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PHOSPHOINOSITOL KINASE FROM GERMINATING MUNG BEAN SEEDS*

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Abstract—Phosphoinositol kinase (adenosine triphosphate–inositolmonophosphate phospho–transferase) has been isolated from cotyledons; about 300-fold purification has been achieved, with a recovery of 11%. The enzyme has a pH optimum at 7·4. It can mediate phosphorylation of lower inositol phosphates to their corresponding higher homologues, ATP being the phosphate donor. ATP can be replaced partially by UTP and PEP. The enzyme requires divalent cations for the reaction. Mn^{2+} has been found to be twice as effective as Mg^{2+}, Ca^{2+} being inhibitory. Phosphoinositol kinase has been found to be different from inositol kinase.

INTRODUCTION

THE PHOSPHORUS metabolism in the seeds during ripening and germination is closely related with phytin metabolism. Germination of the seeds is associated with enzymatic dephosphorylation of phytate by phytase.[1] Synthesis of inositol polyphosphates occurs mainly during the ripening process,[2–4] and also during the germination of seeds.[5]

As to the manner in which phytate is synthesized, there are three possibilities, (i) Stepwise phosphorylation of inositol by kinase type of reactions, (ii) phosphorylation of phosphoinositide intermediates, and (iii) the condensation of smaller phosphorylated units.[6] No experimental evidence is known so far in favour of the third pathway. The second pathway has been established in the animal system,[7–12] especially in brain tissues. Experimental evidence in favour of the first pathway is that myoinositol kinase activity has been detected in yeast hexokinase[13] and in mung bean seedling.[14] There are also reports that phytate is synthesized by the direct phosphorylation of myo-inositol.[15–18]

* Part IV of the series "Metabolism of Inositol Phosphates". For Part III see *Phytochem.* **11,** 495 (1972).

[1] A. DARBRE and F. W. NORRIS, *Biochem. J.* **66,** 404 (1957).
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[12] M. KAI, J. G. SALWAY and J. N. HAWTHORNE, *Biochem. J.* **106,** 791 (1968).
[13] O. HOFFMAN-OSTENHOF, C. JUNGWITH and I. B. DAWID, *Naturwissenschaften* **45,** 265 (1958).
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[18] R. M. ROBERTS and F. LOEWUS, *Pl. Physiol.* **43,** 1710 (1968).

On the basis of ^{32}P-incorporation studies, Mandal and Biswas[5] have earlier showed that phytate is synthesized by a process of stepwise phosphorylation, i.e. higher inositol phosphate is formed by the phosphorylation of its immediate lower homologue. This study, however, awaited the demonstration of an enzyme-system mediating this phosphorylation reaction. The present paper deals with the isolation, purification and partial characterization of this enzyme.

RESULTS

Purification

The enzyme phosphoinositol kinase has been isolated from cotyledons of 24 hr germinated mung bean seeds and purified 300-fold with a recovery of about 11% (Table 1).

TABLE 1. SUMMARY OF PURIFICATION

Purification steps	Protein (mg)	Total unit	Specific activity
1 Crude Homogenate	1422	142	0·1
2 0–40% ammonium sulphate fraction	572	114	0·2
3 Back ammonium sulphate fraction	50	105	2·1
4 DEAE-cellulose chromatography	5	50	10
5 Filtration through Biogel P-200	0·5	15	30

Specific activity has been defined as mμmole IP_4 converted to IP_5 and IP_6 per milligram protein. Protein was estimated by Folin's[19] method. One unit has been defined to be that amount of protein which can convert one mμmole IP_4 to IP_5 and IP_6 under the assay conditions given in the experimental.

Properties of the Enzyme

The enzyme has been found to phosphorylate all the inositol phosphates namely IP,* IP_2, IP_3, IP_4 and IP_5 to their corresponding higher homologues. Inositol kinase activity has however, not been detected with this preparation as using ^{14}C-myoinositol as substrate, no radioactivity could be detected in the inositol–monophosphate fraction. Inositol kinase has earlier been reported from germinating mung bean seeds.[14] In an attempt to locate the inositol kinase in some other fraction during purification of phosphoinositol kinase, it was observed that during back ammonium sulphate fractionation, the phosphoinositol kinase was located in the 20–40% ammonium sulphate fraction, the inositol kinase being retained in 0–20% ammonium sulphate fraction. To resolve the question as to whether the two enzymes are different or not, polyacrylamide gel electrophoresis was used. Proteins in 0–20% and 20–40% ammonium sulphate fraction were subjected to gel electrophoresis. The gels were sliced after completion of electrophoresis and assays were made for inositol kinase and phosphoinositol kinase with the different slices. It was observed that the two enzymes were associated with different protein bands corresponding to different slices,

* IP, IP_2, IP_3, IP_4, IP_5 and IP_6 correspond to inositol mono-, di-, tri-, tetra-, penta- and hexaphosphate respectively.

[19] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).

thereby suggesting their separate entity. Furthermore, the gel-slices of protein in 20–40% ammonium sulphate fraction were assayed for the inositol hexaphosphate–GDP phosphotransferase activity where it was revealed that the latter enzyme was located in another band quite distinct from those for the former two kinases (Fig. 1).

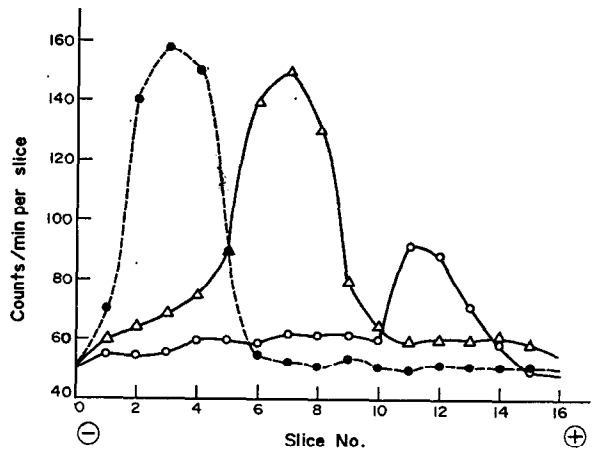

FIG. 1. LOCALIZATION OF PHOSPHOINOSITOL KINASE, INOSITOL KINASE AND INOSITOL HEXAPHOSPHATE–GDP PHOSPHOTRANSFERASE ACTIVITIES IN THE 0–40% AMMONIUM SULPHATE FRACTION. ABOUT 50 μg OF PROTEIN FROM 0–20% AND 20–40% AMMONIUM SULPHATE FRACTIONS WERE APPLIED ON THE SAMPLE GELS OF FIVE DIFFERENT SETS, TWO FOR THE FORMER AND THREE FOR THE LATTER FRACTION. INOSITOL KINASE AND PHOSPHOINOSITOL KINASE WERE ASSAYED BY THE METHOD DESCRIBED IN THE EXPERIMENTAL SECTION. INOSITOL–HEXAPHOSPHATE–GDP PHOSPHOTRANSFERASE WAS ASSAYED BY THE METHOD OF BISWAS AND BISWAS.[20]

Activity is plotted as counts recovered in the inositol-monophosphate (inositol kinase ●—●), inositol penta- and hexaphosphate (phosphoinositol kinase using inositol tetra-phosphate as substrate △—△) and GTP (phosphotransferase; ○—○) fractions.

The kinase reaction has been found to be dependent on the presence of ATP serving as phosphate donor. Without ATP, there is no conversion. With other nucleoside triphosphates namely, UTP, CTP, GTP and *d*ATP, about 40% activity compared to ATP could be detected with UTP and 20% with *d*ATP only. Attempts were made to replace ATP by other phospho-compounds such as phosphoenol–pyruvate, inorganic phosphate, pyrophosphate and polyphosphate. The enzyme activity was halved in presence of phosphoenol–pyruvate. The remaining were proved to be ineffective as phosphate donors (Table 2).

The requirement of divalent cations for the enzyme reaction has been demonstrated. Mg^{2+} is absolutely required for this reaction. However, Mn^{2+} was found to be twice as effective as Mg^{2+}. With Ca^{2+}, an inhibition has been recorded (Table 3). The optimal pH has been recorded at 7·4. The progress of the reaction with increasing time has been followed. It has been observed that with the basic assay system, the conversion is linear with time at least up to 60 min.

DISCUSSION

The reports thus far discussed indicate that the synthesis of phytic acid occurs in the ripening seeds whereas germination is accompanied by the degradation of phytic acid.

[20] S. BISWAS and B. B. BISWAS, *Biochim. Biophys. Acta* **108**, 710 (1965).

TABLE 2. PHOSPHOINOSITOL KINASE ACTIVITY IN PRESENCE OF DIFFERENT NUCLEOSIDE TRIPHOSPHATES AND OTHER PHOSPHOCOMPOUNDS

Conditions of the experiment	Concentration used (μmole/ml)	Specific activity
Complete	0·1	30·0
minus ATP		0
minus ATP plus CTP	0·1	0
minus ATP plus GTP	0.1	0
minus ATP plus UTP	0·1	11·0
minus ATP plus *d*ATP	0·1	5·6
minus ATP plus PEP	0·1	15·0
minus ATP plus $^{32}P_i$	0·1	0
minus ATP plus $^{32}PP_i$	0·8	0
minus ATP plus Poly-^{32}P	0·15	0

^{32}P–PP_i and polyphosphate were prepared as given in the experimental section. Specific radioactivity of P_i, PP_i and Polyphosphate used were $2·7 \times 10^5$ counts/min/μmole P, $4·2 \times 10^5$ counts/min/μmole P and $2·7 \times 10^6$ counts/min/μmole P respectively. Complete reaction mixture was same as shown under enzyme assay. In case of $^{32}P_i$, $^{32}PP_i$ and poly-^{32}P, non-labelled IP_4 was used.

Specific activity as defined in Table 1.

Biswas and Biswas[21] observed that ^{32}P–phytate could be prepared biologically if mung beans are allowed to germinate in presence of $^{32}P_i$; however, this incorporation of $^{32}P_i$ into phytate might be due to the exchange reaction by phytase. Indirect evidence that this was not due to the exchange reaction, came from the fact that when the incorporation of $^{32}P_i$ to phytin was compared at different periods of soaking the maximum specific radioactivity associated with different inositol phosphates was obtained between 24 and 36 hr[5] whereas the phytase level became maximum after 48 hr of soaking.[22] Direct evidence was, however, adduced by studying the exchange reaction using purified phytase and $^{32}P_i$. This was also confirmed by Williams in a different system.[23] The isolation and purification of phosphoinositol kinase from the cotyledons of germinating mung beans further substantiate our previous observations that phytic acid is synthesized in a stepwise fashion.

TABLE 3. REQUIREMENTS OF DIVALENT CATIONS FOR PHOSPHOINOSITOL-KINASE ACTIVITY

Conditions of the experiment	Concentration used (μmole/ml)	Specific activity
Complete	5	30
minus Mg^{2+}		0
minus Mg^{2+} plus Mn^{2+}	2	62
minus Mg^{2+} plus Ca^{2+}	2	11

Conditions were same as in Table 2.

[21] S. BISWAS and B. B. BISWAS, *Sci. Cult.* **34**, 399 (1968).
[22] N. C. MANDAL and B. B. BISWAS, *Plant Physiol.* **45**, 4 (1970).
[23] S. G. WILLIAMS, *Plant Physiol.* **45**, 376 (1970).

It has been shown that the enzyme could recognize IP through IP_5 as substrates and convert them into their higher homologues. IP_4 seems to be the best substrate under the assay conditions tried. But this enzyme cannot phosphorylate myo-inositol. This is also consistent with the fact that another enzyme, myo-inositol kinase, can synthesize myo-inositol monophosphate. Furthermore, IP is also obtained as an intermediate during the biosynthesis of myo-inositol from D-glucose-6-phosphate.[24] From all these evidences, we may formulate the following pathway for the biosynthesis of phytic acid in plants.

$$\text{Myo-inositol} \xrightarrow[\text{ATP, } Mg^{2+}]{\text{MI-kinase}} \text{IP} \xleftarrow{\text{Cyclase NAD, } NH_4^+} \text{D-G-6-P}$$

$$\text{IP} \xrightarrow[\text{ATP, } Mg^{2+}]{\text{Phosphoinositol kinase}} IP_2 \rightarrow IP_3 \rightarrow IP_4 \rightarrow IP_5 \rightarrow IP_6$$

This pathway seems to be comparatively simple in contrast to the pathway proposed by Asada *et al.*[25]

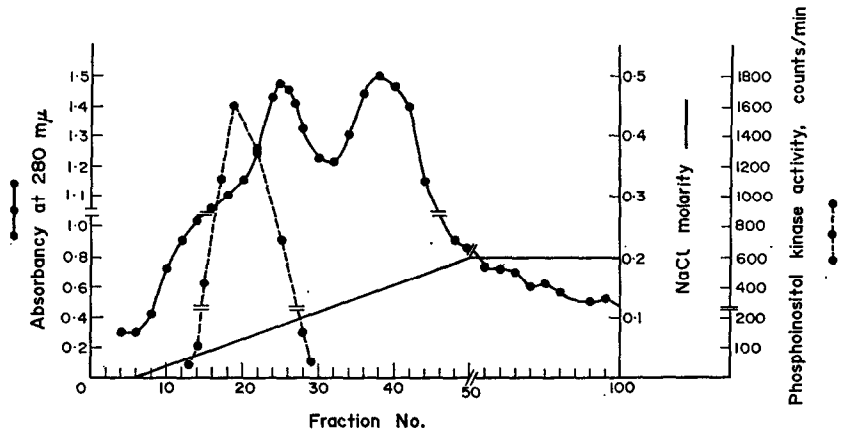

FIG. 2. ELUTION PROFILE OF PHOSPHOINOSITOL KINASE FROM DEAE CELLULOSE COLUMN. DETAILS HAVE BEEN GIVEN IN THE EXPERIMENTAL SECTION.

Phosphoinositol kinase activity (●--●) has been plotted as counts obtained in the IP_5 and IP_6 fraction using IP_4 as substrate. Optical density at 280 nm (●—●) was also plotted.

EXPERIMENTAL

Plant material. Seeds of *Phaseolus aureus* var. B-1 were obtained from seed multiplication Farm, Berhampore, West Bengal and were allowed to germinate aseptically for the requisite period following the method as described by Mandal and Biswas.[22]

Isotopes. ^{14}C-Myoinositol was obtained from Radiochemical Centre, Amersham, England. Labelled phosphate was supplied by DAE, Govt. of India, Trombay.

Purification of the enzyme. About 130 g (fresh wt.) of decoated cotyledons from 24 hr germinated mung bean seeds were homogenized with equal vol. of 0·05 M Tris HCl buffer (pH 7·0) with 0·005 M mercaptoethanol. The supernatant obtained after centrifuging the crude homogenate at 10,000 *g* was made 40% saturated with $(NH_4)_2SO_4$. The pellet was homogenized with 30% and 20% satd. $(NH_4)_2SO_4$ separately.

[24] I. W. CHEN and F. C. CHARALAMPOUS, *Biochem. Biophys. Res. Commun.* **19**, 144 (1965).
[25] K. ASADA, K. TANAKA, and Z. KASAI *Ann. N.Y. Acad. Sci.* **165**, 801 (1969).

The soluble fraction in 20% satd. $(NH_4)_2SO_4$ was found to have maximum activity. The fraction containing the enzyme (about 50 mg) was charged on a DEAE cellulose column (24 × 0·9 cm) equilibrated previously with 0·01 M Tris HCl buffer (pH 7·4) containing 0·005 M mercaptoethanol. Linear gradient elution was carried out between 0 and 0·2 M NaCl concentration with a flow rate of 0·5 ml/min. The different fractions were checked for their absorbancy at 280 nm and also assayed for the enzymatic activity (Fig. 2). The fractions (eluted between 0·05 and 0·08 M NaCl) containing the enzyme were pooled, concentrated and layered on a Biogel P-200 column (6 × 1 cm). The column was washed with 0·05 M Tris HCl buffer (pH 7·4) containing 0·005 M mercaptoethanol; fractions were collected with a flow rate of 1 ml/20 min. The enzyme was eluted just after the void volume had passed. All operations were carried out at 0–4°.

Preparation of ^{32}P *inositol phosphates.* ^{32}P-Labelled inositol phosphates were prepared and fractionated as described earlier.[5]

Enzyme assay. The incubation mixture contained the following in μmoles in a total vol. of 1 ml: Tris HCl buffer (pH 7·4), 100; Mg^{2+}, 5; ATP, 0·1; Mercaptoethanol, 5; ^{32}P–inositol phosphate (usually IP_4), 0·1 and enzyme 100 μg. These were then incubated for 40 min at 37°. The reaction was stopped by adding 0·4 M TCA and kept in cold. The supernatant obtained after centrifugation was neutralized with 1 M NaOH and phytin hydrolysate (2–3 μmole phosphorus) was added as carrier. The different inositol phosphates were separated following the method as described by Mandal and Biswas.[5] Inositol kinase was assayed following the same procedure and using ^{14}C–myoinositol in place of ^{32}P inositol phosphates.

Preparation of ^{32}P*–pyrophosphate and* ^{32}P*–polyphosphate.* ^{32}P–pyrophosphate and polyphosphate were prepared from ^{32}P orthophosphoric acid by the method of Bergman *et al.*[26] 3 mc of ^{32}P–orthophosphoric acid was used for this purpose.

Polyacrylamide gel electrophoresis of proteins. Disc electrophoresis in 5% polyacrylamide gel was performed according to the method of Davis.[27] Electrophoresis was carried out at pH 8·0 for 2 hr in a current of 3 mA/tube. On completion the gels were taken out by rimming with H_2O from a hypodermic needle and gel slices of 2 mm thickness were kept in 0·05 M Tris HCl buffer (pH 7·4) overnight, homogenized, centrifuged and the supernatant taken for enzyme assay. The duplicate sets for each fraction were stained with 1% amido black.

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[26] F. H. BERGMANN, P. BERG and N. DICKMANN, *J. Biol. Chem.* **236,** 1735 (1961).
[27] B. J. DAVIS, *Ann. N.Y. Acad. Sci.* **121,** 404 (1964).

Key Word Index—Phaseolus aureus; Leguminosae; mung bean; phosphoinositol kinase; phytin; inositol