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FREE AND BOUND MYOINOSITOL PHOSPHATES IN MATURE COTTON EMBRYOS

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Abstract—Free myoinositol tetraphosphates, myoinositol pentaphosphate and myoinositol hexaphosphate were detected in mature cotton embryos. Three major isomers of the tetraphosphate were isolated by ion exchange chromatography followed by paper electrophoresis (80 V/cm). No free myoinositol monophosphate, myoinositol diphosphate or myoinositol triphosphate was detected. However, these were found bound to a lipid, presumably as mono-, di- and triphosphoinositides. No evidence was found for the presence of lipid bound higher myoinositol phosphates.

INTRODUCTION

THERE are contradicting reports in the literature on the occurrence of lower myoinositol phosphates in seeds. Preece *et al.*[1] showed that ripe grains of barley contain free myoinositol diphosphate (IP_2), myoinositol triphosphate (IP_3), and myoinositol tetraphosphate (IP_4). On the other hand, Saio[2] reported that no myoinositol phosphates except myoinositol hexaphosphate (IP_6, phytic acid) were present in ripening rice grains. Posternak, *et al.*,[3] Fischler,[4] and Schormüller *et al.*[5] also found no evidence for the presence of lower myoinositol phosphates in ripe wheat grains.

Although phytic acid has been found in all seeds examined, its biosynthesis and role in the developing seed are unknown.[6–8] As part of a program on the biosynthesis of phytic acid, mature cotton embryos were investigated for the presence of lower myoinositol phosphates. The present communication reports the presence of three isomers of IP_4 and an IP_5 in free form, and IP_2 and IP_3 in bound form, probably as di- and triphosphoinositides.

RESULTS AND DISCUSSION

Fractionation of myoinositol phosphates by ion exchange chromatography.

Free myoinositol phosphates were fractionated on a Dowex-1 × 8 (Cl^-) column using linear gradient elution with LiCl. The elution pattern showed six different phosphorus (P)-containing peaks (**1** through **6**) which were obtained at LiCl concentrations of 0·06, 0·34,

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0·41, 0·49 and about 0·6 M, respectively. P and myoinositol contents, and P to myoinositol molar ratios (P/I) for each fraction are given in Table 1. Fraction **1** was found to be mostly orthophosphate (Pi) with only traces of myoinositol monophosphate (IP_1). From the P/I it appears that fractions **2, 3** and **4** are isomers of IP_4, and fractions **5** and **6** are mixtures of IP_5 and IP_6. As expected, the relative distribution of P in the various fractions shows that about 82% of the total P of mature cotton embryos is phytic acid (IP_6). IP_4 and Pi represent only about 7·5% and 0·5%, respectively.

TABLE 1. ANALYSIS OF PHOSPHORUS-CONTAINING FRACTIONS OBTAINED BY DOWEX-1 × 8 (Cl^-) CHROMATOGRAPHY OF A TCA EXTRACT OF COTTONSEED MEAL

Fraction (total vol., ml)	Total P (mmol)	Concentration (mM) Inorganic P (Pi)	Organic P* (P)	Myoinositol† (I)	Molar ratio (P/I)
1 (134)	0·2	1·4	0·1	trace	—
2 (260)	0·44	—	1·7	0·4	4·2
3 (100)	0·64	—	6·4	1·6	4·0
4 (200)	1·6	—	8·0	2·05	3·9
5 (150)	3·3	—	22·0	4·3	5·6
6 (800)	28·8	—	36·0	6·2	5·8
Per cent recovery 94					

* Organic P was determined by subtracting Pi from total P.

† Myoinositol was determined microbiologically and the values reported are the mean of triplicate estimations.

High voltage electrophoresis (HVE) of inositol phosphates

Fractions obtained by ion exchange chromatography were analyzed for myoinositol phosphates by HVE. Electrophoresis was carried out for 20 min at 80 V/cm in 0·06 M oxalate buffer. Fractions **2, 3,** and **4** were not homogeneous and contained three different P-containing components. A P-containing band near the origin (2·5 cm from origin) was found in all the fractions but gave no positive test for inositol. Fraction **5** and fraction **6** each gave only one P-containing band which migrated 20·2 and 20·4 cm towards the anode in 20 min, respectively.

In order to isolate various myoinositol phosphates and their isomers, preparative HVE was carried out on thicker paper. Five P-containing bands (A–E) with relative migration with respect to Pi (R_{pi}) of 2·6, 2·9, 3·1, 3·7 and 3·8, respectively, were eluted from the paper electrophoretogram with deionized H_2O. Fractions obtained in this way contained a substantial amount of oxalate which was removed by chromatography on Dowex 1 × 8 (Cl^-) (see Experimental). For each of the fractions, (A–E), a single P-containing peak, free from oxalate, was obtained at LiCl concentrations of 0·33, 0·39, 0·42 and above 0·5 M, respectively. The values are in close agreement with the values obtained for the myoinositol phosphates in the original extract. P/I and RPi for these fractions and for IP_4, obtained by the action of peanut phytase on phytic acid, and IP_6 are given in Table 2. It can be seen that fractions, A, B and C are isomers of IP_4. Since myoinositol was determined microbiologically using *Kleokera brevis* which is highly stereospecific for myoinositol, and since P/I ratios of all three IP_4 fractions are, in fact, within ±5% of the theoretical P/I value 4, the possibility that any of the IP_4 fraction is a tetraphosphate of substituted inositols such as C-methyl IP_4 or O-methyl IP_4 is highly unlikely. This conclusion was further supported

by the fact that PC of enzymic or acid dephosphorylated products of IP_4 showed only inositol and no substituted inositols or sugars could be detected. Fractions D and E, which gave P/I of 5·3 and 5·6, respectively, are probably mixtures of IP_5 and IP_6. This is not surprising, because IP_5 and IP_6 migrate very close to one another on the electrophoretogram. The moving paper electrophoresis technique described by Tate[9] might be useful in resolving this mixture. Rp_i values of IP_4, obtained by the dephosphorylation of IP_6 with phytase, are quite different from the naturally occurring IP_4 isomers (A, B and C) of cotton embryos. These data show that the IP_4 isomers from mature cotton embryos are probably not the dephosphorylation products of phytase. Tomlinson and Ballou[10] characterized myoinositol 1,2,5,6 tetraphosphate as one of the dephosphorylation products of wheat phytase. Recently it was pointed out by Asada, *et al.*[8] that IP_4, obtained by the action of wheat phytase on phytic acid, is not an intermediate in the biosynthesis of phytic acid in ripening wheat and rice grains. It still remains to be seen if any of the naturally occurring IP_4 isomers are intermediates in the biosynthesis of phytic acid. Incorporation of ^{33}P into IP_4, IP_5 and IP_6, when fed to isolated immature cotton bolls, however, suggests that free IP_4 is an intermediate in the biosynthesis of IP_6.[11]

TABLE 2. COMPARISON OF P TO MYOINOSITOL RATIOS AND ELECTROPHORECTIC MOBILITIES OF PURIFIED IP_4 ISOMERS FROM MATURE COTTON EMBRYOS WITH IP_4 FROM IP_6 DEGRADED BY PEANUT PHYTASE, IP_6 AND Pi*

Fraction† or reference	Molar ratio P/Myoinositol (P/I)	Spot Distance‡ (cm)	Migration relative to Pi (R_{Pi})
Pi		5·3	1·0
A	3·9	14·0	2·6
B	4·1	15·5	2·9
C	3·8	16·5	3·1
D	5·3	20·0	3·7
E	5·6	20·3	3·8
IP_4 (peanut Phytase)	4·0	18·7, 19·1	3·5, 3·6
IP_6	6·1	20·4	3·85

* Conditions for electrophoresis: 0·06 M oxalate buffer, pH 1·55 and 20 min at 80 V/cm on Whatman No. 1 chromatography paper.

† A, B, C, D and E represent the fractions obtained by HVE followed by chromatography on Dowex-1 × 8 (Cl^-). IP_4 and IP_6 are myoinositol tetra- and myoinositol hexaphosphates respectively. Pi is H_3PO_4. IP_4 (peanut phytase) was prepared from IP_6 digestion with peanut phytase.

‡ The spot distance was measured from the starting line to the center of the spot.

No evidence was found for the presence of free IP_2 or IP_3 in cottonseed in agreement with the results obtained by Saio[2] and Nagai and Funahashi[12] with rice grains, by Schormüller *et al.*[5] with wheat grains, and by Sobolev [13] with broad beans, corn sunflower and poppy seeds. Sobolev suggested that phytic acid is synthesized by successive phosphorylation of myoinositol. This may be true; but, if the free lower myoinositol phosphates are the true intermediates for the biosynthesis of IP_6, it might be expected that at least traces of IP_2 and IP_3 would be found in the mature cotton embryos. A recent study by

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Asada *et al.*[8] on the process of IP_6 formation in ripening rice grains shows that IP_6 in seeds is not formed by the progressive phosphorylation of free myoinositol. Similar results have also been reported by Ahuja[14] in the case of developing peas. The absence of IP_2 and IP_3 in the cotton embryo suggests, therefore, that a series of bound myoinositol compounds could be intermediates in the biosynthesis of IP_6.

Evidence for the presence of bound IP_2 *and* IP_3 *in the mature cotton embryo*

Phosphoinositides were extracted from glandless cottonseeds by the method of Dawson and Eichberg[15] and were partially hydrolyzed with HCl to liberate the bound myoinositol phosphates. The liberated myoinositol phosphates were examined by column chromatography on Dowex 1 × 8 (Cl^-) (see Experimental). Elution with a gradient of LiCl gave Pi an IP_2 (P/I = 2·1) and an IP_3 (P/I = 2·8), but no higher inositol phosphates. Even the treatment of the final residue obtained after extraction of free inositol phosphates and phosphinositides with 6 N HCl for 4 hr at 110° did not produce IP_4 or IP_5. Thus it was concluded that lipid-bound IP_4 or IP_5 were not present in mature cotton embryo. It should be noted that it is very important to remove all acid soluble inositol phosphates before HCl treatment, otherwise their degradation products would be obtained. The acid hydrolysate of the first fraction was always found to contain a small amount of myoinositol and a large proportion of Pi. This observation was interpreted to mean that some IP_1 is present which could not be detected because of the presence of large amounts of Pi. These data suggest, but by no means prove, that IP_6 is synthesized by the phosphorylation of a bound lipid-like inositol compound through the IP_3 or perhaps IP_4 stage and then to the IP_6 through the direct phosphorylation of free IP_4 and IP_5. Asada *et al.*[8] and Cosgrove[7] postulated the biosynthesis of IP_6 through phosphoinositide intermediates, in seeds. Recently, Molinari and Hoffmann-Osterhoff[16] obtained an enzyme system from *Lemna gibba* capable of phosphorylating myoinositol into higher phosphorylated products, including IP_6. These results, contrary to our hypothesis, support the stepwise phosphorylation of myoinositol to IP_6. In view of these results we can not completely exclude a pathway involving free IP_2 and IP_3 as intermediates in phytic acid biosynthesis in cotton embryo, although these could not be detected in mature cotton embryo. At present the data available on the biosynthesis of IP_6 are difficult to assess.

EXPERIMENTAL

Materials. Glandless cottonseeds were obtained from the Oilseed Products Research Center, Texas Engineering Experiment Station, College Station, Texas. *Kleokera brevis* yeast culture was obtained from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, U.S.A.

Analytical methods. Inorganic P was determined by the method of Fiske and Subba Row,[17] total P by the method of Bartlett[18] and myoinositol by the method of Campling and Nixon,[19] using *Kleokera brevis* as the test organism.

Ion exchange chromatography of myoinositol phosphates. Myoinositol phosphates (500–600 μmol of P) were chromatographed on a Dowex-1 × 8 (Cl^-), 200–400 mesh, column (1 × 28 in) by linear gradient elution with 0–0·7 M LiCl as described by Grado and Ballou.[20] The rate of elution was about 1 ml per min. 10 × 1 ml Fractions were collected and analyzed for total P. Tubes representing a given peak were pooled. The fractions were evaporated to dryness under red. pres. at 30°, and the residue was extracted with EtOH to remove LiCl. Insoluble

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Li salts were collected by centrifugation and ionized with Dowex-50 (H^+). Samples from each peak were hydrolyzed with 6 N HCl in sealed tubes at 120° for 72 hr. The acid was removed from the hydrolysates by allowing the tubes to stand in an evacuated desiccator over NaOH pellets. The residue was dissolved in H_2O and analyzed for P and myoinositol.

High voltage electrophoresis. (HVE) was carried out on Whatman No. 1 chromatography paper in 0·06 M oxalate buffer, pH 1·55, at 80 V/cm for 20 min as described by Seiffert and Agranoff.[21] P-containing bands on the dried electrophoretogram were identified by the method of Rorem.[22] Preparative HVE was performed on Whatman No. 3 MM paper. Guide strips were cut out and the P-containing bands were located. The strips were cut up and eluted with deionised H_2O. Eluates from each band were neutralized with cyclohexylamine and concentrated to about 5 ml at 35°. The concentrated eluates were chromatographed on Dowex-1 × 8 (Cl^-), as described before, to obtain the myoinositol phosphates.

Extraction of myoinositol lipids. Cottonseed meal was extracted successively with neutral $CHCl_3$: MeOH mixtures, to remove the neutral lipids and relatively non-polar phospholipids, and 20% TCA soln, to remove the free inositol phosphates.[21] Finally, the meal was extracted with a mixture of $CHCl_3$–MeOH–conc. HCl (250:125:2) to extract the polar phospholipids.[14]

Acid hydrolysis of myoinositol lipids. A myoinositol lipid fraction was neutralized with 1 N LiOH and evaporated to dryness at 20°. The residue was partially hydrolyzed with 6 N HCl in a sealed ampoule at 110° for 10 min to liberate the bound myoinositol phosphates. The acid hydrolysate was extracted with Et_2O to remove the free fatty acids. The aq. phase was neutralized with satd LiOH, and the myoinositol phosphates were precipitated as Ba salts from 80% EtOH.[18] The Ba salts were then decomposed with Dowex-50(H^+) ion exchange resin to obtain the free acids.

Extraction of myoinositol phosphates: One kg of *n*-hexane defatted cottonseed meal was extracted at 20° with 4 vol. of 20% TCA for 1 hr. The residue was removed by centrifugation at 2600 *g* for 20 min. The clear supernatant was neutralized (pH 8) with satd LiOH, treated with excess of Ba acetate to ppt inositol phosphates and the ppt was separated by centrifugation. The supernatant was concentrated to 100 ml at 40° and the soluble Ba-salt of inositol monophosphate was ppt by adding 4 vol. of EtOH. The ppt was centrifuged, washed with 80% EtOH and pooled with the ppt of Ba inositol phosphates obtained in the preceding step. The combined ppt was then dissolved in 3N HCl and centrifuged to remove any insoluble matter. The clear supernatant was adjusted to pH 8 with 10 N-LiOH and the salts of Ba inositol phosphates were precipitated with EtOH as before. The ppt was centrifuged and decomposed with Dowex-50 × 8 (H^+), 50 mesh, to obtain inositol phosphates in soln in the free acid form for further fractionation and purification by ion exchange chromatography and HVE.

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