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LOCALIZATION OF ENZYMES CATALYSING KAURENE BIOSYNTHESIS IN IMMATURE PEA SEEDS*

RONALD C. COOLBAUGH and THOMAS C. MOORE

Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon 97331, U.S.A.

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Abstract-Comparative assays of (-)-kaurene accumulation in cell-free extracts of whole pea (*Pisum sativum* L. cv. Alaska) seeds, isolated seed coats, cotyledons, and embryonic shoot-root axes revealed that the enzymes catalysing the synthesis of (-)-kaurene from mevalonic acid-2-^{14}C are apparently localized exclusively in the cotyledons. Differential centrifugation of extracts showed that the enzymes catalysing (-)-kaurene biosynthesis occur in the soluble fraction. The enzymes responsible for squalene (identification tentative) biosynthesis were present in both the seed coat and the cotyledon extracts.

INTRODUCTION

SEEDS of many species are known to accumulate much greater quantities of gibberellins (GA's) during certain stages of development than commonly are found in the vegetative parts of older plants.[1] However, the evidence for the localization of GA's in the various parts of developing leguminous seeds, as obtained by extraction and bioassay procedures, is somewhat contradictory.[2,3]

West *et al.* have investigated extensively many aspects of the pathway of GA biosynthesis in extracts of *Echinocystis macrocarpa* seeds[4] and have shown that the enzymes responsible for kaurene biosynthesis are localized in the soluble fraction of the liquid endosperm of those seeds.[5,6] Dennis and West' further demonstrated that the enzymes responsible for the oxidation of kaurene occur in the microsomal fraction of the liquid endosperm of *Echinocystis macrocarpa* seeds. Graebe[8] reported that the enzymes responsible for kaurene biosynthesis also are contained in the soluble fraction of the liquid endosperm of immature *Cucurbita pepo* seeds. These data support the extraction data[3] which indicated that GA synthesis in *Echinocystis macrocarpa* seeds may be localized in the liquid endosperm. Anderson and Moore[9], Graebe[10] and Coolbaugh and Moore[11] reported on the incorpora-

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tion of mevalonic acid into kaurene in extracts of immature pea (*Pisum sativum* L.) seeds and fruits, but did not investigate in detail localization of the enzymes.

In view of the conflicting data on extractable GA-like materials in immature leguminous seeds, the absence of evidence for the localization of enzymes catalysing kaurene biosynthesis in these seeds, and the anatomical differences between leguminous and *Echinocystis macrocarpa* seeds during certain stages of development, these investigations were conducted to determine the distribution of enzymes catalysing the synthesis of kaurene in isolated parts of pea seeds.

RESULTS AND DISCUSSION

Localization of Enzyme Activities for Kaurene and Presumptive Squalene Biosynthesis in Cell-free Extracts of Different Parts of Immature Pea Seeds

Enzyme preparations from whole seeds, isolated cotyledons, seed coats and embryo axes (all from one randomized group of freshly harvested pea seeds) were assayed for kaurene biosynthesis by methods described in Fig. 1 and in the Experimental section. Most if not all of the kaurene synthesis observed in extracts of these seeds occurred in extracts of isolated cotyledons, with little or none occurring in the seed coat or in the embryo axis homogenates (Fig. 1).

More kaurene accumulated in the extracts of isolated cotyledons than in those of whole seeds. By direct testing of seed coats and on the basis of results presented in a subsequent paper,[12] it is concluded that oxidation of kaurene by enzymes originally present in seed coats or embryo axes did not contribute to this difference. Rather, the authors believe this result to be mainly due to competition among enzymes in two or more biosynthetic pathways for common substrates and to disruption of physiological controls when the natural products and enzymes from one seed part are combined with the components of another seed part. Squalene biosynthesis is a pathway which competes for substrates in these reactions. The radioactive product which migrates to R_f 0·35 on the chromatograms developed in hexane (Fig. 1) has been tentatively identified as squalene[13] and is by far the most abundant product of these reactions. This product was formed in extracts of both seed coats and cotyledons, and it accumulated more in extracts of isolated seed coats than in extracts of whole seeds (Fig. 1).

Although the data for the embryo axis homogenates are not presented in Fig. 1, the dpm/mg protein in the kaurene region of these chromatograms were only 38, as compared to the 2300 in the whole seed extracts. This is considered to be insignificant incorporation since, in a second experiment, the amount of radioactive material behaving chromatographically like kaurene was even less in spite of the fact that the enzyme extract was concentrated by grinding the embryo axis tissues in a smaller volume of buffer. Thus, the failure to observe mevalonic acid-2-^{14}C metabolism in cell-free extracts of embryo axes may have been due to an inherent incapability of these preparations to metabolize mevalonic acid or to the low protein content of these extracts. Since more than 100 per cent of the radioactivity incorporated into kaurene and squalene in extracts from whole seed preparations was observed in the extracts from isolated cotyledons and seed coats, respectively, it seems highly unlikely that the enzymes present in the embryo axis tissues contributed significantly to the metabolism of mevalonic acid in the whole seed extracts.

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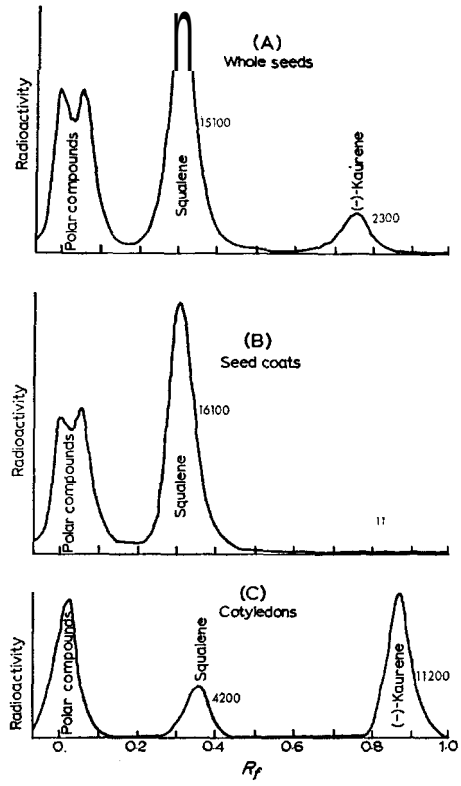

FIG. 1. TRACINGS OF STRIP CHART SCANS OF RADIOCHROMATOGRAMS OF PRODUCTS OF MEVALONIC ACID-2-^{14}C METABOLISM IN EXTRACTS OF DIFFERENT PARTS OF IMMATURE PEA SEEDS.

Pea seeds of approximately half-maximum fresh weight were used to prepare enzyme extracts from: (A) whole seeds; (B) isolated seed coats; (C) isolated cotyledons; and isolated embryo axes. Each sample of tissues (40 whole seeds, 6·3 g; 40 seed coats, 3·7 g; 40 pairs of cotyledons, 1·2 g; and 40 embryo axes, 25 mg) was rinsed twice with buffer, homogenized in 6·4 ml of potassium phosphate buffer and centrifuged at 40,000 g for 15 min. The resultant supernatants were used as the enzyme extracts and were incubated in complete reaction mixtures (see Experimental section) for 75 min at 30". Analyses were by TLC and liquid scintillation counting. The numbers beside the peaks represent the actual dpm/mg protein present in those areas of the chromatograms.

The results on localization of the enzymes responsible for kaurene biosynthesis in different parts of pea seeds differ rather strikingly from comparable data for *Echinocystis macrocarpa* seeds. In wild cucumber seeds, kaurene is formed in the liquid endosperm, while squalene synthesis appears to be exclusively localized in the embryonic tissues.[5] In pea seeds, kaurene biosynthesis is localized in the cotyledons, whereas the synthesis of squalene occurs both in the seed coat and in the cotyledon extracts. These differences become even more intriguing when it is realized that the early morphological development of pea seeds also includes the formation of liquid endosperm. As described by Hayward,[14] the pea embryo occupies a small part of the embryo sac during early embryogenesis, the remainder being filled with liquid (free-nuclear) endosperm. By the time the seed is 11-12 days old,

[14] H. E. HAYWARD, *The Structure of Economic Plants,* MacMillan, New York (1948).

the endosperm is entirely digested, and the seed consists of the embryo and the seed coat. The pea seeds utilized in these investigations were 12-16 days of age.

In extracts of *Echinocystis macrocarpa* the highest incorporation of mevalonic acid into the lipid fraction occurred in seeds when the cotyledons were not yet visible and the seed cavity was mostly filled with endosperm, and the activity declined as the seeds matured and the cotyledons formed.[5] Cell-free extracts of pea seeds exhibit a relatively low capacity for kaurene biosynthesis during early morphological development when liquid endosperm is present. In fact, no evidence was obtained in these investigations for kaurene biosynthesis in the liquid endosperm of pea seeds. The activity in pea seeds increases to a maximum when the seeds are approximately half-maximum fresh weight and the cotyledons nearly fill the seed cavity and then decreases as the seeds mature.[11] Thus, so far as can be determined on the basis of collective studies to date, GA biosynthesis in developing seeds of *Pisum sativum* L. and *Echinocystis macrocarpa* Greene occurs in anatomically quite dissimilar parts of the seeds, but parts which, in both kinds of seeds, are most commonly associated with a nutritive function.

Of course the incorporation of mevalonic acid into kaurene or kaurenoic acid in cell-free enzyme extracts does not necessarily mean that these intermediates are converted to GA's in these tissues. However, it is of interest to note that the data on extractable GA content in legume and *Echinocystis macrocarpa* seeds[3] correlate with the results of Graebe *et al.*[5] and those of the present paper concerning localization of kaurene synthesizing enzymes.

Determination of the Soluble Versus Particulate State of the Enzymes Catalysing Kaurene Biosynthesis

The data from four independent experiments on the differential centrifugation of enzyme extracts (Table 1) indicated that in extracts of whole seeds the activity for kaurene biosynthesis increased with increasing centrifugal force used to prepare the enzymes up to 40,000 *g*

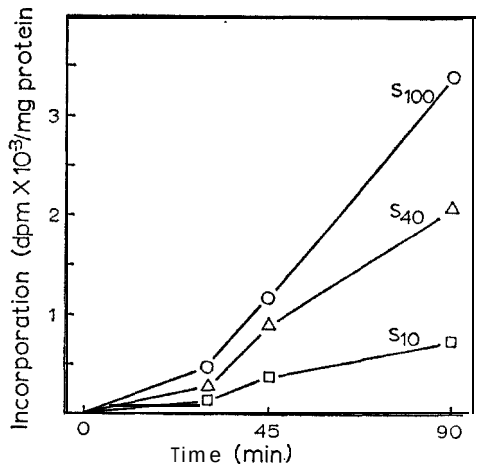

FIG. 2. TIME COURSES OF INCORPORATION OF MEVALONIC ACID-2-^{14}C INTO KAURENE BY SUPERNATANT FRACTIONS OF COTYLEDON EXTRACTS.

Crude homogenate of isolated cotyledons was successively centrifuged at 10,000 *g* for 15 min, 40,000 *g* for 15 min and 100,000 *g* for 60 min, and portions of the resultant supernatant fractions (S_{10}, S_{40}, S_{100}, respectively) were assayed for kaurene biosynthesis using 0·75 ml of each enzyme extract for the indicated times.

and then decreased in the 100,000 *g* supernatant. In extracts of isolated cotyledons this activity continued to increase even in the 100,000 g supernatant (Table 1). The latter result was found to be consistent throughout a 90-min incubation (Fig. 2).

TABLE 1. COMPARATIVE ACCUMULATION OF KAURENE IN DIFFERENTIALLY CENTRIFUGED ENZYME EXTRACTS FROM WHOLE IMMATURE PEA SEEDS AND EXCISED COTYLEDONS

Fraction	DPM incorporated/mg protein			
	Whole seed extract		Isolated cotyledon extract	
	Experiment 1	Experiment 2	Experiment 3	Experiment 4
S_{10}	150	1150	700	250
S_{40}	650	4650	2050	850
S_{100}	650	2850	3200	1250

Crude homogenates of whole seeds or isolated cotyledons were centrifuged at 10,008 *g* for 15 min and the supematants (S_{10}) separated into two portions; one was held in an ice bath while the other was centrifuged at 40,080 g for 15 min. Half of the 40,008 g supematant (S_{40}) was held in an ice bath while the other half was centrifuged at 100,000 g for 60 min. Reaction mixtures were prepared in duplicate and incubated for 75 min at 30°, except for those in Experiment 4, which were incubated for 90 min. Radioactivity was measured by liquid scintillation counting. Data are means of duplicate determinations rounded to the nearest 50 dpm.

Greater accumulation of kaurene with increasing centrifugal force, as observed with the cotyledon extracts, indicates that the enzymes catalysing kaurene biosynthesis are soluble. This result has been reported previously by Upper and West[6] for kaurene synthase activity from *Echinocystis macrocarpa*. The increasing activity in the present study is considered to be due to the combined effects of particulate substances competing for substrate or intermediates with the kaurene-synthesizing enzymes and particulate substances contributing to the disappearance of kaurene after it is formed.

Decreased kaurene biosynthesis in the 100,000 *g* supernatant of extracts from whole seeds is unexpected but highly reproducible. Whatever the reason for this result, it has been shown the enzyme extracts prepared from isolated cotyledons are responsible for most if not all of the kaurene synthesis observed in extracts of whole pea seeds and that the enzymes for the incorporation of mevalonic acid into kaurene in these extracts are present in the 100,000 g supernatant.

EXPERIMENTAL

Source *and purity of reagents.* Mevalonic acid-2-^{14}C lactone (sp. act. 5·86 mc/m-mole) in benzene solution was purchased from Amersham/Searle Corp., Des Plaines, Illinois. The benzene was removed under N_2 and the lactone was hydrolysed by treating overnight with 100% excess NaOH, after which the mevalonic acid was diluted with H_2O to a concentration of approximately 0·01 μc/μl. Adenosine triphosphate (ATP) was purchased from Sigma Chemical Company, St. Louis, Missouri. Constituents of the liquid scintillation counting solutions, 2,5-diphenyloxazole (PPO), *p*-bis-2′-(5′phenyloxazolyl)-benzene (POPOP) and triphenyl-p-terphenyl were purchased from Packard Corporation. Toluene-^{14}C was purchased from Packard Corporation, Palo Alto, California. Silica gel G was purchased from Brinkmann Instruments Company, Westbury, New Jersey. All other chemicals were reagent grade, and all organic solvents were redistilled before use.

Culture of plants. 'Alaska' peas (*Pisum sativum* L.; W. Atlee Burpee Company, Riverside, California) were grown in a greenhouse where the light and temperature regime consisted of a 16-hr photoperiod, at approximately 20–27° and a light intensity of 8600–10,760 lx, and an 8-hr nyctoperiod at approximately 16–18°. The natural light intensity was supplemented with and the photoperiod extended by Gro-Lux fluorescent lamps. Seeds were planted in water-saturated vermiculite in plastic pots. The plants were irrigated alternately with water and complete nutrient solution throughout the periods of culture. After 8-10 days, the seedlings in each pot were thinned to leave 10 quite uniform plants. The fruits were harvested and the seeds taken from the pods between the 12th and the 16th day after flowering.

Preparation of enzyme extracts. In experiments on the localization of enzymes, pea seeds of approximately half-maximum fresh weight were used. Each sample of whole seeds or excised seed parts was rinsed twice with buffer. homogenized in K_2HPO_4–KH_2PO_4 buffer (0·1 M; pH 7·1) containing 50 μg/ml each of streptomycin sulfate and-penicillin G, in a chilled mortar and pestle, and the homogenates were centrifuged for 15 min at 40,000 ***g***. The resultant supernatants were used as enzyme extracts. In experiments on the differential centrifugation of enzymes, crude homogenates were prepared as described above for whole seeds or isolated cotyledons and initially centrifuged 15 min at 10,000 ***g***. All centrifugations were done at 0–4° in a Sorvall RC2-B refrigerated centrifuge, with the exception of the 100,000 ***g*** centrifugations which were done in a Spinco Model L refrigerated preparative ultracentrifuge, with a No. 40 angle head rotor.

Reaction conditions andproduct isolation. Acomplete reaction mixture routinely contained 0·09 μmole of mevalonic acid-2-^{14}C. O-75 ml of enzyme extract. 0·5 μmoles of ATP. 3 μmoles of $MnCl_2$, and 0·6 mlof 0·1 M K phosphate buffer at pH 7·1 in a total of 1·6 ml. Duplicate reaction mixtures were'incubated for 75 min at 30". The reactions were stopped by adding 3 ml of acetone, and the reaction mixtures were extracted twice with l-ml portions of benzene. The combined organic extracts were then evaporated under reduced pressure. Each residue was extracted 3 x with 0·2 ml of acetone, and the entire 0·6 ml of extract was applied to a 5 x 20 cm glass plate coated with a 250 μ layer of silica gel G. TLC chromatograms were developed routinely in hexane. After the solvent front had advanced 15 cm from the origin, the plates were removed from the solvent and scanned to locate the radioactive products.

Radioassay procedures. TLC chromatograms were scanned to detect radioactive compounds with a Packard Radiochromatogram Scanner, Model 7201. The appropriate bands of silica gel were removed from the chromatograms and placed in liquid scintillation vials each of which contained 10 ml of counting solution. The liquid scintillation fluid routinely used contained 0·3 ma of *p*-bis-2′-(5′-phenyloxazolyl)-benzene and 30 mg of triphenyl-p-terphenyl per 10 ml of toluene. The radioactivity measurements were made with a Packard Tricarb Liquid Scintillation Spectrometer, Model 3375. The liquid scintillation data are expressed in disintegrations per minute (dpm); the counting efficiency was approximately 85 %.

Protein determinations. Protein determinations were made on portions of each enzyme preparation by a method of Lowry *et al.*[15] using bovine serum albumin as a standard.

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