KAURENE SYNTHETASE FROM PLASTIDS OF DEVELOPING PLANT TISSUES[1]

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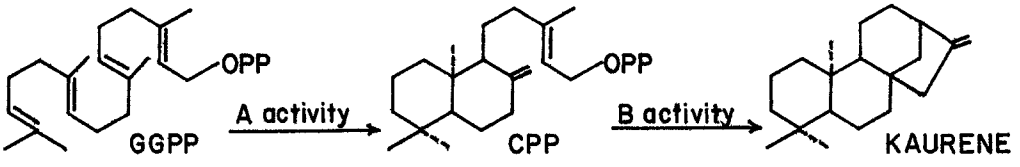
SUMMARY: Kaurene synthesis from copalyl pyrophosphate is present in the plastid fraction from all three sources of developing plant tissue studied. Isolated plastids from Echinocystis macrocarpa endosperm have kaurene synthetic capacity from either geranylgeranyl pyrophosphate or copalyl pyrophosphate. However, organelle preparations from etiolated pea shoot tips or developing castor bean endosperm have either no or a very limited ability to synthesize kaurene from geranylgeranyl pyrophosphate. These results suggest that the first committed step in gibberellin biosynthesis, geranylgeranyl pyrophosphate conversion to copalyl pyrophosphate, may be limited under certain stages of plant development.

Kaurene synthetase catalyzes the reactions of GGPP[4] to CPP, the "A" activity, and CPP to ent-kaurene, the "B" activity (1):



Kaurene synthesis is the first committed step in gibberellin biosynthesis (2,3) and therefore should be important in hormonal regulation of plant development. Recently, Green et al. (4) have demonstrated GGPP biosynthesis from IPP in the proplastid and mitochondrial fractions from developing castor bean endosperm. Kaurene synthetase has been characterized from higher plant and fungal sources, but only under conditions which would have released the enzyme from organelles.

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[4] Abbreviations: GGPP, all-trans-geranylgeranyl pyrophosphate; CPP, trans-copalyl pyrophosphate; IPP, isopentenyl pyrophosphate.

The present study was undertaken to determine the subcellular localization of kaurene synthetase in rapidly developing tissues.

MATERIALS AND METHODS:

Echinocystis macrocarpa seeds were obtained from fresh fruit picked in the Santa Monica Mountains in Southern California. Endosperm (50 ml) was diluted to 75 ml with 10 mM TES·KOH buffer (pH 7.5), 1 mM DTT, 1 mM $MgCl_2$ with a final sucrose concentration of 0.5 M. The chilled liquid endosperm was squeezed through one layer of miracloth and centrifuged at 500 g for 5 min. After centrifugation at 10,000 g for 15 min, the supernatant yielded a 10,000 g soluble fraction (S_{10}) and a particulate fraction (P_{10}). The P_{10} was resuspended in three ml of 10 mM TES·KOH buffer (pH 7.5), 1 mM DTT, 1 mM $MgCl_2$ and 0.5 M sucrose. Two and one half ml of this suspension were layered on a buffered, discontinuous sucrose gradient and centrifuged as described by Reid et al. (7). The gradients were collected dropwise from the bottom in 1.6 ml fractions.

Etiolated Progress #9 pea seedlings were prepared by growing seeds for six days at 30° C in the dark. The shoot tips (17.4 gm, 1-2 cm. in length) were homogenized in 35 ml of 0.05 M TES·TRIS buffer (pH 7.2), 1 mM DTT, 1 mM $MgCl_2$ and 0.5 M sucrose and the S_{10}, P_{10} and sucrose-gradient fractions were prepared as described for E. macrocarpa except the P_{10} was resuspended in two ml of extraction medium and the gradient was collected in 1.1 ml fractions. Fresh immature castor bean seeds were obtained from the UCLA botanical garden. Combined embryonic and endosperm tissues (6.32 gm) were homogenized in 12 ml of extraction medium (8). The S_{10}, P_{10} and sucrose-gradient proplastid and mitochondrial fractions were prepared according to Green et al. (4).

[2-^{14}C]-GGPP was synthesized according to Upper and West (9); [1-$^{3}H_2$]-CPP was prepared by the same general procedure from [1-$^{3}H_2$]-copalol (a generous gift from Dr. Robert Coates, Department of Chemistry, University of Illinois, Urbana). The assay mixture for total kaurene synthetase ("A" + "B") consisted of 3.2 nmoles [2-^{14}C]-GGPP (14,400 dpm), 5 µmoles $MgCl_2$, 10 µmoles KH_2PO_4, and 75 µmoles TRIS·HCO_3 buffer (pH 7.2) in a total volume of 1.0 ml. The assay mixture for kaurene synthetase -"B" consisted of 2.4 nmoles [1-$^{3}H_2$]-CPP (108,000 dpm), 2.5 µmoles $MgCl_2$, 5 µmoles KH_2PO_4 and 30 µmoles TRIS·HCO_3 buffer (pH 7.2) in a final volume of 0.5 ml. Kaurene was identified by co-chromatography with authentic kaurene on thin layer chromatograms (6). One unit of enzyme activity was defined as one pmole kaurene formed per minute at 30° C.

Triose phosphate isomerase assays were conducted as previously described (4). Cytochrome oxidase activity of freeze-thawed enzyme samples was assayed by measuring the decrease in absorbance at 550 nm from a one ml incubation mixture containing initially 20 nmoles cytochrome c, reduced by $NaHSO_3$, 100 µmoles KH_2PO_4 buffer (pH 7.2) and 10 µl of each gradient fraction.

RESULTS AND DISCUSSION:

Enzyme activities in the fractions of a discontinuous sucrose gradient prepared with the P_{10} fraction from E. macrocarpa endosperm are shown in Figure I. The distribution of cytochrome oxidase indicates that mitochondria are centered about fraction 16 with little mitochondrial contamination of other fractions. Triose phosphate isomerase activity serves as a marker for plastids; the plastids are in a zone centered in fraction 7, but there is con-

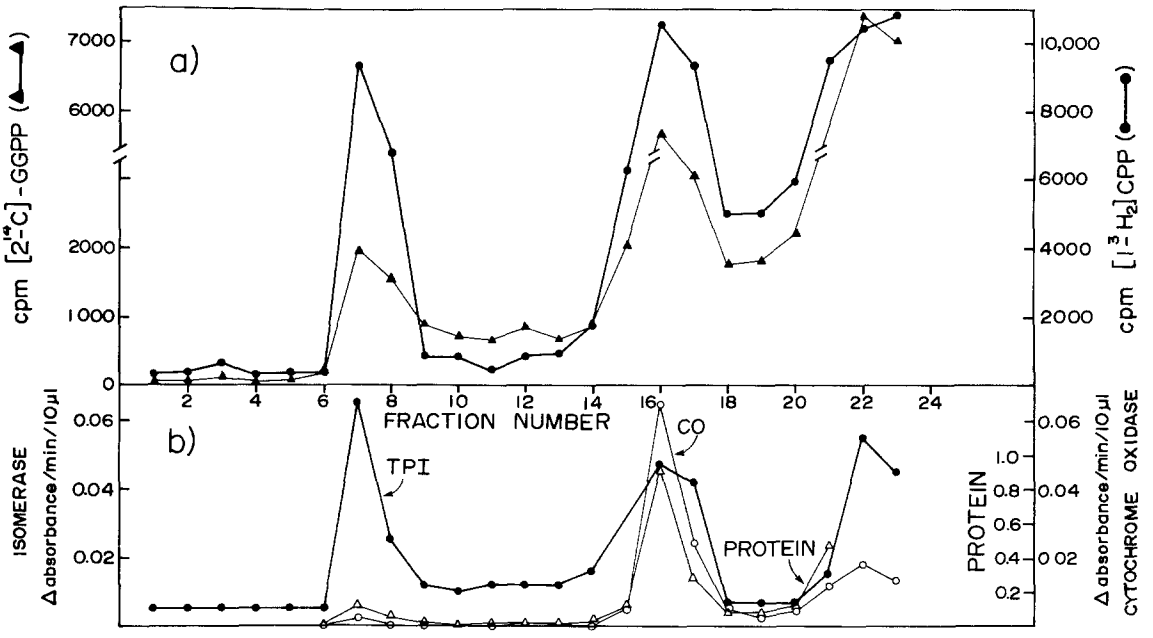

Figure I Sedimentation velocity separation of E. macrocarpa organelles on a discontinuous sucrose gradient. a) Benzene-extractable products from [2-^{14}C]-GGPP (▲-▲) and [1-$^{3}H_2$]-CPP (●—●). b) Marker enzymes and protein content: cytochrome oxidase (CO), triose phosphate isomerase (TPI), and 280 nm adsorbance of each fraction diluted 1:5 with water (Protein).

siderable contamination of the mitochondrial fraction with plastids. The top of the gradient (fraction 21-23) presumably contains enzymes released from broken organelles or present as contaminants from the S_{10} in the preparation of the P_{10} fraction. The plastid and mitochondrial fractions have the capacity to convert both GGPP and CPP to benzene-extractable products. In the plastid fraction kaurene represents 76% of these products formed from GGPP and 82% of these products from CPP. The corresponding figures for the mitochondrial fraction are 82% and 89% (Table I). The remaining extractable products are presumably alcohols released from the substrates by phosphatase action.

Table II indicates a close correlation in the distribution of triose phosphate isomerase activity and kaurene synthetase activity from both substrates. Thus it is concluded that the plastids are the primary site of the particulate kaurene synthetase activity. The particulate kaurene synthetase comprises a significant

TABLE I

Kaurene Synthetase in Sucrose Density Gradient Fractions

Enzyme Source		CPM Incorporated into Kaurene from [a,b]	
		[2-^{14}C]-GGPP	[1-$^{3}H_2$]-CPP
E. macrocarpa	plastid fraction (Tube No 7)	1189 cpm	3226 cpm
	mitochondrial fraction (Tube No 16)	2386 cpm	5333 cpm
Etiolated Pea Shoot Tips	plastid fraction	6 cpm	2024 cpm
	mitochondrial fraction	4 cpm	10 cpm

[a] Assay conditions are described in the text.

[b] Volume of each fraction assayed/incubation time:
E. macrocarpa plastid and mitochondrial -"A" (100 µl/60'), "B" (50 µl/30');
Pea plastid and mitochondrial -"A" (200 µl/60'), "B" (200 µl/60')

portion of the total kaurene synthetic activity from crude homogenates (Table III).

Identical gradients prepared with etiolated pea shoot-tip particulate material reveals marker enzyme profiles similar to those of E. macrocarpa. The kaurene synthetase "B" activity profile is also similar. Therefore, a major portion of the organelle-derived "B" activity from etiolated pea shoot tips is found in the plastids (Table II). However, "A" activity could not be detected in either the plastid or mitochondrial fractions (Table I). "A" activity is also absent in the crude homogenate, S_{10}, and P_{10} preparations (Table III).

Results similar to those seen with peas are found with the endosperm from developing castor bean seeds. The P_{10} and plastid fractions efficiently convert CPP to kaurene; however, the P_{10} has only a limited capacity for the synthesis of kaurene from GGPP as shown in Table III.

The kaurene synthetase distribution between the soluble (S_{10}) and particulate (P_{10}) fractions from the three developing tissues is presented in Table III. The estimation of enzyme activity in the S_{10} is probably low at

TABLE II

Enzyme Distribution Between Plastid and Mitochondrial Fractions

	Total Units of Activity			
	Kaurene Synthesis from		Triose Phosphate Isomerase	Cytochrome Oxidase
	[2-^{14}C]-GGPP pmoles/min	[1-$^{3}H_2$]-CPP pmoles/min	µmoles/min	µmoles/min
. macrocarpa endosperm				
plastid fraction	84.7	322	2.28	0.0113
mitochondrial fraction	170	531	3.11	0.502
lastid/mitochondrial ratio)	(0.50)	(0.61)	(0.73)	(0.023)
:iolated Pea Shoot Tips				
plastid fraction	0	17.5	5.30	0.156
mitochondrial fraction	0	0.086	1.52	13.6
.astid/mitochondrial ratio)	-	(203†)	(3.5)	(0.011)

Other experiments indicate a more usual ratio is 4.0.

TABLE III

Subcellular Distribution of Kaurene Synthetase

		Total Units of Activity (pmoles/min) (% of Total Activity)			
		Kaurene Synthesis from			
		[2-^{14}C]-GGPP		[1-$^{3}H_2$]-CPP	
E. macrocarpa endosperm	soluble (S_{10})	1.0	(80%)	6.0	(81%)
	particulate (P_{10})	0.25	(20%)	1.4	(19%)
Etiolated Pea Shoot Tips	soluble (S_{10})	0		0.080	(73%)
	particulate (P_{10})	0		0.029	(27%)
Developing Castor Bean	soluble (S_{10})	0.0080†		0.056	(20%)
	particulate (P_{10})	0.0046†		0.23	(80%)

† These figures correspond to 100 cpm kaurene or less and we feel that these values are not reliable enough to predict the levels of enzyme activity present.

the high protein levels (1-2 mg) required for the conversion of GGPP or CPP to kaurene because there is a slight inhibition of the rate, probably due to non-specific protein adsorption of the detergent-like substrates (13). The exact distribution of cytosolic- and organelle-derived enzyme is impossible to determine because of organelle damage during tissue manipulation. However, the data suggest that a significant portion of the kaurene synthetic activities is in the plastids from the particulate fraction.

A number of observations in recent years have pointed to the chloroplast as one site for at least a portion of the gibberellin biosynthetic pathway (10-12). The data presented here provide the initial evidence that a key enzyme in gibberellin biosynthesis is found in a fraction which contains only plastids. This, along with evidence that MVA kinase (14) and isopentenyl pyrophosphate isomerase and prenyl transferase activities leading to GGPP biosynthesis (4) are found in plastid fractions, supports the concept of one site of localization of kaurene biosynthesis in plastids. However, since the tissues examined are not photosynthetic, it is not clear what relationship these results have to kaurene synthesis in the chloroplasts of mature leaf tissue from the same sources.

The explanation at the molecular level for the lack of "A" activity in two of these developing systems at a time when the "B" activity is readily detected is uncertain at the present time. But it suggests that the conversion of GGPP to CPP may be a critical point in the control of the biosynthesis of gibberellins in these and perhaps other systems. Presently it is impossible to determine at what developmental stages kaurene synthesis from MVA is expressed in the plastids.

Knowledge of the organelle distribution of gibberellin biosynthetic activities should contribute to the understanding of the important questions of the mode of hormone action and its relation to plant development.

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