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FARNESYL PYROPHOSPHATE AND GERANYLGERANYL PYROPHOSPHATE SYNTHETASES DURING *CUCURBITA PEPO* GERMINATION*

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Key Word Index—*Cucurbita pepo*; Cucurbitaceae; pumpkin; farnesyl pyrophosphate synthetase; geranylgeranyl pyrophosphate synthetase.

Abstract—Although the geranylgeranyl pyrophosphate synthetase activity was very low compared with farnesyl pyrophosphate synthetase activity on imbibition of pumpkin seed, the former increased markedly and the latter decreased as germination proceeded.

INTRODUCTION†

IN A PREVIOUS paper,[1] we reported the separation of geranylgeranyl-PP synthetase and farnesyl-PP synthetase from pumpkin fruit and showed that the geranylgeranyl-PP synthetase free of farnesyl-PP synthetase catalyzed the condensation of isopentenyl-PP with either farnesyl-PP, geranyl-PP or dimethylallyl-PP to give geranylgeranyl-PP as the single product. Comparative studies of these two prenyltransferases responsible for the chain-elongations up to C_{15} and C_{20} in isoprenoid biosynthesis are of fundamental importance. We have now shown that there is a marked difference in the change in activity of these two enzymes during pumpkin-seed germination.

RESULTS AND DISCUSSION

Ammonium sulfate fractions of cotyledon extracts from completely etiolated plants were prepared and their capacity to incorporate isopentenyl-[1-^{14}C] pyrophosphate into farnesyl-PP or geranylgeranyl-PP was investigated.

The previous study showed that farnesyl-PP synthetase can be assayed by incubating radioactive isopentenyl-PP and geranyl-PP in the presence of Mg^{2+}, whereas geranylgeranyl-PP synthetase is almost insensitive to this incubation.[1] Therefore, such an incubation was justified in assaying farnesyl-PP synthetase in the present study. Although an assay system with radioactive isopentenyl-PP plus geranyl-PP in the presence of Mn^{2+} is more sensitive than one with farnesyl-PP instead of geranyl-PP for geranylgeranyl-PP synthe-

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† *Abbreviations used*: farnesyl-PP, 2,6-*trans,trans*-farnesyl pyrophosphate; geranylgeranyl-PP, 2,6,10-*trans, trans,trans*-geranylgeranyl pyrophosphate; geranyl-PP, geranyl pyrophosphate; dimethylallyl-PP, 3,3-dimethylallyl pyrophosphate; isopentenyl-PP, Δ^3-isopentenyl pyrophosphate.

[1] OGURA, K., SHINKA, T. and SETO, S. (1972) *J. Biochem.* **72**, 1101.

tase, the farnesyl-PP system was used for the assay of this enzyme in order to eliminate the possibility of detecting farnesyl-PP synthetase.

Table 1 shows the variation found in a typical experiment in the conversion of radioactive isopentenyl-PP into acid-labile materials under these conditions. The farnesyl-PP synthetase activity was predominant in the enzyme from imbibed seeds, and decreased with time. Conversely, the activity of geranylgeranyl-PP synthetase, which had been almost negligible initially became dominant after 7 days. Although maximum activity for geranylgeranyl-PP synthetase occurred from 3 to 5 days after germination in several experiments, the change of relative activity of these two enzymes was reproducible.

TABLE 1. VARIATION IN PRENYLTRANSFERASE ACTIVITIES IN A 35–55% AMMONIUM SULFATE FRACTION OF *Cucurbita pepo* COTYLEDONS DURING GERMINATION

Period of germination (days)	Activities in nmol product/mg protein/hr	
	FPP synthetase*	GGPP synthetase†
0	6·23	0·17
2	4·97	0·56
3	1·25	1·34
4	0·34	0·51
7	0·11	0·64

* Activities when isopentenyl-[1-^{14}C]-PP and geranyl-PP were used in the presence of $MgCl_2$.

† Activities when isopentenyl-[1-^{14}C]-PP and farnesyl-PP were used in the presence of $MnCl_2$.

The method of preparation of the enzymes and the composition of the incubation mixture are described in the Experimental section.

For further evidence for the developmental variation of the two enzymes, incubation with radioactive isopentenyl-PP and geranyl-PP in the presence of Mn^{2+}, which could detect both of these two enzymes, was also carried out, and the products were analyzed. As potassium fluoride was added to inhibit phosphatase present in the crude enzyme, alkaline phosphatase treatment was not effective, and hence the radioactive materials obtained by the acid treatment of the products were identified by radio–gas chromatography (GC–RC). It is well established that the acid hydrolyses of farnesyl-PP and geranylgeranyl-PP give mainly a mixture of nerolidol and farnesol and a mixture of geranyllinalool and geranylgeraniol, respectively.[2,3]

As shown in Table 2, a clear difference in the variation of these two enzymes was observed. The major materials derived from the incubation with the enzyme from imbibed seeds were C_{15} prenols, nerolidol and farnesol. However, those from the incubation with the enzyme from 7-day-old cotyledons were C_{20} prenols, geranyllinalool and geranylgeraniol. A mixture of C_{15} and C_{20} prenols was formed from the products derived from the incubation with the enzyme of 2-day-old cotyledons.

[2] GOODMAN, DE W. S. and POPJÁK, G. (1960) *J. Lipid Res.* **1**, 286.
[3] OSTER, M. O. and WEST, C. A. (1968) *Arch. Biochem. Biophys.* **127**, 112.

Skilleter and Kekwick[4] have shown that the incorporation of isopentenyl-PP into phytol is stimulated by light in the leaves of *Phaseolus vulgaris*.

TABLE 2. RADIO-GAS CHROMATOGRAPHY OF PRENOLS LIBERATED BY THE ACID TREATMENT OF THE PRODUCT FORMED BY THE INCUBATION OF ISOPENTENYL-[1-^{14}C]-PP AND GERANYL-PP IN THE PRESENCE OF $MnCl_2$ WITH A 35–55% AMMONIUM SULFATE FRACTION OF *Cucurbita pepo* COTYLEDONS DURING GERMINATION

Period of germination (days)	Detector response (peak ht, cpm)			
	Nerolidol	Farnesol	Geranyllinalool	Geranylgeraniol
0	572	295	76	27
2	550	211	294	110
4	196	87	561	213
7	61	33	541	150

The methods of gaschromatographic analysis were described in Experimental section.

To examine the effect of light, 8-day-old etiolated seedlings were exposed to light, and the farnesyl-PP synthetase and geranylgeranyl-PP synthetase activities were assayed. However, the effect of light appeared to be insignificant (Table 3).

West and Oster[3] have reported that geranylgeranyl-PP is formed predominantly on incubation of mevalonate in the presence of Mg^{2+} with crude extracts from *Echinocystis macrocarpa* endosperm. In our experiment with the enzyme of pumpkin seeds, however, the formation of geranylgeranyl-PP was too small as compared with the formation of farnesyl-PP. to be detected when either Mg^{2+} or Mn^{2+} was used.

TABLE 3. EFFECT OF LIGHT ON THE FARNESYL-PP AND GERANYLGERANYL-PP SYNTHETASE ACTIVITIES

Period (hr)	Incorporation of isopentenyl-[1-^{14}C]-PP into				
	Farnesyl-PP		Geranylgeranyl-PP		
	Exp. 1		Exp. 1		Exp. 2*
	In light (dpm)	In dark (dpm)	In light (dpm)	In dark (dpm)	In light (dpm)
0	1152	—	2618	—	3050
3	1212	1329	2295	1739	3176
6	1201	1093	3494	2376	3298
12	1249	998	3047	2624	2815

* The data do not correspond to the period indicated on the left column, but correspond to 0, 2, 5 and 10 hr, respectively.

Green and Baisted[5] have examined the variation in farnesyl-PP synthetase activity during pea-seed germination in relatively short-term experiments, and suggested that farnesyl-PP synthetase is the rate-limiting enzyme in squalene synthesis from mevalonate.

Our finding that the developmental changes in farnesyl-PP synthetase and geranylgeranyl-PP synthetase activities are not parallel supports our earlier proposal that chain elongations up to C_{15} and C_{20} are independent and that the synthesis of geranylgeranyl-PP

[4] SKILLETER, D. N. and KEKWICK, R. G. O. (1970) *Phytochemistry* **9**, 153.
[5] GREEN, T. R. and BAISTED, D. J. (1972) *Biochem. J.* **130**, 983.

from C_5 does not require the supplement with farnesyl-PP provided by farnesyl-PP synthetase. These results might also reflect the requirement for dormancy and growth regulated by abscisic acid and gibberellic acid. The present study suggests also that 3 to 7-day-old pumpkin cotyledons are a better source than pumpkin fruit for the preparation of geranylgeranyl-PP synthetase free of farnesyl-PP synthetase.

EXPERIMENTAL

Materials. Isopentenyl-[1-^{14}C]-PP (1·2 μCi/μmol), geranyl-PP and farnesyl-PP were the same preparations used in the previous study.[1]

Enzyme preparation. Pumpkin (*Cucurbita pepo*) seeds were soaked for 48 hr in H_2O and planted in vermiculite. The seeds were grown in the dark with regular watering at 25–27°. The cotyledons (20 g) were homogenized for 1 min in 0·05 M Tris–HCl buffer (50 ml), pH 7, in a chilled Waring Blender. The homogenate was squeezed through four layers of cheesecloth and centrifuged at 105 000 *g* for 2 hr. The fraction precipitating between 35 and 55% saturation with $(NH_4)_2SO_4$ was dissolved in minimum vol. of 0·05 M Tris–HCl buffer, pH 7, and this soln was immediately used for the assay. Enzyme preparation was carried out at 4°. Protein concn were determined by measurement of absorbance at 280 and 260 nm.

Assay of prenyltransferase. The amounts of isopentenyl-[1-^{14}C]-PP incorporated into farnesyl-PP and geranylgeranyl-PP were measured in a manner similar to those described previously.[1,6] The incubation mixture for geranylgeranyl-PP synthetase contained 25 nmol isopentenyl-[1-^{14}C]-PP (1·2 μCi/μmol), 25 nmol farnesyl-PP, 5 μmol $MnCl_2$, 50 μmol Tris–HCl buffer, pH 7, 50 μmol KF, 10 μmol iodoacetamide and the enzyme soln (0·1 ml), in a final vol. of 1 ml. After 5 min preincubation in the absence of substrates to inhibit phosphatase and isopentenyl pyrophosphate isomerase, farnesyl-PP and isopentenyl-PP were added, and the incubation was continued for 60 min at 37°. The reaction was stopped by heating the incubate for 5 min at 100°, and nonpolar fractions were removed by extraction with 4 ml of Et_2O. The aq. phase was acidified with 0·1 ml 6 N HCl and heated at 53–55° for 10 min. The mixture was then made alkaline with 0·15 ml 6 N NaOH, and extracted with 4 ml of hexane. The hexane extracts were washed with H_2O and 2 ml aliquots of the extracts were counted for radioactivity in a toluene scintillator. For measurement of farnesyl-PP synthetase activity, the incubation mixture contained the same as that for the assay of geranylgeranyl-PP synthetase activity except that farnesyl-PP and $MnCl_2$ were substituted by geranyl-PP and $MgCl_2$, and the enzyme activity was similarly measured. The error in these assays was less than 5%.

GLC analysis. Carrier farnesol, nerolidol, geranylgeraniol and geranyllinalool were added to the petrol extracts of prenols liberated by the acid treatment of the enzymatic reaction product. The petrol soln was then conc to a small vol. and was separated by GC–RC. The separations were made with linear temp. gradient (4°/min) 130–200° on a 1 m column packed with 1·5% OV 17 on Chromosorb W, with He at 30 ml/min.

Light treatment. Eight-day-old etiolated seedlings were illuminated for specified periods with light of a 20 W Toshiba white fluorescent tube. The greenish cotyledons were homogenized immediately, and the enzyme activities were compared with those in control experiments with etiolated seedlings.

[6] OGURA, K., NISHINO, T. and SETO, S. (1968) *J. Biochem.* **64**, 197.