillus subtilis* (*natto*) N-77

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An extracellular phytase from *Bacillus subtilis* (*natto*) N-77 was purified 322-fold to homogeneity with the specific activity of 8.7 units per mg protein by ultrafiltration, and a combination of Sephadex G-100 and DEAE-Sepharose CL-6B column chromatographies. The molecular weight of the purified enzyme was estimated to be 36 kDa on gel filtration and 38 kDa on SDS-polyacrylamide gel electrophoresis, suggesting that the native enzyme is a monomeric protein. The enzyme had the isoelectric point of pH 6.25, and Ca^{2+} requirement for the production and activity, the K_m value of 0.5 mM, and the activation energy of 9.87 kcal/mol for sodium phytate. The enzyme proved to be fairly specific for phytate and was most active at pH 6.0—6.5 and 60°C. Its activity was greatly inhibited by reagents and metal ions such as EDTA, Zn^{2+}, Cd^{2+}, Ba^{2+}, Cu^{2+}, Fe^{2+}, and Al^{3+}.

Phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate) is the major storage form of phosphorus in cereals and legumes, representing 18 to 88% of the total phosphorus.[1] Phytate form of phosphorus is not readily utilizable by monogastric animals[2] and results in contribution to phosphorus pollution problems in areas of intensive livestock production. The interaction of phytic acid with essential dietary minerals, protein, or vitamins is considered to be one of the primary factors limiting the nutritional values of cereals and legumes in man and animals.[3] Attempts[4-7] have been made to hydrolyze dietary phytate by phytases to improve the feed quality and to decrease in the amount of phosphorus excreted by animals. Partially purified microbial phytase preparations have been reported in *Aerobacter aerogenes*,[8] *Pseudomonas* sp.,[9] *Aspergillus niger*,[10] and *Aspergillus oryzae*.[11] However, characteristics of homogeneous enzyme preparations have been elucidated only in a few microorganisms such as *Aspergillus terreus*,[12,13] *Aspergillus ficuum*,[14] and *Bacillus subtilis*.[15]

Bacillus subtilis (*natto*) strains grow well on steamed soybeans, which are rich in phytate, without any additional nutrition, and can actively ferment those to *natto*, a traditional soybean cheese in Japan. Therefore, I attempted to survey whether *B. subtilis* (*natto*) strains produce potential hydrolytic enzymes for phytate in soybeans, and have found that most of the *B. subtilis* (*natto*) isolates from commercial *natto* samples actively produced phytase.

This paper describes purification and characterization of *B. subtilis* (*natto*) phytase in relation to other microbial phytases.

Materials and Methods

Chemicals. Phytic acid, dodecasodium salt hydrate, was purchased from Aldrich Chemical Company, Inc. (Milwaukee). Sodium tripolyphosphate anhydrous, sodium pyrophosphate, *p*-nitrophenylphosphate disodium salt, sodium glycerophosphate, and sodium β-glycerophosphate were obtained from Wako Pure Chemical Industries, Ltd. (Osaka). All other chemicals were of the analytical grade commercially available.

Microorganism and cultivation. Bacillus subtilis (*natto*) N-77 is one of isolates capable of producing phytase from commercial *natto* samples. The strain N-77 was classified as *B. subtilis* according to "Bergey's Manual Vol. 2".[16] However, the strain N-77 was identified as *B. subtilis* (*natto*) because of the following characteristics: viscous substance production on steamed soybeans or media supplemented with glutamate and sucrose,[17] biotin requirement for the growth,[17] and harboring two plasmids of approximately 35 and 3.5 MDa in which γ-glutamyltranspeptidase for synthesis of the viscous substance is encoded.[18,19]

Spore suspension of the strain was aerobically cultivated for phytase production at 37°C in 1,000-ml Erlenmeyer flasks containing 500 ml of heart infusion (HI) broth (Difco Laboratories, Detroit) supplemented with 10 mM $CaCl_2 \cdot 2H_2O$, 1% glucose, 1% D-mannose, and 0.5% yeast extract (hereafter referred to as the Broth). HI broth (Difco) was used for the simple reason that its small content of phosphate was convenient for the phytase assay, and is not the best medium for phytase production.

Enzyme assay. Phytase activity was routinely meassured by incubating 150 μl of enzyme preparation with 600 μl of 2 mM sodium phytate in 0.1 M Tris–HCl buffer (pH 7.0) supplemented with 2 mM $CaCl_2 \cdot 2H_2O$ (hereafter referred to as the Buffer). At the end of an appropriate incubation (15—30 min) at 37°C, the reaction was stopped by adding 750 μl of 5% trichloroacetic acid, and the liberated inorganic orthophosphate (Pi) was photometrically measured at 700 nm by following the production of phosphomolybdate with 1.5 ml of the color reagent, which was prepared daily by mixing four volumes of 1.5% ammonium molybdate solution in 5.5% sulfuric acid and one volume of 2.7% ferrous sulfate solution. One unit of phytase was defined as the amount of enzyme required to liberate one μM Pi per min under the assay conditions. The specific activity was expressed in units of enzyme activity per mg protein.

Protein measurement. Protein was measured with BCA Protein Assay Reagent (Pierce, Rockford) according to recommendations by the supplier with bovine albumin fraction V (Pierce) as the standard.

Purification of phytase. The five following steps in the purification were done at room temperature, as the enzyme had a high thermal stability.

Step 1. Ultrafiltration. After aerobic cultivation at 37°C for 7 days, a 200-ml sample of the culture supernatant was concentrated to 1/50 of the original volume by ultrafiltration through a Diaflo YM3 membrane (Amicon corporation, Danvers). The insoluble residue was eliminated by centrifigation.

Step 2. First gel filtration through Sephadex G-100. The concentrated culture filtrate was passed through a Sephadex G-100 column (Pharmacia LKB, Uppsala, 2.2 × 42 cm) which had been equilibrated with the Buffer. Fractions (3.1 ml each) were collected by eluting with the Buffer, and the enzyme active fractions were pooled and concentrated by ultrafiltration.

Step 3. Second gel filtration through Sephadex G-100. The active fraction (about 3 ml) was again put through the Sephadex G-100 column in the same way as in Step 2.

Step 4. DEAE-Sepharose CL-6B column chromatography. The active fractions obtained in Step 3 were put onto a column (1.9 × 30 cm) of DEAE-Sepharose CL-6B (Pharmacia LKB) equilibrated with the Buffer.

The column was throughly washed with the Buffer, and the adsorbed enzyme was eluted from the column with a 300-ml linear gradient of zero to 0.4 M NaCl in the Buffer. The active fractions were pooled and concentrated by ultrafiltration.

Step 5. Third gel filtration through Sephadex G-100. The concentrated enzyme solution was put through the Sephadex G-100 column in the same way as in Step 2. The active fractions were combined and concentrated by ultrafiltration, and used as the purified phytase preparation (stored at 4°C) for further studies.

Molecular weight estimation. The molecular weight was estimated by gel filtration on a Sephadex G-100 column using a kit for Gel filtration Calibration (Pharmacia Fine Chemicals, Uppsala), and by 0.1% sodium dodecyl sulfate–5—20% gradient polyacrylamide gel electrophoresis (SDS–PAGE) using a kit of SDS–PAGE Molecular Weight Standards (Bio-Rad Laboratories, Richmond) according to recommendations by the suppliers.

Isoelectric point estimation. The isoelectric point (p*I*) was estimated by PAGE with 6.25% Ampholine (pH 3.5—10) in a gel rod (0.5 × 10 cm) using a kit for Isoelectric Focusing Calibration (Pharmacia LKB) according to recommendations by the supplier.

Results

Phytase production

B. subtilis (*natto*) N-77 was aerobically cultivated at 37°C in the Broth. Phytase activity was detected at the late log phase and reached the maximum level 5 days after the start of cultivation. It was found that Ca^{2+} and D-mannose greatly stimulated the enzyme production with an optimum concentration of 5 mM and 1% in the Broth, respectively. The addition of Ca^{2+} to the supernatant cultured without Ca^{2+} did not accomplish any acceleration of the enzyme activity, showing that Ca^{2+} is needed to produce the enzyme or to stabilize the enzyme produced.

Purification and homogeneity of the enzyme

A typical purification of *B. subtilis* (*natto*) N-77 phytase is summarized in Table I. A Ca^{2+}-supplemented buffer (the Buffer in Materials and Methods) was used in all steps of purification, because the enzyme proved to require Ca^{2+} with an optimum concentration of 2 mM for the activity. The enzyme was purified 322-fold from the culture supernatant with 14.6% recovery. The purified enzyme showed a single protein band on SDS–PAGE (Fig. 1) and on an isoelectric focussing rod gel with the specific activity of 8.7 U/mg for sodium phytate hydrolysis.

Estimation of molecular weight and isoelectric point

The molecular weight of the enzyme was estimated to be 36 and 38 kDa by gel filtration and SDS–PAGE, respectively (Fig. 1). Hence, it is assumed that the native phytase is a monomer. The p*I* value was estimated to be 6.25.

Effects of pH and temperature on activity and stability

The enzyme was found to be most active in the range of pH 6.0 to 6.5 at 37°C, and stable over the range of pH 5.0 to 11.0 when treated at various pHs for 20 hr at 25°C (Fig. 2). The optimum temperature for the enzyme activity was 60°C, and the enzyme was stable up to 50°C when treated in the Buffer for 15 min (Fig. 3). The hydrolysis rate of phytate was almost doubled when the temperature was

Table I. Summary of Purification of *B. subtilis* (*natto*) N-77 Phytase

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)
Culture supernatant	22.0	814.8	0.027	1
Ultrafiltration	22.2	453.1	0.049	1.8
1st Sephadex G-100	16.25	41.7	0.39	14.4
2nd Sephadex G-100	9.39	9.2	1.02	37.8
DEAE-Sepharose CL-6B	3.97	0.51	7.78	288.1
3rd Sephadex G-100	3.22	0.37	8.70	322.2

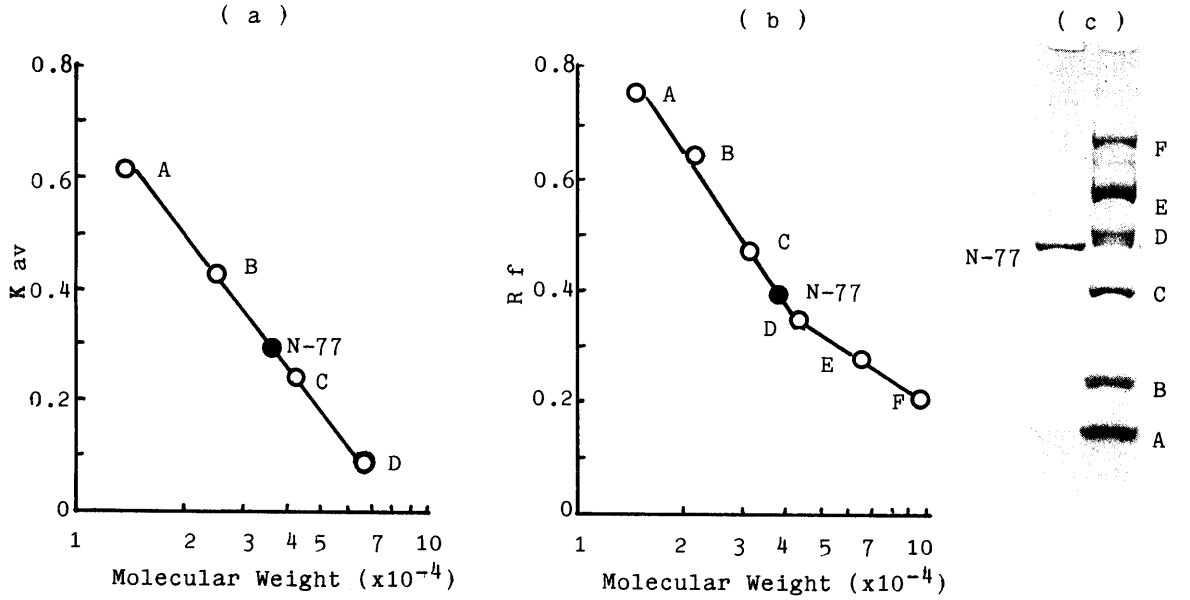

Fig. 1. Molecular Weight Estimation of *B. subtilis* (*natto*) N-77 Phytase by Gel Filtration (a) and SDS–PAGE (b, c).

(a) Gel filtration was done by Sephadex G-100 column chromatography with known protein standards: A, ribonuclease *A* (13.7 kDa); B, chymotrypsinogen *A* (25.0 kDa); C, ovalbumin (43.0 kDa); D, bovine serum albumin (67.0 kDa); N-77, N-77 phytase.
(b, c) SDS–PAGE was done by using 0.1% SDS–5—20% gradient polyacrylamide gel with known protein standards: A, hen egg white lysozyme (14.4 kDa); B, soybean trypsin inhibitor (21.5 kDa); C, bovine carbonic anhydrase (31.0 kDa); D, hen egg white ovalbumin (42.7 kDa); E, bovine serum albumin (66.2 kDa); F, rabbit muscle phosphorylase (97.0 kDa).

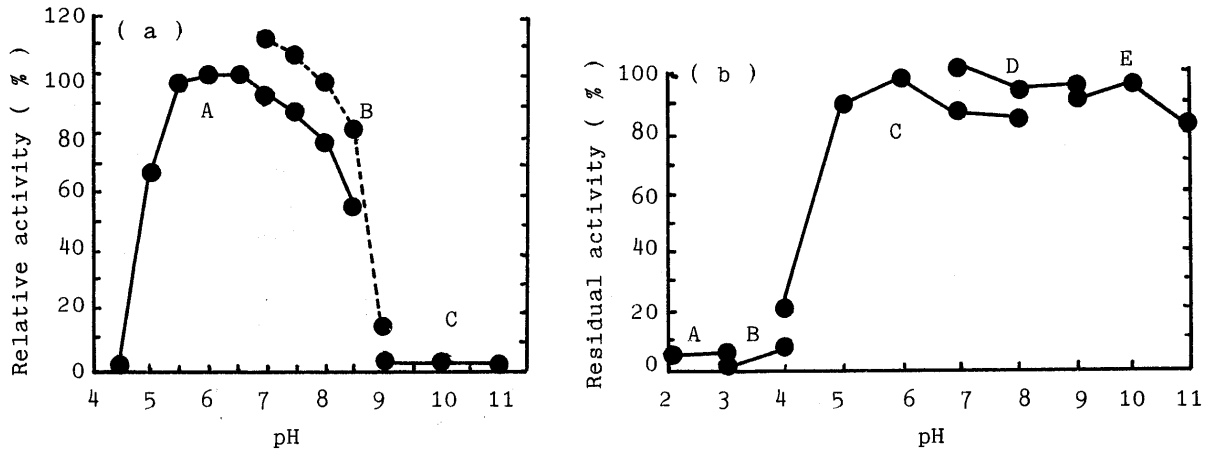

Fig. 2. Effects of pH on Activity and Stability of *B. subtilis* (*natto*) N-77 Phytase.

Effects of pH on the enzyme activity (a) and on the enzyme stability (b) were examined as described in the text. The 0.1 M buffers used were as follows: A, maleic acid–Tris–NaOH; B, Tris–HCl; C, glycine–NaOH in (a), and A, glycine–HCl; B, formic acid–NaOH; C, maleic acid–Tris–NaOH; D, Tris–HCl; E, glycine–NaOH in (b).

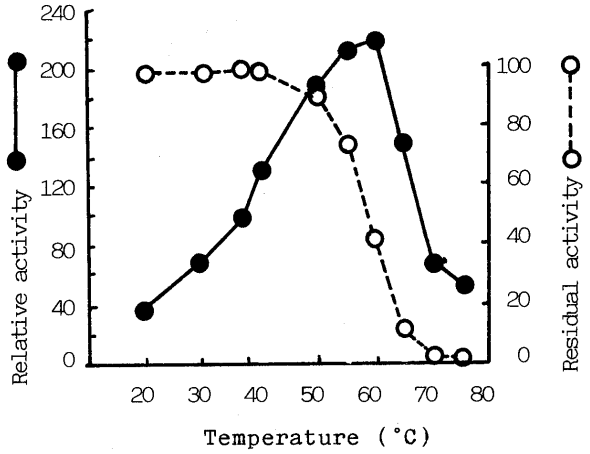

Fig. 3. Effects of Temperature on Activity and Stability of *B. subtilis* (*natto*) N-77 phytase.

Details of the experiments were given in the text.

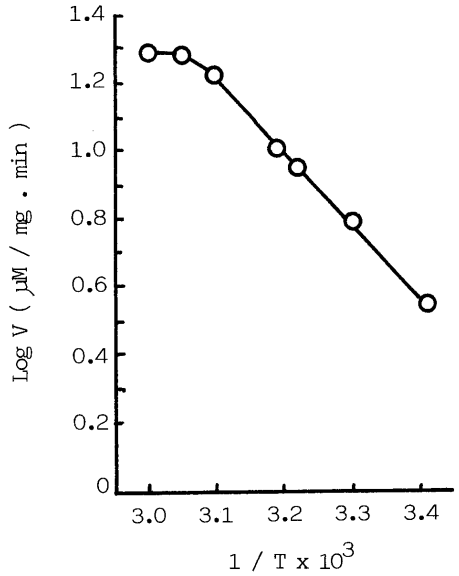

Fig. 4. Arrhenius Plot of the Effects of Temperature on the Activity of *B. subtilis* (*natto*) N-77 phytase.

The enzymic reaction was done for 15 min at various temperatures. Each enzyme activity was assayed as described in the text.

Table II. Substrate Specificity of *B. subtilis* (*natto*) N-77 Phytase

Substrate (2 mM)	Relative velocity (%)
Sodium phytate	100
Sodium tripolyphosphate	21.1
Sodium pyrophosphate	4.2
Sodium *p*-nitrophenylphosphate	0
Sodium glycerophosphate	0
Sodium *β*-glycerophosphate	0

elevated from 37°C to 50°C for 15 min of reaction.

Substrate specificity

The reaction velocity of hydrolysis of phosphate compounds by the enzyme is shown in Table II. The enzyme proved to be fairly specific for phytate, but had no activity on *p*-nitrophenylphosphate, which is a general substrate for acid phosphatase.

Kinetics of enzyme reaction

Effects of substrate concentration on the enzyme activity showed simple Michaelis–Menten kinetics, and the Lineweaver–Burk plot indicated 0.50 mM K_m value for sodium phytate. Temperature effects on hydrolysis of sodium phytate was investigated, and the activation energy calculated from the Arrhenius plot was 9.78 kcal/mol (Fig. 4).

Effects of reagents and metal ions

The effects of reagents and metal ions on the enzyme activity were examined using sodium phytate as a substrate (Table III). The enzyme activity was greatly inhibited by EDTA, Zn^{2+}, Cd^{2+}, Ba^{2+}, Cu^{2+}, Fe^{2+}, and Al^{3+}, and moderately inhibited by Mg^{2+}, Hg^{2+}, Mn^{2+}, Ni^{2+}, and Co^{2+} at 5 mM concentration. Sodium azide, phenylmethylsulfonyl fluoride, and *p*-chloromercuribenzoic acid showed little effect on the enzyme activity at 5 mM concentration.

Enzymic hydrolysis of phytate in grains

The enzyme effectively hydrolyzed phytate in 15% (w/v) suspension of ground soybean flour, ground rapeseed flour, or rice bran, and liberated Pi of 7.84, 4.34, or

Table III. Effects of Reagents and Metal Ions on the Activity of *B. subtilis* (*natto*) N-77 Phytase

Addition (5 mM)	Relative activity (%)
None	100
NaN_3	104.1
PMSF	100.0
PCMB	98.6
$MgCl_2$	55.8
$HgCl_2$	54.8
$MnCl_2$	17.7
$NiCl_2$	16.4
$CoCl_2$	16.1
$ZnCl_2$	4.0
$CdCl_2$	4.0
$BaCl_2$	4.0
$CuCl_2$	4.0
$FeSO_4$	4.0
$AlCl_3$	4.0
EDTA	1.5

PMSF, phenylmethylsulfonyl fluoride; PCMB, *p*-chloromercuribenzoic acid.

2.77 μM/ml, respectively when treated with 1.1 U/ml of the purified enzyme in the Buffer at 50°C for 6 hr.

Discussion

Microbial phytases have been investigated, but most of the data were obtained with partially purified enzyme preparations. A few reports of homogeneous enzyme preparations have been made on *Aspergillus terreus*,[12,13] *A. ficuum*,[14] and *B. subtilis*.[15] There are some difficulties in comparing the inherent differences among various phytases because of the differences in experimental conditions and in purity of the enzyme preparations. Phytases of *Aspergillus* species origin have been reported to have a large molecular weight, 214 kDa (a homohexamer) in *A. terreus*,[13] 85—100 kDa (a monomer) in *A. ficuum*,[14] and 200 kDa in *A. niger*,[10] their broad substrate specificity, and enzymic activity in the acidic range of pH 3.5 to 5.5.[10,13,14] On the other hand, *B. subtilis* (*natto*) N-77 phytase was shown to have a smaller molecular weight of 36—38 kDa (a monomer) (Fig. 1), a fairly high specificity for phytate (Table II), and enzymic activity in the neutral range of pH 5.5 to 8.5 (Fig. 2). Some similarities between phytases from *B. subtilis*[15] and *B. subtilis* (*natto*) were shown in their molecular weight, and Ca^{2+} requirement for the activity. However, some differences between them were also shown: *B. subtilis* phytase[15] showed two isozymes on electrophoresis, maximum activity at pH 7.5, and 0.05 mM K_m, while *B. subtilis* (*natto*) N-77 phytase showed a single protein band on electrophoresis, maximum activity at pH 6.0 to 6.5, and 0.5 mM K_m. Regarding the substrate specificity, *B. subtilis* phytase[15] was highly phytate-specific, while N-77 phytase had a fairly high specificity for phytate with some hydrolytic activity on inorganic compounds such as tripolyphosphate and pyrophosphate (Table II). Matsuzaki *et al.*[20] also reported differences between α-amylases of *B. subtilis* and *B. subtilis* (*natto*) with respect to their molecular weight, substrate specificity, electrophoretic mobility, and optimal pH range for the activity. Preliminary results showed that *B. subtilis* (*natto*) N-77 hydrolyzed phytate in steamed soybeans during the fermentation process to *natto*, and so may improve the nutritional value of soybeans. For industrial production of *B. subtilis* (*natto*) phytase, further work is needed to identify more effective and less expensive media, and cultural conditions.

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