Phytochemistry, Vol. 20, No. 8, pp. 1815–1817, 1981.
Printed in Great Britain.

0031–9422/81/081815–03 $02.00/0

TWO FORMS OF PHOSPHOINOSITOL KINASE FROM GERMINATING MUNG BEAN SEEDS*

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(*Received* 18 *April* 1980)

Key Word Index—*Phaseolus aureus*; Leguminosae; mung bean; myo-inositol phosphates; phosphoinositol kinases.

Abstract—Phosphoinositol kinase, the key enzyme responsible for the biosynthesis of higher inositol phosphates has been isolated from the cotyledons of mung beans germinated for 24 hr and has been resolved into two different forms, phosphoinositol kinase A and phosphoinositol kinase B. Both forms were purified to homogeneity and characterized. The K_m values for ATP with phosphoinositol kinase A (1.78×10^{-4} M) and phosphoinositol kinase B (3.12×10^{-5} M) showed that phosphoinositol kinase B had a greater affinity for ATP. ATP could be partially replaced as phosphate donor by UTP and phosphoenolpyruvate in the case of phosphoinositol kinase A but not in the case of phosphoinositol kinase B.

INTRODUCTION

Inositol phosphates have been shown to accumulate in developing seeds [1–3], in maturing potato tuber [4] and in coconut water [5]. This phospho compound has been found to be synthesized in seeds mainly during the later stages of ripening [6] and is considered to be the end product of phosphorus metabolism. The germination of seeds involves the breakdown of inositol phosphates by phytase-catalysed dephosphorylation [7–9]. Though this compound is widely distributed throughout the biological system, until recently, very little was known about the biosynthesis of higher inositol phosphates. As a result of ^{32}P-incorporation studies in germinating mung bean seeds, Mandal and Biswas [10] showed that the higher inositol phosphates arise by direct phosphorylation of lower inositol phosphates. The full understanding of the process, however, awaited the demonstration of the enzyme system(s) catalysing the phosphorylation reactions. In a previous communication [11], only one form of enzyme was detected. By adopting a different procedure, the multiple forms of this enzyme have been recently detected in our laboratory. The present communication reports some of the characteristics of these two forms of enzyme from the cotyledons of mung bean seeds germinated for 24 hr.

RESULTS AND DISCUSSION

Multiple forms of phosphoinositol kinase

Phosphoinositol kinase (PI-kinase) was isolated and purified through ammonium sulphate saturation, DEAE-cellulose column chromatography and Sephadex G200 gel filtration as described previously [12]. In an attempt to locate the PI-kinase activity in the polyacrylamide gel, the active fractions obtained from Sephadex G200 gel filtration were concentrated and subjected to PAGE.† The gels were sliced into 2 mm thick slices and each slice was assayed for PI-kinase activity. One gel tube, on staining with 0.02% Coomassie Brilliant Blue R250, showed (Fig. 1a) two major and one minor protein bands. When assayed, the enzyme activity was found to be associated with both major protein bands (Fig. 1b), indicating the existence of multiple forms of phosphoinoistol kinase.

Resolution of the multiple forms of phosphoinositol kinase

The demonstration of the existence of two forms of PI-kinase in the PAGE studies was followed by an attempt to resolve these activities. As the two forms of enzyme (as indicated by PAGE) were co-eluted from the Sephadex G200 column, it was decided to try and separate them by charge rather than by MW. Accordingly, the Sephadex G200 fractions were dialysed against 10 mM Tris–HCl buffer, pH 7.4, containing 5 mM 2-mercaptoethanol, and loaded onto a DEAE-Sephadex A_{25} column (1 × 6 cm), previously equilibrated with the same buffer. A linear gradient between 0 and 0.2 M NaCl was set up using 15 ml of the above buffer in the mixer and 15 ml of 0.2 M NaCl (in buffer) in the reservoir. As shown in Fig. 2, two active fractions, termed PI-kinase A and PI-kinase B, were eluted with two different salt concentrations. The electrophoretic mobilities of these two forms corresponded with the values obtained in the previous experiment. That the two forms are not due to cross-contamination has been confirmed by a rechromatography of the two forms on the same column under the same conditions.

*Part 13 in the series "Metabolism of Inositol Phosphates". For Part 12 see De, B. P. and Biswas, B. B. (1979) *J. Biol. Chem.* **254**, 8717.

†Abbreviations: I-1-P, myo-inositol-1-monophosphate; I-2-P; myo-inositol-2-monophosphate; MES, 2[*N*-morpholino]-ethane sulfonic acid; IP_4, IP_5, IP_6, tetra-, penta- and hexa-phosphates of myo-inositol respectively; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis.

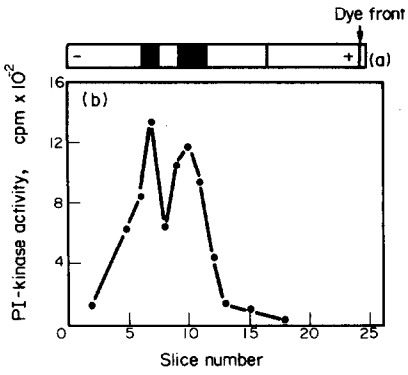

Fig. 1. (a) PAGE (5%) of Sephadex G200 purified PI-kinase. (b) Localization of PI-kinase activities on 5% PAGE. About 50 μg of Sephadex G200 purified enzyme was loaded onto the gel. PI-kinase activity has been expressed as counts obtained in IP_5 and IP_6 fractions using [^{32}P]IP_4 (6.8 × 10^2 cpm/nmol) as substrate.

Characterization of phosphoinositol kinases

Electrophoretic mobility. To test the purity of PI-kinase A and PI-kinase B, the enzymes were subjected to PAGE under non-denaturing conditions. Both forms gave a single protein band with the mobility of PI-kinase B being a little higher than that of PI-kinase A. However, in the presence of SDS, PI-kinase A was found to consist of two subunits (MW 82000 and 62000) and PI-kinase B three subunits (MW 82000, 39500 and 34000): one subunit (MW 82000) appeared to be common to both forms.

Effect of pH *on the activity of the two forms.* The effect of pH on the PI-kinase activity was tested using Tris–MES buffer (pH range covered 6.0–8.5). The pH optimum of PI-kinase B was 7.0 whilst that of PI-kinase A was 7.4.

Effect of sodium chloride. PI-kinase B activity was inhibited by increasing concentrations of sodium chloride (reduced by 25% in presence of 20 mM NaCl), but no such inhibition was observed with PI-kinase A. This effect might be due to some charge effect at the active site of the PI-kinase B, e.g. increasing concentration of sodium chloride might suppress the ionization at the active site.

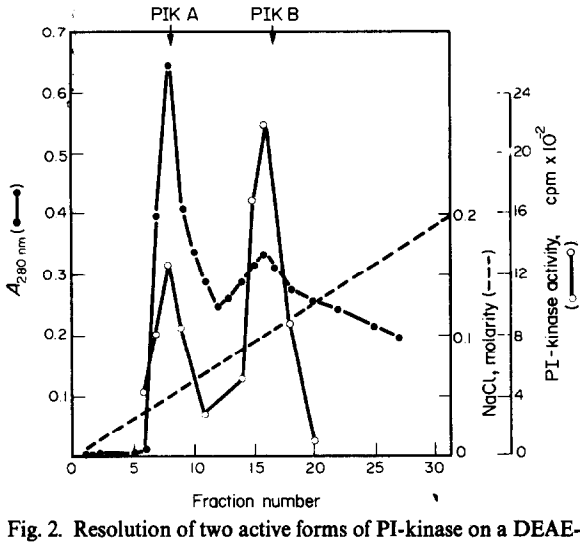

Fig. 2. Resolution of two active forms of PI-kinase on a DEAE-Sephadex A_{25} (1 × 6 cm) column. ●——●, Absorbance at 280 nm; ○——○, PI-kinase activities expressed as counts obtained in IP_5 and IP_6 fractions using [^{32}P]IP_4 (3.6 × 10^2 cpm/nmol) as substrate; PIK A and PIK B, PI-kinase A and PI-kinase B, respectively.

Phosphorylation of different myo-inositol monophosphates by the two enzymes. To study the phosphorylation of myo-inositol monophosphates by both forms of enzyme, I-1-P and I-2-P were used as substrates and [Y-^{32}P] ATP as the phosphate donor. In this experiment, IP_5 and IP_6 fractions were isolated and collected. Both PI-kinase A and PI-kinase B were found to phosphorylate I-1-P stepwise to IP_5 only. The synthesis of IP_6 could be achieved if I-2-P was used as the substrate (Table 1). The observations from different laboratories [13–15] point to the fact that the inositol monophosphate is a more immediate precursor of IP_6 in plants than inositol itself. If this is true, one would expect inositol-1-phosphate, the product of the synthase action [16], to accumulate at a stage where inositol hexaphosphate is to be synthesized. Whether ripening mung bean seeds accumulate inositol-1-phosphate or not has not yet been determined. The production of IP_5 from I-1-P, the product of the synthase, with both of these forms rather suggests that *in vivo* there might be another enzyme system which can phosphorylate IP_5 to IP_6. In fact, the enzyme (IP_6-ADP-phosphotransferase), responsible for the synthesis of IP_6 from IP_5, has been isolated from germinating mung bean seeds.

Effect of phosphate donors. The two forms of enzyme utilized ATP as the phosphate donor but the affinity of the two forms towards ATP seemed to be different. Thus the K_m values for ATP with PI-kinase A (1.78 × 10^{-4} M) and PI-kinase B (3.12 × 10^{-5} M) indicated the greater affinity of PI-kinase B towards ATP.

The physiological reasons for the existence of these two forms of phosphoinositol kinase in germinating mung bean seeds is not yet clear. There might be advantages of having two forms of this enzyme in the seeds, particularly during maturation. What is interesting is that only one form of the enzyme (PI-kinase A) has been detected in the embryo of mung bean seeds germinated for 24 hours, whereas two forms are detectable in the cotyledon. The possibility of the proteolytic conversion of form A to

Table 1. Effect of PI-kinase on different inositol monophosphates

Substrate used	Enzyme	nmol ^{32}P-incorporated from [γ-^{32}P]ATP/mg protein/40 min	
		IP_5	IP_6
I-1-P	PI-kinase A	8.3	0
I-1-P	PI-kinase B	12.7	0
I-2-P	PI-kinase A	4.1	7.5
I-2-P	PI-kinase B	12.8	18.0

I-1-P and I-2-P (0.1 μmol each) were incubated at 37° for 40 min with 0.1 μmol [γ-^{32}P]ATP (1.5 × 10^3 cpm/nmol), 100 μmol Tris–HCl, pH 7.4, 5 μmol 2-mercaptoethanol, 5 μmol Mg^{2+} and 50 μg enzyme protein. After the termination of the reaction with 1 ml 0.4 M TCA, the mixture was neutralized with 1 M NaOH and loaded onto a Dowex-1-Cl^- (0.5 × 8 cm) column. Unreacted [γ-^{32}P]ATP was washed through with 0.2 M KCl in 0.02 M HCl. IP_5 was eluted with 150 ml 0.4 M HCl and IP_6 with 40 ml 1 M HCl. The incorporation of ^{32}P was linear throughout the incubation period.

form B has been carried out by subjecting a mixture of cotyledon extract and embryo enzyme to the purification and by testing the effect of the protease inhibitor, phenyl methyl sulphonyl fluoride, and the polyphenol oxidase inhibitor, polyvinyl pyrrolidone on the isolation of the enzymes from cotyledons. Thus, it is plausible that two forms of this enzyme might utilize different energy sources for phosphorylation. In order to find out whether ATP can be replaced by other phosphate donors in these enzymatic reactions, the experiments were performed with different phosphate donors [GTP, CTP, UTP and phosphoenol pyruvate (PEP)]. Though the two forms of enzyme utilized ATP as the phosphate donor, PI-kinase A was found to be partially active with PEP and UTP as the phosphate donors (Table 2). It appears, at present, that even at the depletion of ATP, phosphorylation of inositol phosphates might continue in the seeds. The existence of these two forms of enzyme, or isoenzymes, in the germinating seeds might reflect a situation developed during maturation of seeds.

EXPERIMENTAL

Plant materials. Mung bean seeds (*Phaseolus aureus* var. B-1) were supplied from the seed multiplication farm, Berhampore, West Bengal, India.

Radiochemical. [^{32}P]Orthophosphoric acid was obtained from Bhabha Atomic Research Centre, Bombay, India.

Isolation of inositol phosphates. [^{32}P]Inositol phosphates were isolated and purified according to the method of ref. [10].

Assay of phosphoinositol kinase. Phosphoinositol kinase was routinely assayed by the method of ref. [11].

Preparation of [γ-^{32}P]*ATP.* This was prepared according to the method of ref. [17].

Preparation of I-1-P. I-1-P was prepared from glucose-6-phosphate following the method of ref. [16].

Polyacrylamide gel electrophoresis (PAGE). This was performed according to the method of ref. [18]. Gels were sliced and eluted as described earlier [12]. The protein was stained with 0.02% Coomassie Brilliant Blue R250 and destained with a soln containing 5% MeOH and 7.5% HOAc. PAGE in the presence of SDS was carried out as described in ref. [19].

Acknowledgement—This work was supported in part by a United States Department of Agriculture Grant FG-In-547.

Table 2. Effect of phosphate donors on PI-kinases

Condition of the experiment	PI-kinase A activity	PI-kinase B activity
1. Complete system (with 0.1 mM ATP)	39.0	47.6
2. Complete system −ATP	0	0
3. Complete system −ATP+PEP	22.3	0
4. Complete system −ATP+UTP	4.3	0
5. Complete system −ATP+GTP	0	0
6. Complete system −ATP+CTP	0	0

PI-kinase A and PI-kinase B activities have been expressed as nmol [^{32}P]IP_4 (5.8×10^2 cpm/nmol) converted/mg protein/40 min. The concentration of each of the phosphate donors (PEP, UTP, GTP and CTP) was 0.1 mM.

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