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Purification and Properties of Phytate-Specific Phosphatase from *Bacillus subtilis*†

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An enzyme which liberates P_i from *myo*-inositol hexaphosphate (phytic acid) was shown to be present in culture filtrates of *Bacillus subtilis*. It was purified until it was homogeneous by ultracentrifugation, but it still showed two isozymes on polyacrylamide gel electrophoresis. The enzyme differed from other previously known phytases in its metal requirement and in its specificity for phytate. It had a specific requirement for Ca^{2+} for its activity. The enzyme hydrolyzed only phytate and had no action on other phosphate esters tested. This *B. subtilis* phytase is the only known phytate-specific phosphatase. The products of hydrolysis of phytate by this enzyme were P_i and *myo*-inositol monophosphate. The enzyme showed optimum activity at pH 7.5. It was inhibited by Ba^{2+}, Sr^{2+}, Hg^{2+}, Cd^{2+}, and borate. Its activity was unaffected by urea, diisopropylfluorophosphate, arsenate, fluoride, mercaptoethanol, trypsin, papain, and elastase.

Phytase (EC 3.1.3.8) hydrolyzes *myo*-inositol hexaphosphate with the release of one or more phosphoric acid groups. A crude preparation of phytase was first obtained by Suzuki et al. from rice bran (cited in reference 18). The presence of phytases has been reported in plants, animal tissues, and microorganisms (1, 2, 5, 9, 16, 18, 23–26, 29). The phytases that have so far been purified are, however, nonspecific and act not only on phytate but also on other phosphate esters (16, 18, 22, 26, 28). None of them has an absolute requirement for cations for its activity. The present paper deals with an enzyme occurring in culture filtrates of *Bacillus subtilis* which hydrolyzes phytate and differs in its specificity and properties from other previously known phytases. It acts only on inositol polyphosphates and not on any other phosphate esters tested. It has a specific requirement for Ca^{2+} for its activity. The purification, properties, and kinetics of this specific phytase are described in this paper. A preliminary note on this work has been published (21).

MATERIALS AND METHODS

All chemicals used were of analytical grade. The biochemicals were from Sigma Chemical Co. (St. Louis, Mo.); the chemicals used for polyacrylamide gel electrophoresis were obtained from Eastman Kodak Co. (Rochester, N.Y.); casein (commercial) was obtained from Polson (Anand, India); casein hydrolysate (A & H "Vitamin free") came from Allen and Henburys (London, U.K.); peptone was from Difco Laboratories (Detroit, Mich.); and Sephadex G-100 (40 to 120 μm) was obtained from Pharmacia Fine Chemicals (Bromma, Sweden). Wheat bran was purchased locally. Amberlite IRP-64 (100 to 325 mesh), from Rohm and Haas (Philadelphia, Pa.), was washed by the method of Hirs et al. (12), and DEAE-cellulose (Sigma) was washed according to Peterson and Sober (20).

Sodium phytate type V, prepared from corn (Sigma), was purified by ion-exchange chromatography by the method of Nagai and Funahashi (19). The total phosphorus content after digestion was 10.88%; the inorganic phosphorous content was negligible. Most of the experiments were carried out with sodium phytate (type V) without further purification. *myo*-Inositol hexaphosphate is more correctly designated as *myo*-inositol hexakis (dihydrogen phosphate); however, for brevity it will be referred to as *myo*-inositol hexaphosphate.

Total and inorganic phosphorus were determined by the method of Fiske and Subba Row (8). Protein was routinely determined by the methods of Warburg and Christian (27) and, in purified enzyme preparations, Lowry et al. (17), using crystalline bovine serum albumin as the standard. Microbiological assay of inositol was carried out by the method of Beadle (3). Routine blanks, standards with different amounts of inositol, and a recovery experiment (a known amount of inositol added to the experimental sample) were run along with the experimental assays.

The following methods were used: carbohydrate (Gottschalk and Ada [11]); free sulfhydryl groups (Ellman [7]); disulfide groups (Cavallini et al. [4]); tyrosine and tryptophan (Goodwin and Morton [10]). Amino acid analysis (13) was carried out in an automatic amino acid analyzer using 3-mg samples of purified enzyme hydrolyzed by 6 N HCl in evacuated, sealed tubes at 110°C for 24 and 72 h.

Phytase activity. The reaction mixture contained Tris-hydrochloride (200 μmol), $CaCl_2$ (1 μmol), sodi-

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um phytate (0.67 μmol) (equivalent to 4 μmol of phytate phosphorus), and enzyme at pH 7.5 in 2 ml. Under these conditions there was no precipitation of calcium phytate even after long storage or heating and cooling. It was not determined whether phytate from sources other than corn would be precipitated by Ca^{2+} under these conditions as stated by Hoff-Jørgensen (14). After incubation at 30°C for 10 min, the solution was deproteinized by the addition of 2 ml of 10% trichloroacetic acid, and the P_i liberated was determined in a sample (after centrifugation in the case of crude enzyme samples). The enzyme was dialyzed for 12 to 24 h against 0.01 M Tris-hydrochloride buffer (pH 7.5) containing 1 mM $CaCl_2$ and diluted with the same buffer, if necessary, for assay. Blanks were run for P_i content in enzyme and substrate, but these levels were negligible. There was also no nonenzymic hydrolysis of the substrate under the conditions of assay. The amount of enzyme taken was such that the P_i liberated did not exceed 20% of the initial esterified phosphorus.

A unit of phytase activity was defined as the amount of enzyme that liberated 1 μmol of P_i per min under the above conditions. The specific activity of the enzyme was defined as the number of units per milligram of protein.

Production of enzyme. The stock culture on nutrient agar was subcultured on wheat bran extract-peptone agar (containing 10% aqueous extract of wheat bran and 1% peptone) and incubated overnight at 37°C. Wheat bran extract was prepared by autoclaving 1 kg of wheat bran in 10 liters of water at 120°C for 60 min, cooling, and squeezing through muslin cloth. It contained 22 g of solids per liter. The organism grew as a flat whitish pellicle in 20 to 22 h. The inoculum was transferred to 500-ml Erlenmeyer flasks containing 100 ml of medium of the following composition: wheat bran extract, 100 ml; $(NH_4)_2SO_4$, 0.04 g; $MgSO_4 \cdot 7H_2O$, 0.02 g; casein, 1 g; KH_2PO_4, 0.05 g; K_2HPO_4, 0.04 g; pH 6 to 6.2. Before inoculation, $CaCl_2$ solution, which was separately sterilized, was added aseptically to each flask to give a final concentration of 0.2%. The flasks were incubated on a rotary shaker (220 rpm) at 28°C. Maximum enzyme activity was obtained at the end of 72 h (0.15 to 0.24 U/ml). The fermented broth was pooled from several flasks and was clarified by centrifugation in a Sharples Super Centrifuge at 13,000 × *g*. The clear, amber-colored supernatant liquid was precipitated with ethanol as soon as possible as described below.

Purification of the enzyme. All operations were carried out at 0 to 4°C unless otherwise stated. The concentration of $CaCl_2$ in the enzyme solution was 1 mM in all the steps except during Amberlite treatment, when it was 5 mM. Ca^{2+} is required not only for the activity of the enzyme but also for its stability. The enzyme is very stable after precipitation with ethanol and acetone, but only in the presence of calcium. (A protease is also present in the culture fluid which hydrolyzes the enzyme very rapidly if calcium is chelated with EDTA or removed by dialysis or treatment with cation-exchange resins).

To 4.5 liters of the enzyme 13.5 liters of ethanol (−20°C) was added with constant stirring. Stirring was continued for 30 min, and the mixture was then allowed to settle for 1 h. Most of the clear supernatant liquid was siphoned off, and the remaining liquid was centrifuged for 10 to 15 min at 1,000 × *g*. The precipitate was washed successively with ethanol and acetone and then dried in vacuo at 0°C over P_2O_5 and paraffin wax. About 20 g of dry powder was obtained; its phytase activity was unchanged even after several months of storage at 0°C (fraction I).

A 20-g sample of enzyme was made into a paste with 20 ml of 1 M acetate buffer (pH 5.5) and then homogenized with 220 ml of the same buffer. It was centrifuged for 45 min at 1,000 × *g*, and the residue was reextracted with 220 ml of the same buffer. The combined extracts were reprecipitated by the addition of 550 ml of acetone (−20°C) with stirring. The precipitate was collected as before, washed with acetone, and then reextracted as before with two 100-ml lots of 1 M acetate buffer (pH 5.0). The combined extracts were dialyzed against 0.01 M acetate buffer (pH 5.0) for 24 h with five changes of 5 liters each of the same buffer (fraction II).

Acetate and $CaCl_2$ concentrations of the enzyme solution were made 0.2 M and 5 mM, respectively. The solution was then adsorbed batchwise on 200 g of Amberlite IRP-64 previously equilibrated with the same buffer. The resin was stirred for 25 to 40 min and then filtered under suction on Whatman no. 1 filter paper. It was suspended in the same buffer with stirring for 30 min and again filtered under suction. The washing was repeated twice. The washed resin was suspended in 600 ml of water, and the pH of the

TABLE 1. Effect of cultural conditions on phytase production by *B. subtilis*[a]

Supplements	Incubation (h)	Phytase activity (U/100 ml)
Glucose, casein	90	0
Glucose, casein, inositol	90	0
Glucose, casein, inositol, yeast extract	90	14
Maltose, casein hydrolysate, phytate	48	6
Starch, casein hydrolysate, phytate, yeast extract	48	10
Wheat bran extract, casein hydrolysate, phytate	24	17
Wheat bran extract, casein hydrolyzate, phytate	48	24
Wheat bran extract, casein hydrolyzate, phytate	90	22
Casein, phytate, wheat bran extract	48	15
Casein, phytate, wheat bran extract	72	24
Casein, phytate, wheat bran extract	90	24
Casein, wheat bran extract	72	24

[a] A 100-ml volume of medium contained $(NH_4)_2SO_4$ (0.04 g), $MgSO_4 \cdot 7H_2O$ (0.02 g), KH_2PO_4 (0.05 g), K_2HPO_4 (0.04 g), and $CaCl_2$ (0.20 g) at pH 6 and the listed supplements at the following levels: casein or casein hydrolysate, 1 g; yeast extract, 0. 5 g; sodium phytate, 0.06 g; inositol, 0.005 g; glucose, maltose, or potato starch, 5 g. The mixture was kept on a shaker at 28°C for different periods.

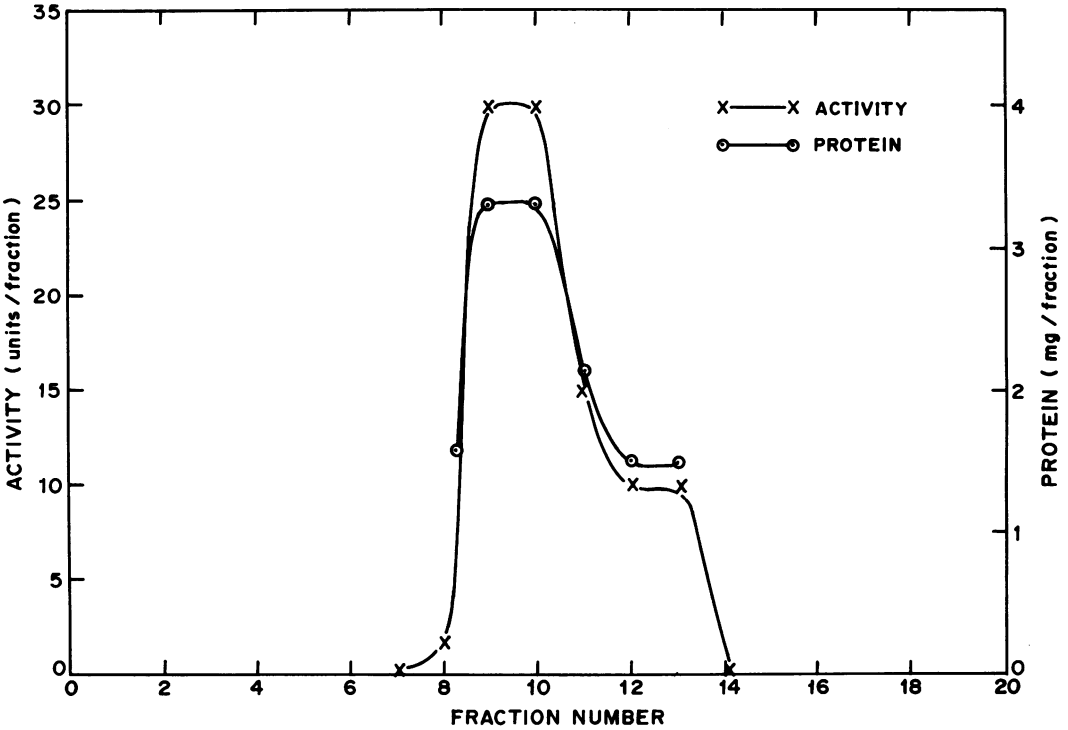

FIG. 1. Chromatography of phytase on DEAE-cellulose.

mixture was raised to 6.0 to 6.2 by adding 300 to 320 ml of 2 M Tris base. The mixture was then stirred for 40 min and filtered under suction. The resin was reextracted twice with 800- to 900-ml volumes of 0.01 M acetate buffer–5 mM $CaCl_2$ (pH 6.0). The combined eluates were pooled and lyophilized until the final volume was about 250 ml. The concentrated enzyme solution was dialyzed against 1 mM Tris-hydrochloride (pH 7.5)–1 mM $CaCl_2$ for 24 h with three changes of 5 liters each of the same buffer. The dialyzed enzyme was lyophilized and again dialyzed against 5 mM Tris-hydrochloride (pH 7.5)–1 mM $CaCl_2$ (fraction III).

The enzyme (volume, 35 ml; 480 U) was loaded on a DEAE-cellulose column (21 by 4.4 cm) previously equilibrated with 5 mM Tris-hydrochloride buffer (pH 7.5)–1 mM $CaCl_2$. The flow rate was 1.5 ml/min. The column was washed with 200 ml of 5 mM Tris-hydrochloride (pH 7.5)–1 mM $CaCl_2$. The enzyme was eluted with 100 mM Tris-hydrochloride (pH 7.5)–1 mM $CaCl_2$, and 30-ml fractions were collected without application of external pressure. Twelve enzyme fractions having a specific activity of 8.5 or more units per mg of protein were pooled, frozen, and lyophilized to dryness (Fig. 1). The residue was dissolved in 5 ml of 10 mM Tris-hydrochloride (pH 7.5)–1 mM $CaCl_2$ and dialyzed for 12 to 15 h against three changes of the same buffer (fraction IV).

RESULTS AND DISCUSSION

Production of enzyme. Six *B. subtilis* strains were screened for their activity in a medium containing glucose, casein hydrolysate, sodium phytate, and inorganic salts (at the concentrations shown in Table 1). At the end of 72 h, three cultures showed no phytase activity and the others showed 0.02, 0.04, and 0.15 U, respectively, per ml of culture filtrate. The culture showing maximum activity was *B. subtilis* 2712 (designated 2051 in earlier catalogs) from the National Collection of Industrial Microorganisms, located in our laboratory; this culture was used for further work.

The effect of different carbohydrates and inducers on phytase production is shown in Table 1. There was no enzyme production in a glucose + casein medium or starch + casein medium (not shown). Supplementation with inositol failed to induce the enzyme, but phytate and yeast extract or wheat bran extract (both of which contain phytate) induced the enzyme. Phytase was also produced with maltose or starch as a carbon source when one of the three inducers was also added to the medium. Significant enzyme production was observed within 24 h with casein hydrolysate as the main nitrogen source, whereas with casein maximum enzyme formation was obtained after 72 h. A medium containing wheat bran extract, casein, and salts was used for further work on producing phytase for purification.

Properties of the purified enzyme. The purifi-

TABLE 2. Purification of *B. subtilis* phytase

Fraction no.	Purification stage	Vol (ml)	Total activity (U)	Total protein (mg)	Sp act (U/mg of protein)	Recovery of enzyme (%)	Purification (fold)
	Culture filtrate	4,500	900			100	
I	Ethanol precipitate	450	810	3,700	0.22	90	1
II	Acetone precipitate	380	800	1,000	0.80	89	4
III	Amberlite IRP-64 treatment	35	480	240	2.00	53	9
IV	DEAE-cellulose column chromatography	20	290	34	8.53	32	39

cation of the enzyme was about 40-fold with respect to the alcohol precipitate of the culture filtrate, and the final specific activity was 8.5 to 9 U/mg (Table 2). The purified enzyme was colorless, and the ratio of the absorbance at 280 nm to that at 260 nm was 1.8.

The enzyme was very stable and could be stored at −20°C for at least a year without significant loss in activity. At 0°C in 24 h the enzyme did not lose any activity at pH levels ranging from 5 to 8. At pH 7.5 in 0.1 M Tris-hydrochloride–1 mM $CaCl_2$ it lost only 17% of its activity at 60°C in 1 h and 30% at 70°C in 10 min. In 0.1 N HCl the enzyme lost 60% of its activity in 20 min at 25°C, but at 0°C it was completely inactivated in 20 min. In 0.1 N KOH, however, it lost only 10% of its activity in 2 h at 0°C, whereas in the same period it lost 60% of its activity at 25°C. The relative cold lability of the enzyme at low pH is noteworthy.

Ultracentrifugation. The ultracentrifugal determination of the molecular weight of the enzyme was carried out by the Archibald method (15). The partial specific volume was assumed to be 0.725 cm^3/g. Enzyme of maximum specific activity showed only one symmetrical peak (Fig. 2) with $s_{20,w}$ = 3.5. The molecular weight was 36,500.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was carried out with 7% acrylamide gels at pH 8.5 in 0.02 M Tris-glycine buffer (6) for 90 to 120 min (3 to 4 mA per gel tube). After staining with Amido Black 10B for protein, the gel showed two protein bands very close to each other (Fig. 3). Since the two bands may be an artifact due to the absence of Ca^{2+} in the buffer, the experiment was repeated with 1 mM of $CaCl_2$ added to the buffer and gel; two proteins were again observed. To verify whether both the bands had phytase activity, the gel after electrophoresis was incubated with 10 ml of enzyme assay mixture for 30 min and then in 10 ml of the phosphate reagent of Fiske and Subba Row (8) for 10 to 15 min. Both the bands corresponding to protein bands were stained blue, showing that both of them had phytase activity. In another experiment, after the electrophoretic run the gel was cut into small sections (corresponding to the positions of the stained protein bands on a second gel run simultaneously) and eluted with 10 mM Tris-hydrochloride (pH 7.5)–1 mM $CaCl_2$. The solutions were concentrated and again loaded on a polyacrylamide gel. It was found that each band gave only one protein band on electrophoresis. Hence, they were not artifacts produced during gel electrophoresis. The amounts obtained of the two isozymes were insufficient for further characterization.

About 1.3 ml of enzyme (185 U) was loaded on a Sephadex G-100 column (100 by 24 mm) previously equilibrated with 50 mM Tris-hydrochlo-

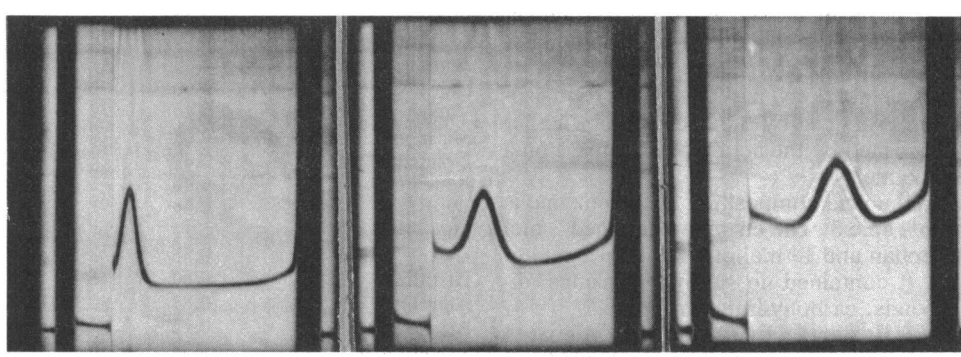

FIG. 2. Ultracentrifugal pattern of purified phytase. Protein concentration, 0.83% in 50 mM Tris-hydrochloride (pH 7.5)–50 mM $CaCl_2$; 59,780 rpm; 23°C; phase plate 60°; synthetic boundary cell; photographs at 32, 96, and 160 min.

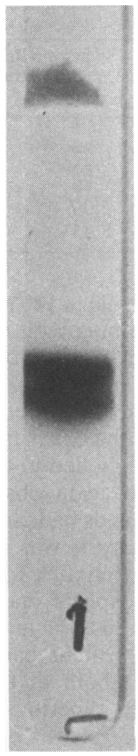

FIG. 3. Polyacrylamide gel electrophoresis of purified phytase. Experimental conditions were as described in the text. The black band near the top (cathodic end) is due to opaque spacer gel (5% acrylamide and 1.25% N,N'-methylene-bis-acrylamide).

ride (pH 7.5)–1 mM $CaCl_2$ and eluted with the same buffer. Fractions (3 ml) were collected, assayed for enzyme and protein, concentrated, and used for gel electrophoresis. Only a single protein peak was obtained with no increase in specific activity, and all of the active fractions showed two bands on electrophoresis. No separation of the proteins was observed on Sephadex. The crude extract after ethanol precipitation also showed two enzymatically active bands. It is not known whether the two isozymes are produced by the bacteria or whether they are formed by the action of a protease on a single enzyme.

The amino acid composition of the enzyme is shown in Table 3. The enzyme contained 3 mol of tryptophan and 14 mol of tyrosine per mol of enzyme. It contained no sulfhydryl groups, disulfide bonds, carbohydrate, or inositol.

Kinetics. Enzyme activity was proportional to time and enzyme concentration, provided the total amount of phosphate hydrolyzed did not exceed 20% of the phytate phosphorus.

The relative activities of the enzyme at 20, 30, 40, 45, 50, 55, and 60°C were 100, 190, 360, 480, 600, 710, and 860, respectively. The energy of activation was calculated to be 11,600 cal (ca. 48.6 kJ) per mol. The enzyme showed a pH optimum at 7 to 7.5 with a sharp drop in activity above pH 8 due possibly to the precipitation of calcium and phytate. Apparent K_m values at pH 5.5, 6, 6.5, and 7.5 were found to be 15×10^{-5}, 10×10^{-5}, 6×10^{-5}, and 5×10^{-5} M, respectively. The K_m for phytate at pH 7.5 and 0.5 mM $CaCl_2$ (the degree of hydrolysis being limited to 5% of the total initial ester phosphorus) was 3.5×10^{-5} M. In addition to inositol hexaphosphate, other polyphosphates formed by enzyme reaction are further hydrolyzed by the enzyme. Since these compounds are also hydrolyzed during the reaction and all of them also bind Ca^{2+}, the kinetics of the reaction are complex and no further analysis of the data was made.

Metal requirement. Dialysis against EDTA resulted in loss of activity with some enzyme preparations. This was found to be due to traces of proteolytic activity still present in the purified enzyme. When the enzyme was treated with 0.1 mM diisopropylfluorophosphate for 4 h and then dialyzed against 10 mM EDTA in 10 mM Tris-hydrochloride buffer (pH 7.5), there was no loss of enzyme activity. After dialysis the enzyme showed no activity in the absence of Ca^{2+}. The requirement for Ca^{2+} was specific and there was no activation by Ba^{2+}, Zn^{2+}, Mg^{2+}, or Mn^{2+}.

Inhibitors. Enzyme activity was unaffected by treatment with 8 M urea for 10 h at 0°C or with

TABLE 3. Amino acid composition in phytase

Amino acid	Amino acid (μmol/mg) of enzyme	Residues[a] (mol)
Aspartic acid	1.60	58.4
Threonine	0.35	12.8
Serine	0.34	12.4
Glutamic acid	1.08	39.4
Proline	0.33	12.0
Glycine	0.99	36.1
Alanine	0.79	28.8
Half-cystine	0.00	0.00
Valine	0.44	16.1
Methionine	0.08	2.9
Isoleucine	0.49	17.8
Leucine	0.46	16.8
Tyrosine[b]	0.38	13.9
Phenylalanine	0.25	9.1
Lysine	0.56	20.4
Histidine	0.80	29.2
Ammonia	0.88	32.1
Arginine	0.22	8.0
Tryptophan[b]	0.08	3.0

[a] These calculations were made on the basis of a molecular weight of 36,500.

[b] Estimated spectrophotometrically.

trypsin, papain, or elastase. There was no loss of activity in the presence of 4 mM P_i and 1 mM diisopropylfluorophosphate, arsenate, molybdate, fluoride, *p*-chloromercuribenzoate, *o*-phenanthroline, or mercaptoethanol. Ba^{2+}, Sr^{2+}, Hg^{2+}, and Cd^{2+} at 5×10^{-4} M concentration (under the standard assay conditions) inhibited the enzyme by 64, 70, 15, and 90% respectively. Borate at 0.06 M inhibited the enzyme by 65%.

Products of hydrolysis of phytate. The products of enzyme reaction were determined as follows: 150 ml of 0.1 M Tris-hydrochloride (pH 7.5)–1 mM $CaCl_2$, sodium phytate (300 μmol of phytate phosphorus), and 100 U of purified enzyme were incubated at 30°C until samples showed no further increase in P_i. The enzyme was inactivated by boiling, and after filtration the filtrate was made 0.1 M with respect to HCl and passed through a Dowex 1 × 8 column (chloride form, 200 to 400 mesh, 350 by 24 mm). The column was washed with water and subsequently eluted by stepwise increase in HCl concentration from 0.1 N to 1 N. The eluates were analyzed for P_i and organic phosphate. Free and bound phosphorus were present only in the 0.1 N HCl eluates. The initial eluates of this fraction contained P_i (249 μmol), and the later eluates contained organic phosphate (48 μmol). The fractions containing organic phosphate were pooled and adjusted to pH 5.0 with NaOH solution. Saturated barium acetate solution was added, and the resulting trace of precipitate was removed. The clear liquid was passed through a Dowex-50 (H^+ form) column (200 by 20 mm) to remove Ba^{2+}. The effluents and washings were collected and neutralized to pH 7. They were found to contain 0.29 μmol of inositol and 0.34 μmol of total phosphorus per ml of hydrolysate and no free P_i. Hence the molar ratio of phosphorus to inositol was 1.17, corresponding to an inositol monophosphate. A sample of the solution, containing 1 μmol of phosphorus, was incubated with 5 U of the enzyme in the presence of the enzyme and Ca^{2+} at pH 7.5 for 48 h under the usual assay conditions. There was no liberation of P_i, indicating that the inositol monophosphate is the end product of the hydrolysis. Inositol monophosphate was the only inositol phosphate eluted by 0.1 N HCl; higher concentrations of HCl were required to elute inositol polyphosphates. These results show that the reaction of *B. subtilis* phytase is:

$$\text{Inositol hexaphosphate} \xrightarrow{\text{phytase} + Ca^{2+}} \text{Inositol monophosphate} + 5\ P_i$$

The inositol monophosphate was not further characterized.

Specificity. The action of purified phytase on several phosphate esters was tested. The reaction mixture contained 200 μmol of Tris-hydrochloride (pH 7.5), 1 μmol of $CaCl_2$, substrate corresponding to 5 μmol of phosphorus, and 0.4 U of enzyme in 2 ml. It was incubated for 1 h at 30°C. The action of phytase was also determined with these substrates in the absence of Ca^{2+} by using enzyme dialyzed against Tris-hydrochloride (pH 7.5) without calcium chloride. The following compounds were tested: α- and β-glycerophosphate, glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, 3-phosphoglyceric acid, *p*-nitrophenyl phosphate, phenyl phosphate, AMP, ADP, ATP, NAD, NADH, yeast adenylic acid, and pyrophosphate. Controls were run for determining initial P_i with each substrate. There was no detectable liberation of P_i with any of these compounds, though more enzyme and a longer period of incubation were used than with in the assay with phytate. This *B. subtilis* phytase is the only known phytase reported to be specific for phytate.

In view of its high specificity, this phytase is suitable for the estimation of *myo*-inositol hexaphosphate for which no simple and rapid method of estimation is available. It remains to be determined whether the enzyme acts on all the isomeric inositol polyphosphates.

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