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AN INHIBITOR OF PHOSPHOINOSITOL KINASE FROM UNGERMINATED MUNG BEAN SEEDS*

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Key Word Index—*Phaseolus aureus*; Leguminosae; mung bean; phosphoinositol kinase; natural inhibitor.

Abstract—A protein inhibitor of phosphoinositol kinase has been detected in the later stages of ripening of mung bean seeds. This has been isolated and purified from the ungerminated seeds. It migrated as a single protein band when subjected to polyacrylamide gel electrophoresis. The MW of the inhibitor is approx. 86 000. The phosphoinositol kinase inhibition has been found to be dependent on the protein concentration of the purified inhibitor. It seems that 1 molecule of the inhibitor is necessary to inhibit 1 molecule of enzyme. The nature of the inhibition has been found to be non-competitive, the K_i of which is around $1{\cdot}47 \times 10^{-6}$ M. The enzyme inhibitor complex dissociates on gel electrophoresis without any loss of enzyme activity.

INTRODUCTION

SYNTHESIS of inositol polyphosphates occurs during ripening[1-3] and germination of seeds.[4] Phosphoinositol kinase from germinating mung bean seeds has already been reported from this laboratory.[5] In the later stages of ripening mung bean seeds the activity of phosphoinositol kinase is negligible and no activity is discernible in the fully matured seeds. However, during germination, the activity seems to re-appear for a short period. This led us to investigate the mechanism by which the enzyme is regulated in the ripening and germinating seeds. In the present paper we report an inhibitor for phosphoinositol kinase.

RESULTS

When the activity of phosphoinositol kinase was investigated in ripening mung bean seeds, it was found that the enzyme is detectable at a stage 7 days after flowering (stage I) and increases thereafter, reaching its maximum at a stage 21 days after flowering (stage IV). Following this a steady decline was found in the phosphoinositol kinase (PI-kinase) activity and about 5 weeks after flowering (stage VI) it almost reaches the base-level (Table 1). On the other hand, while in the partially purified preparation from the ungerminated seeds PI-kinase activity could not be detected,[6] the enzyme could be detected in the seeds germinated for 12, 24 and 36 hr (0·5, 2·0 and 0·23 nmol IP_4 converted to IP_5 and IP_6/mg protein respectively).

* Part VII in the series "Metabolism of Inositol Phosphates." For Part VI see *Phytochem.* **12,** 315 (1973).

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TABLE 1. PHOSPHOINOSITOL KINASE ACTIVITY AT DIFFERENT STAGES OF RIPENING OF MUNG BEAN SEEDS

Stages	Days after flowering	PI-kinase activity (nmol IP_4 converted to IP_5 and IP_6/mg protein)	Stages	Days after flowering	PI-kinase activity (nmol IP_4 converted to IP_5 and IP_6/mg protein)
I	7 ± 1	1·5	IV	21 ± 2	7·5
II	14 ± 2	5·0	V	30 ± 2	1·9
III	18 ± 2	6·5	VI	36 ± 2	0·3

The enzyme was isolated and purified from mung bean seeds of the respective stages (see Experimental). The enzyme assay[5] made with 200 μg enzyme from the ammonium sulphate fraction.

To determine whether the regulation is by an inhibitor, the enzyme fraction of stage IV of the ripening seeds was incubated in the presence of various concentrations of enzyme fraction of stage V. Instead of being additive, it was found that the activity was reduced (Table 2). The enzyme of stage IV was then incubated in the presence of the crude protein extract obtained from the ungerminated seeds. An inhibition of the phosphoinositol kinase activity was again observed. The PI-kinase isolated and purified from the mung beans germinated for 24 hr was also found to be inhibited by the extract from ungerminated seeds. A concentration of 1·3 mg crude protein totally inhibited PI-kinase from ripening as well as germinating seeds (Table 2).

TABLE 2. INHIBITION OF PHOSPHOINOSITOL KINASE BY THE EXTRACT OF MUNG BEAN SEEDS AT DIFFERENT STAGES OF DEVELOPMENT

System	PI-kinase activity (nmol IP_4 converted to IP_5 and IP_6/mg protein)	System	PI-kinase activity (nmol IP_4 converted to IP_5 and IP_6/mg protein)
1 Assay with stage IV enzyme	6·0	5 Assay with DEAE purified enzyme of germinating seeds	12·0
2 Assay with stage V enzyme	1·0	6 System 1 + crude protein from ungerminated seeds	0
3 Assay with enzyme of stage IV and enzyme of stage V	1·7	7 System 5 + the same crude protein	0
4 Assay with enzyme of stage IV and enzyme of stage V	0·3		

About 100 μg of stage IV enzyme was used in each of the systems 1, 3 and 4. Systems 2, 3 and 4 contained 100, 50 and 100 μg respectively of stage V enzyme. About 200 μg of enzyme was used in system 5. Systems 6 and 7 contained about 1·3 mg of protein from ungerminated seeds. Other details were as in Table 1. The enzyme preparation in each case was stored for 15 days prior to assay.

It seemed possible that this inhibition of PI-kinase activity was due to phytase, ATPase or protease that might be present in the crude protein from ungerminated seeds. However, when assayed, all the three enzymes were found to be absent in the extract from ungerminated seeds. Furthermore, the purified enzyme, the purified inhibitor and the enzyme-inhibitor complex have been found to be free of phytase and ATPase activities.

Nature of the Inhibitor

The inhibitor has been found to be non-dialyzable and precipitable by ammonium sulphate and trichloroacetic acid. Moreover, the absorption spectrum, polyacrylamide gel electrophoresis and subsequent staining of inhibitor with amido black suggest that the inhibitor is a protein.

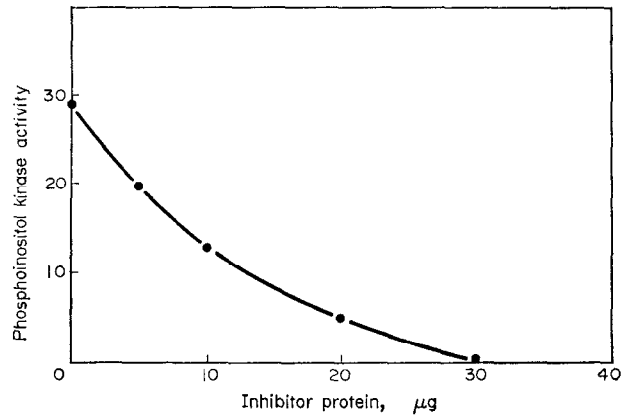

FIG. 1. PI-KINASE ACTIVITY IN THE PRESENCE OF DIFFERENT CONCENTRATIONS OF THE PURIFIED INHIBITOR.
Details of the enzyme assay has been referred to in the experimental section. Phosphoinositol kinase activity has been plotted as nmol IP_4 converted to IP_5 and IP_6 per mg protein.

The PI-kinase inhibition was found to be dependent on the protein concentration of the purified inhibitor, which increases in a curvilinear manner. Using 50 μg enzyme, 25–30 μg of the inhibitor can fully inhibit the enzyme activity under the conditions studied (Fig. 1).

The nature of the inhibition was then studied. Using different concentrations of IP_4* the PI-kinase activity was determined in the presence and absence of the inhibitor. When the Lineweaver–Burk plot was made, two straight lines were obtained indicating that the inhibition was non-competitive. The non-competitive nature was further investigated (Fig. 2). K_s and K_i have been found to be $3{\cdot}6 \times 10^{-4}$ M and $1{\cdot}47 \times 10^{-6}$ M (considering the MW to be 86 000) respectively.

Effect of preincubation of the Enzyme and the Inhibitor

The enzyme (50 μg) and the inhibitor (8 μg) were incubated at 37° in the presence of buffer alone for different periods of time. The other components were added after the requisite period of pre-incubation and PI-kinase activity assayed as usual. It was found that the inhibitor complexes with the enzyme within the first 10 min of preincubation, giving 60% inhibition. After 2 and 5 min the inhibition was 30 and 45% respectively. Beyond 10 min, further increase in the preincubation time has little effect on the inhibitory activity. The enzyme and the inhibitor used under standard conditions without preincubation recorded 50% inhibition of the PI-kinase activity. Preincubation of the enzyme with the inhibitor thus increases the efficiency of inhibition.

* IP_4—Inositol tetraphosphate.

Behavior of the Enzyme-inhibitor Complex on Polyacrylamide Gel Electrophoresis

The enzyme-inhibitor complex dissociates when subjected to gel electrophoresis, as indicated in the Experimental, and two bands one corresponding to the position of the enzyme and the other corresponding to the inhibitor were detectable. When assayed,[5] from the duplicate set, the enzyme was found to correspond to band 1 and that more than 90% of the original enzyme activity before complexing with the inhibitor was detected in that region. This indicates that by complexing with the inhibitor, the enzyme *per se* is not changed and full enzyme activity can be restored when the inhibitor is separated.

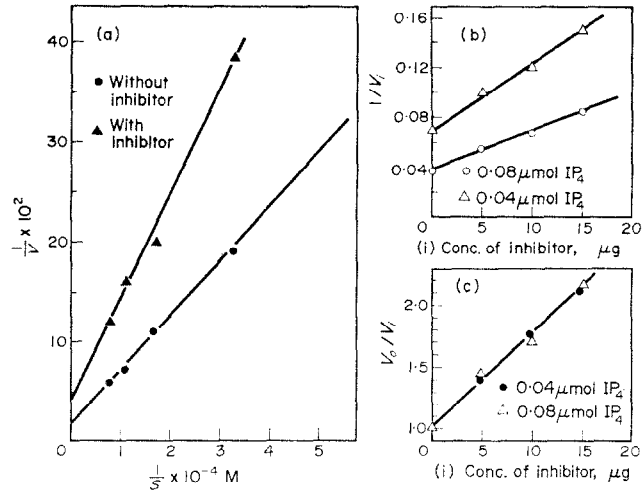

FIG. 2. PLOTS SHOWING THE NATURE OF PI-KINASE INHIBITION BY THE INHIBITOR.
(a) Lineweaver–Burk plot. V has been expressed as nmol IP_4 converted to IP_5 per mg protein and [S] in M. About 50 μg of enzyme and 12 μg inhibitor were used in each case. (b) Plot of the reciprocal of inhibited rate (V_i) against inhibitor (i) concentration using different IP_4 concentration. V expressed as in (a); enzyme used 50 μg. (c) Plot of the ratio of V without inhibitor (V_0) to that with inhibitor (V_i) against different inhibitor concentration. V expressed as in (a); enzyme used 50 μg.

MW of the Inhibitor

About 20 μg of the purified inhibitor was subjected to polyacrylamide gel electrophoresis in the presence of 1% sodium dodecyl sulphate for MW determination (see Experimental). Only one protein band was detected. When the mobility was compared with that of the proteins of known MW, the MW of the inhibitor was found to be approx. 86 000.

DISCUSSION

Though there is a postulate that the germination of seed is associated with the degradation of phytin,[7–10] the possibility of the synthesis of phytin even during germination has been asserted[4] and the enzyme PI-kinase has been isolated and purified from the germinating seeds.[5] This enzyme has been found to operate during ripening of mung bean

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seeds. The enzyme activity increases up to a stage 21 days after flowering, beyond which a sharp decline was noticed followed by almost zero value 5 weeks after flowering. On the other hand, while in the partially purified preparation from ungerminated seeds PI-kinase could not be detected, the enzyme could be detected in seeds germinated for 24 hr. This reappearance of the PI-kinase might be due to several reasons, (a) by the activation of the pre-existing enzyme which presumably was inactivated during the ripening process, (b) by the synthesis of PI-kinase *de novo*, which presupposes that PI-kinase disappears during the later stages of ripening, and (c) by the removal of an inhibitor which apparently accumulates during the later stages of ripening. That the last possibility is operative in the present system has been confirmed by the detection of an inhibitor in the developing as well as ungerminated seeds (Table 2).

The stoichiometric studies on the inhibition show that for the total inhibition of PI-kinase the ratio of enzyme to the inhibitor required is 1:0·5–0·6. The inhibitor does not compete with the inositol phosphates and the K_i for this non-competitive inhibition has been found to be $1{\cdot}47 \times 10^{-6}$ M. Since the MW of the enzyme is 177 000,[11] it is apparent that 1 molecule of inhibitor can inhibit 1 molecule of enzyme. The enzyme-inhibitor complex is labile since it can also be dissociated by polyacrylamide gel electrophoresis. That the inhibitor itself is not a specific protease has been proved by the fact that when the enzyme inhibitor complex was subjected to polyacrylamide gel electrophoresis, the enzyme could be separated without any loss of activity.

Reports of a number of enzyme systems are known which are inactivated by specific protein. Among these are the trypsin inhibitor from soybean,[12] chymotrypsin inhibitor from potato,[13] RNase inhibitor from liver,[14] protease inhibitor from different sources[15,16] invertase inhibitor from maize endosperm[17] and DNase inhibitor from mouse liver.[18] The observations in the present paper suggest that the failure to detect PI-kinase activity in the ungerminated seeds[6] is not due to its absence, but due to the presence of an inhibitor.

EXPERIMENTAL

Sources of plant material, radioisotopes and details of the methods of isolation and purification of the enzyme PI-kinase, preparation of ^{32}P-inositol phosphates, enzyme assay, and polyacrylamide gel electrophoresis have been reported earlier.[5] Plants of *Phaseolus aureus* were raised at the Institute ot Shyamnagar. The emergence of flowerbuds in each plant was noted and the respective flowers marked. Pods were collected and the PI-kinase isolated and purified as reported earlier.[5]

Purification of the inhibitor. Ungerminated mung bean seeds were powdered in a pestle and mortar and the powder soaked for 1 hr in 0·05 M Tris-HCl buffer (pH 7·4) containing 0·005 M mercaptoethanol. The resulting pulp was homogenized in a Sorval Omnimixer with the same buffer. The homogenate was passed through double layered cheese-cloth and the filtrate centrifuged at 10 000 *g* for 15 min. The supernatant obtained was made 50% saturated with $(NH_4)_2SO_4$ and the ppt was homogenized with 50 and 20% satd. $(NH_4)_2SO_4$ separately. The soluble fraction in 20% satd. $(NH_4)_2SO_4$ was then made 90% satd. with $(NH_4)_2SO_4$. The ppt. was dissolved in the required vol. of 0·05 M Tris-HCl buffer (pH 7·4) containing 0·005 M mercaptoethanol and dialyzed overnight. The dialyzed protein was separated on a DEAE-cellulose column (22 × 1 cm) previously equilibrated with 0·01 M Tris-HCl buffer (pH 7·4) containing 0·005 M mercaptoethanol by linear gradient elution between 0 and 0·1 M NaCl concentration. Fractions were collected with a flow rate of 1 ml/3 min and their absorbancy at 280 nm was measured. When assayed, the protein in fraction 28 was found to exhibit PI-kinase activity and fraction 40 the inhibitor activity. The inhibitor pooled

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from the 39th to the 40th tube gave a single protein band when subjected to polyacrylamide gel electrophoresis. When assayed, the inhibitor activity was found to correspond to the protein band.

Assay for the inhibitor. The inhibitor was assayed by determining the degree of inhibition of PI-kinase in the presence of the inhibitor by comparison with a PI-kinase system without inhibitor. The standard assay system contained the following in μmol in a total vol. of 1 ml: Tris-HCl (pH 7·4), 100; ATP, 0·1; Mg^{2+}, 5; mercaptoethanol, 5; inositol phosphate (usually IP_4), 0·1; and enzyme, 50 μg.

Assay for phytase and ATPase. Assay for phytase and ATPase were by the method of Mandal *et al.*[19]

Polyacrylamide gel electrophoresis of the enzyme inhibitor complex. Three tubes each with 50 μg of enzyme, 30 μg of the inhibitor and 50 μmol of Tris-HCl buffer pH 7·4 were incubated for 10 min at 37°. Under these conditions, the complex does not exhibit any enzyme activity. The contents of the other tubes were then dialyzed against 0·001 M Tris-HCl buffer (pH 7·4) for 2 hr in the cold. Each dialyzed fraction was concentrated under vacuum in the cold to 50 μl. After polyacrylamide gel electrophoresis in 5% acrylamide[5] at pH 8·0 for 2 hr, the gels were taken out and slices of 2 mm thick were cut from one gel. These were kept in 0·05 M Tris-HCl buffer (pH 7·4) overnight, homogenized and the supernatant from each was assayed for the PI-kinase activity using IP_4 (specific activity 4×10^2 cpm/nmol as substrate. The duplicate was stained in 0·2% coomassie blue overnight and then destained electrophoretically with a solution of MeOH:7·5%. HOAc (1:1).

Polyacrylamide gel electrophoresis of proteins in presence of sodium dodecyl sulphate for determination of MW. The method followed was that of Weber and Osborn.[20] Other conditions were as described earlier.[11]

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