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PURIFICATION AND MODE OF ACTION OF PHYTASE FROM *PHASEOLUS AUREUS**

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Abstract—Phytase isolated from germinated mung bean cotyledons was further purified and migrated as a single protein band in polyacrylamide gel electrophoresis. The MW obtained by SDS polyacrylamide gel electrophoresis is 158000. *Sn*-3-glycerylphosphocholine (*Sn*-3-GPC) has a regulatory role on phytase; it is promotive below 30 μM and inhibitory at higher concentrations. The inhibition is competitive with a K_i of 6×10^{-5} M. The K_m for myo-inositol hexaphosphate is 4×10^{-4} M. In the dephosphorylation of phytate the phosphate from position 6 is liberated first, followed by those at positions 5 and 4, or 1 and 3. The phosphate at position 2 is stable.

INTRODUCTION

THE DEPHOSPHORYLATION of phytate by phytase[1,2] (myo-inositol hexaphosphate phosphohydrolase, E.C. 3.1.3.8) in germinating seeds has recently attracted many workers.[1-11] This enzyme is widely distributed not only in various higher plants but also in animal tissues[12] as well as in many species of fungi.[3] In our laboratory it has been shown that phytase is not present in the cotyledons of ungerminated mung bean seed but it appears during germination *de novo*.[1,13] This enzyme was subsequently isolated and partly characterized.[1] The present paper deals with further purification, characterization, and mode of action of the enzyme. The stimulation as well as inhibition of phytase activity by *Sn*-3-glycerylphosphocholine (*Sn*-3-GPC) is also recorded.

RESULTS

Purification and isolation of phytase from polyacrylamide gel after electrophoresis

Phytase prepared by Mandal *et al.*[1] (first bentonite step in Table 1) after electrophoresis on polyacrylamide gels showed two protein bands, the major for phytase and the minor

* Part VIII in the series "Metabolism of Inositol Phosphates". For Part VII see *Phytochemistry* **12**, 321 (1973).

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for phosphatase activity. Attempts to purify phytase from the phosphatase by gel filtration using Biogel P200 column (1·5 × 25 cm) were not successful. The MW of phytase and phosphatase were found to be *ca* 160000.[1] Since gel filtration was unsuitable for separating the two enzymes, an attempt was made to isolate them by polyacrylamide gel electrophoresis on a large scale as described earlier.[1] The gel slices were resuspended in 0·05 M Tris–HCl, pH 7, and homogenized for 4 min by the Sorvall Omnimixer at 0° and kept cold. After 6 hr the homogenized fractions were centrifuged at 10000*g* for 1 hr. The supernatant was filtered through a Büchner funnel under a vacuum. The filtrate was dialyzed overnight against Tris–HCl buffer pH 7. Each fraction was assayed for phytase and phosphatase. The phytase was found in fraction 18 and phosphatase in 21. The preparation of phytase thus obtained migrated as a single band when subjected to polyacrylamide gel electrophoresis. This preparation was used for subsequent experiments.

TABLE 1. SUMMARY OF FURTHER PURIFICATION

Purification steps	Protein (mg)	Sp. act.		Total unit	
		Phosphatase	Phytase	Phosphatase	Phytase
1 First Bentonite	8	13	32	104	256
2 Second Bentonite	6	14	40	84	240
3 Polyacrylamide gel fractionation	3.5	—	64	—	224

One unit of enzyme has been defined as that amount of protein which can liberate 1 μmol P_i/hr from respective substrate under experimental conditions. The assay procedure and earlier purification steps were those of Mandal *et al.*[1]

Molecular weight of phytase

To determine the MW of phytase, the enzyme (30 μg) was subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) (see Experimental). Only one protein band was detected for phytase. The distance through which the protein moved was determined, and from the curve obtained with standard proteins as described earlier, the MW of phytase was calculated to be *ca* 158000. However, the presence of subunits of identical nature has been ruled out by the MW determined by passing through Biogel P200 in absence of SDS.[1]

Effect of Sn-3-*GPC and other lipids on phytase activity*

Sn-3-GPC at low concentration (below 30 μM) has been found to promote the phytase activity by 30% over the control, whereas at higher concentration it is inhibitory. Complete inhibition occurs at 0·5 mM. Other lipids and phospholipids tested were stimulatory to some extent but not inhibitory at high concentration.

Methyl stearate, Na-tauroglycocholate, methyl laurate, methyl myristate, methyl palmitate (0·1–0·5 μmol), and lysolecithin and lysophosphatidyl ethanolamine (50–200 μg), were tested using the method of Mandal *et al.*[1] Activity was 10–30%-greater than in the control.

The nature of inhibition by Sn-3-*GPC*

From a Linweaver Burke plot, the nature of inhibition of phytase by *Sn*-3-GPC was shown to be competitive. This was verified with two different concentrations of phytate (0·5 and 1 μmol/ml). K_i calculated from these plots was 6×10^{-5} M.

The mode of dephosphorylation of IP_6 by phytase

The question arises whether dephosphorylation of myo-inositol hexaphosphate by phytase isolated from germinating mung bean seed is a stepwise process or not. Myo-Inositol-6-^{32}P-hexaphosphate prepared by the procedure described under Experimental was hydrolysed by phytase liberating non-labelled intermediates, and labelled P_i (Table 2). Similarly when inositol 5-^{32}P-hexaphosphate was hydrolysed by phytase IP_5 and P_i were radioactive and the decrease in counts in the IP_5 fraction was associated with an increase in counts in the P_i fraction. No radioactivity was detected during the time course experiment in other products IP_4, IP_3, IP_2 and IP_1. Alternatively when inositol-3-^{32}P-hexaphosphate was used as substrate for phytase the reaction products IP_5, IP_4, IP_3 and P_i were radioactive but no counts were detected in IP_2 and IP_1 indicating that prior to cleavage of the phosphate group at position 3, either that at position 4 or 1 might be cleaved. It appears from the data in Table 2 that cleavage of phosphate in IP_6 starts from position 6 and this is followed by positions 5 and 4, 1 and 3 or 1 and 4.

TABLE 2. MODE OF DEPHOSPHORYLATION OF SPECIFICALLY ^{32}P-LABELLED INOSITOL HEXAPHOSPHATE BY PHYTASE

Substrate	Time (min)	cpm in inositol phosphate IP_6	IP_5	IP_4	IP_3	IP_2	IP_1	P_i
6-^{32}P-IP_6	0	400	—	—	—	—	—	—
	5	300	—	—	—	—	—	95
	10	212	—	—	—	—	—	185
	15	100	—	—	—	—	—	296
	20	10	—	—	—	—	—	385
5-^{32}P-IP_6	0	400	—	—	—	—	—	—
	5	240	150	—	—	—	—	6
	10	140	170	—	—	—	—	82
	15	12	220	—	—	—	—	160
	20	—	160	—	—	—	—	241
	25	—	90	—	—	—	—	305
	30	—	15	—	—	—	—	382
3-^{32}P-IP_6	0	598	—	—	—	—	—	—
	10	342	200	50	—	—	—	—
	20	90	135	210	80	—	—	80
	30	10	60	190	160	—	—	175
	40	—	10	100	150	—	—	337
	50	—	—	22	82	—	—	488

IP_6 (6-^{32}P), IP_6 (5-^{32}P) and IP_6 (3-^{32}P) were prepared by PI-kinase reaction with the substrate IP_5 (6-OH), IP_5 (5-OH) and IP_5 (3-OH) respectively using β-γ-^{32}P-ATP as phosphate donor. The reaction mixture was passed through Dowex-1 (Cl^-) column (0·5 × 8 cm) and the phytase product (P_i and inositol phosphates) were eluted with different concentration of HCl. The specific activity (cpm/μmol phosphate) of 6-^{32}P-IP_6, 5-^{32}P-IP_6 and 3-^{32}P-IP_6 were respectively 820, 900 and 1000 and the input were 410, 400 and 600 cpm respectively.

DISCUSSION

Phytase isolated from the cotyledons of germinating mung bean seeds has optimal activity at pH 7·5,[1] but phytase isolated from other sources is active at an acidic pH. However, the induced phytase in *Bacillus subtilis* has a pH optimum at 7. Recently F_2 phytase isolated from wheat bran[14] was shown to have a pH optimum at 7·2. Since phytase from

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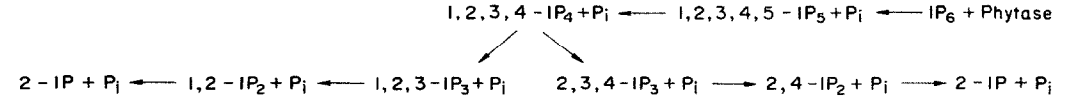
different sources is only partly purified and is invariably associated with acidic phosphatase and pyrophosphatase, the pH optimum of phytase might be influenced by the presence of these enzymes. In order to distinguish our preparation, extensive purification of phytase was undertaken. From Biogel filtration and polyacrylamide SDS gel electrophoresis it appears that the enzyme contains only subunits of MW 158000. This is the most extensively purified preparation of phytase reported so far. Tomlinson and Ballou[15] showed that a partially purified phytase preparation degraded phytase by two distinct pathways. Taking this observation as well as the report of the occurrence of acidic phytase from plants, it was of interest to know whether the pH of this phytase could be changed in the presence of other components or factors. Recently a report appeared that lysolecithin could activate phytase from wheat bran.[11] However, no change in pH optimum or mode of degradation of phytate was observed. Similarly we tested the effect of different lipids on the phytase system. It has been found that *Sn*-3-GPC at different concentrations can either activate or inactivate the enzyme, but no shift in pH optimum has been recorded.

The mode of action of phytase in our case was studied using a different approach. We prepared specifically labelled inositol hexaphosphate by using appropriate substrates and the enzyme phosphoinositol kinase.[16] It has been observed that the degradation of phytate is a stepwise process. The degradation starts from position 6 followed by positions 5 and 4, 1 and 3 or 1 and 4. Moreover, this pattern is not influenced by the presence of *Sn*-3-GPC. Thus the steps of degradation of inositol hexaphosphate by the phytase described here may be as follows since the elimination of ^{32}P at specific positions was studied without determination of L or D forms.



This mode of degradation is somewhat different from that reported for other systems.[14,17–21] Although some of the properties of the present enzyme are similar to those of the F_2 phytase from wheat bran,[14] other characteristics such as MW, inhibition by P_i and *Sn*-3-GPC and mode of action are distinct from the latter. This is suggestive that mung bean phytase is different from F_2 wheat bran phytase.

EXPERIMENTAL

Material. Seed of *Phaseolus aureus* var. B-1 were obtained from Seed Multiplication Farm, Berhampore, West Bengal. *Sn*-3-GPC and other lipids were obtained from Biochemical Unit, V.P. Chest Institute, Delhi. 2-P-inositol was obtained from Sigma Chemical Co., U.S.A. IP_5 (6-OH), IP_5 (5-OH), IP_5 (3-OH) and IP_5 (2-OH) were kindly donated by Dr. M. E. Tate, University of Adelaide, South Australia.

Purification of phytase. The isolation procedure was that of Mandal *et al.*[1] Phytase was treated twice with bentonite; the supernatant contained the enzyme with a slight contamination of phosphatase. This preparation was used for the gel electrophoresis. Phytase was isolated from the 5% polyacrylamide gel after electrophoresis as described in the text.

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Assay procedure. The procedure was that of Mandal *et al.*[1] Incubation was normally carried out at 37° for 30–60 min. The liberated P_i was estimated by the ascorbic acid method.[22]

Polyacrylamide gel electrophoresis of proteins. Disc electrophoresis in 5% polyacrylamide gel, performed according to the method of Davis,[23] was carried out at pH 8·0 for 2 hr with a current of 3 mA/tube. On completion the gels were taken out by rimming with H_2O from a hypodermic needle and 2-mm thick gel slices were kept in 0·05 M Tris–HCl buffer, pH 7, overnight; homogenized, centrifuged and the supernatant taken for enzyme assay. The duplicate sets were stained with 1% amido black or 0·2% coomassie blue.

Polyacrylamide gel electrophoresis of proteins in presence of sodium dodecyl sulphate for determination of MW. The method followed was that of Weber and Osborn.[24] Fifty microlitre samples (*ca* 30 μg) were applied to 5% gels containing 0·1% SDS and subjected to electrophoresis for 6 hr at room temp. at 5 mA/tube. The gels were taken out after the completion of run and stained in 0·2% coomassie blue overnight. The gels were destained electrophoretically. The standard proteins used as markers were β-amylase (150000) bovine serum albumin (69000) and egg white albumin (42000).

Phosphoinositol kinase and preparation of specifically labelled IP_6. Phosphoinositol kinase (PI-kinase) from germinated mung bean seed was isolated and purified by the procedure of Lahiri Majumder *et al.*[16] Specifically labelled IP_6 was also prepared by their procedure. IP_6 (6-^{32}P), IP_6 (5-^{32}P), IP_6 (3-^{32}P) were prepared by PI-kinase reaction with the substrate IP_5 (6-OH), IP_5 (5-OH) and IP_5 (3-OH) respectively using β-γ-^{32}P-ATP as phosphate donor. The specifically labelled IP_6 after elution with HCl from a short Dowex-1 column (8 × 0·5 cm) was concentrated in vacuum desiccator in the cold.

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