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Distribution of a Fatty Acid Cyclase Enzyme System in Plants[1]

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ABSTRACT

Extracts from tissues of 24 plant species were tested for the enzyme that catalyzes the conversion of 13-L-hydroperoxy-*cis*-9,15-*trans*-11-octadecatrienoic acid to the cyclic fatty acid 12-oxo-*cis*-10,15-phytodienoic acid. The enzyme was detected in 15 of the 24 tissues examined, and was demonstrated in seedlings, leaves, and fruits.

Recently, Zimmerman and Feng (10) reported the synthesis of a cyclic, prostaglandin-like fatty acid from (9,12,15)-linolenic acid, catalyzed by an enzyme in flaxseed. The compound, 8-[2-(*cis*-pent-2'-enyl)-3-oxo-*cis*-cyclopent-4-enyl]octanoic acid, is analogous to the prostaglandins of the A-type, and the common name 12-oxo-*cis*-10,15-phytodienoic acid was proposed to avoid the cumbersome systematic nomenclature. The structure of the cyclic fatty acid is shown in Figure 1. In another report we showed (9) that other polyunsaturated fatty acids in addition to (9,12,15)-linolenic acid could be utilized as substrates in the synthesis of cyclic fatty acids. Fatty acids with 18, 20, or 22 carbons could be enzymically converted to cyclic compounds provided they were unsaturated at the *n*-3,6,9 positions. We further showed that an *n*-6 hydroperoxide of the fatty acid was an intermediate in the biosynthetic pathway (Fig. 1). The hydroperoxide is also an intermediate in the synthesis of another fatty acid, 12-oxo-13-hydroxy-*cis*-9,15-octadecadienoic acid, catalyzed by a hydroperoxide isomerase enzyme (11). Here, we report the presence of the enzyme that catalyzes the synthesis of a cyclic fatty acid from the 13-hydroperoxide of (9,12,15)-linolenic acid in a wide variety of plant sources.

MATERIALS AND METHODS

Chemicals. Linolenic acid and soybean lipoxygenase (21,000 units/mg) were purchased from Sigma Chemical Company.[2] 9-Hydroxy-hexadecanoic acid, obtained from Serdary Research Laboratories, Inc., London, Ontario, was converted to 9-oxo-hexadecanoic acid by treatment with Jones' reagent (chromium trioxide in dilute H_2SO_4) (1). Platinum oxide (Adam's catalyst) was purchased from Matheson, Coleman, and Bell, Norwood, Ohio, and the silicone phase DC LSX-3-0295 for GC from Applied Science Laboratories, Inc., State College, Pa.

Plant Materials. Seeds of the following plants were germinated in moist paper toweling in the dark for 6 days at 27 C, except for muskmelon seeds, which were grown for 7 days: alfalfa, *Medicago sativa* L., var. Travois; barley, *Hordeum vulgare* L., var. Dickson; beets, *Beta vulgaris* L., var. Ruby Queen; corn, *Zea mays* L., var. NK199 (Northrup King); cucumber, *Cucumis sativus* L., var. Straight Eight; flax, *Linum usitatissimum* L., var. Summit; lettuce, *Lactuca sativa* L., var. Grand Rapids; mung bean, *Vigna radiata* (L.) Wilczek var. radiata; muskmelon, *Cucumis melo* L., var. Iroquois; oat, *Avena sativa* L.; pea, *Pisum sativum* L., var. Alaska; pole bean, *Phaseolus vulgaris* L., var. Kentucky Wonder; radish, *Raphanus sativus* L., var. Early Scarlet Globe (red variety) and var. White Icicle (white variety); soybean, *Glycine max* (L.) Merr., var. Merit; sunflower, *Helianthus annuus* L., var. Sundak; watermelon, *Citrullus lanatus* (Thunb.) Matsum. & Nakai, var. Charleston Gray; and wheat, *Triticum aestivum* L., var. Thatcher. Apple, *Malus sylvestris* Mill., var. Red Delicious, and leaves of New Zealand spinach, *Tetragonia tetragonioides* (Pall.) Ktze., were obtained at a local produce market. Fresh eggplant fruit, *Solanum melongena* L., and potato tubers, *Solanum tuberosum* L., were obtained from a local garden the day of the experiment. Needles of Colorado spruce, *Picea pungens* Engelm., and leaves of staghorn sumac, *Rhus typhina* L., were picked from trees near the university just prior to the experiment.

Preparation of Enzyme Solutions. For seedlings, the intact plants without seed coats were used. Potato tubers and the fleshy portion of the fruits of apple and eggplant (seeds removed) were diced prior to enzyme extraction. Each plant tissue was frozen with liquid N_2 in a mortar and ground with a pestle. When the tissue thawed, the extraction medium containing 1.5% Triton X-100 in 0.05 M K-phosphate (pH 7.0) was added. One ml of buffer was added for each gram of plant tissue. The tissue was ground in the extraction medium, filtered through four layers of cheesecloth, centrifuged at 12,000*g* for 10 min, and the supernatant used as the enzyme source.

Enzyme Assay. The substrate for the fatty acid cyclase enzyme. 13-L-hydroperoxy-*cis*-9,15-*trans*-11-octadecatrienoic acid, was prepared *in situ* for each assay by reacting 0.74 ml of an 8 mM (9,12,15)-linolenic acid-Tween 20 substrate solution (7) with 2 mg of soybean lipoxygenase in 23.5 ml of 0.8 mM borate buffer (pH 9.0). After 45 min the mixture was adjusted to pH 7.2 by the addition of 5.9 ml of 0.2 M K-phosphate (pH 7.0) containing 600 μmol of 9-oxo-hexadecanoic acid as an internal standard. The enzyme reaction was initiated by the addition of 0.8 to 4.0 ml of the plant extract and stopped after intervals ranging from 0.5 to 15 min by addition of 7.5-ml portions of the reaction mixture to 10 ml of chloroform-methanol solvent (2:1, v/v). Then the pH was adjusted to 3.9 with 1 M citric acid and the products partitioned into the chloroform phase with gentle stirring under N_2. After 10 min an additional 10 ml of chloroform was added and the extraction continued for 30 min. The chloroform phase was separated and evaporated under reduced pressure. The fatty acid products were dissolved in ethyl ether, converted to methyl esters with diazomethane, and hydrogenated by bubbling H_2 through 2 ml of a methanolic solution of the sample for 15 min with 1 mg of platinum oxide as catalyst. The hydrogenation step allowed the

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[2] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

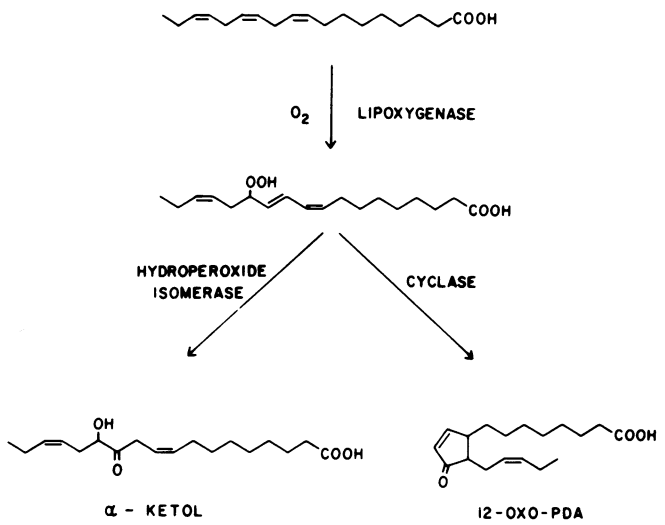

FIG. 1. Reactions catalyzed by lipoxygenase, hydroperoxide isomerase, and the fatty acid cyclase enzyme.

separation of the cyclic fatty acids from the α-ketol product of hydroperoxide isomerase. The products were separated by GC with a glass column (2 m × 2 mm, i.d.) containing DC LSX-3-0295 on 100/120 mesh Gas-chrom Q. The column was temperature programmed from 170 to 220 C at 2 C/min with a helium flow rate of 20 ml/min. The cyclic fatty acid in the gas chromatogram was identified in each case by characteristic mass fragments in its mass spectrum, which was obtained in a separate analysis by GC-MS. Characteristic fragments were m/e 83, 153, 240, and 279 (10). The quantities of cyclic fatty acid and α-ketol at each time interval were calculated from the concentration of the internal standard and plotted *versus* time. The reaction rate was determined from a graphical plot of concentration *versus* time.

Mass Spectrometry. Mass spectra were obtained with a Hewlett-Packard 5992A GC-MS system with the column prepared and programmed as described above. The mass spectrometer was operated at 70 ev.

RESULTS AND DISCUSSION

Each of the plant tissues listed under "Materials and Methods" was measured for its capacity to synthesize 12-oxo-PDA[3] and α-ketol. The results of the rate studies are shown in Table I. Of the 24 plant tissues examined, 15 were able to synthesize 12-oxo-PDA from 13-hydroperoxy linolenic acid at a significant rate. The rates are expressed as μmol of 12-oxo-PDA synthesized/min·g fresh weight. In many of the rate studies, two cyclic fatty acid components with identical mass spectra were present in the gas chromatogram. These two compounds have been shown to be diastereomers (unpublished data) and were added together to compute the total quantity of cyclic fatty acid synthesized. It should be pointed out that no attempt was made to maximize enzyme activity with seedling development for any of the seedlings studied. All of the seedlings were examined for enzyme activity at 6 to 7 days after planting, so it is likely that some of the plants investigated may have had higher activities at other stages of development. Similarly, no attempt was made to determine the optimum pH for synthesis of 12-oxo-PDA or α-ketol for each of the tissues examined. All of the enzyme assays were conducted at pH 7.2. When those extracts which catalyzed cyclic fatty acid synthesis were heated at 100 C for 5 min prior to the reaction, there was no synthesis of 12-oxo-PDA or α-ketol, demonstrating the enzymic nature of the reactions.

[3] Abbreviations: 12-oxo-PDA: 12-oxo-*cis*-10,15-phytodienoic acid; α-ketol: 12-oxo-13-hydroxy-*cis*-9,15-octadecadienoic acid.

Table I. *Survey of Plants for Cyclic Fatty Acid and α-Ketol Synthesis*

The reaction conditions were described under "Materials and Methods." Seedlings were grown in the dark at 27 C and were harvested 6 to 7 days after planting.

	Fatty Acid Cyclase Rate	Total 12-Oxo-PDA Synthesized[1]	Hydroperoxide Isomerase Rate	Total α-Ketol Synthesized[1]
	μmol/min·g fresh weight	*μmol*	*μmol/min·g fresh weight*	*μmol*
Alfalfa seedling	0	0	0	0
Apple fruit	0	0	0	0
Barley seedling	0.85	0.72	2.0	1.2
Bean seedling	0	0	0	0
Beet seedling	0.055	0.15	0.039	0.1
Colorado spruce needles	t[2]	t[2]	t[2]	t[2]
Corn seedling	0.77	0.68	2.9	1.6
Cucumber seedling	0.024	0.44	0.024	0.40
Eggplant fruit	0.48	0.33	0.48	0.41
Flax seedling	1.9	1.2	2.6	2.7
Lettuce seedling	1.9	0.81	2.7	1.5
Mung bean seedling	0.027	0.68	0.01	0.18
Muskmelon seedling	0.013	0.48	0.017	0.64
Oat seedling	0.43	0.68	0.60	1.1
Pea seedling	t[2]	t[2]	t[2]	t[2]
Potato tuber	0.63	0.32	0.63	0.72
Radish seedling, red	0	0	0	0
Radish seedling, white	0.065	0.13	0.056	0.20
Soybean seedling	0	0	0	0
Spinach leaves	2.0	0.28	2.6	0.42
Staghorn sumac leaves	t[2]	t[2]	t[2]	t[2]
Sunflower seedling	2.5	1.0	9.1	2.9
Watermelon seedling	0	0	0	0
Wheat seedling	1.4	0.71	1.6	1.1

[1] Measurements were made after the reaction had been completed, usually 1 to 5 min after the addition of enzyme. The reaction volume was 30 ml.

[2] t = trace; 12-oxo-PDA was not apparent in the gas chromatogram, but could be measured by selected ion monitoring of the mass fragment m/e 83 with GC-MS.

The cyclase enzyme and hydroperoxide isomerase were present in a wide range of plant tissues. The two enzymes were found in both monocotyledons and dicotyledons and in the seedlings of lipid-storing as well as carbohydrate-storing seeds. They were also present in potato tubers, in eggplant fruit (but not apple fruit), and in spinach leaves, but were only barely detectable in leaves of staghorn sumac or spruce needles. The highest activities for the two enzymes were observed in sunflower seedlings. This tissue was able to synthesize 12-oxo-PDA at a rate of 2.5 μmol/min·g fresh weight and α-ketol at a rate of 9.1 μmol/min·g fresh weight.

The enzyme rate data of Table I can be misleading, however. For example, the initial rate of the cyclase was approximately the same for flax seedlings (1.9 μmol/min·g fresh weight) and for spinach leaves (2.0 μmol/min·g fresh weight). Yet, at the completion of the reaction the total 12-oxo-PDA synthesized by the extract from flax seedlings was 1.2 μmol, whereas the total from the extract of spinach leaves was only 0.28 μmol. Endogenous materials in the enzyme preparation could have influenced the results. Most important, however, is that fatty acid cyclase can be demonstrated in a variety of plant tissues. Similar results were observed for the synthesis of the α-ketol.

The extracts of apple, cucumber, eggplant, mung bean, muskmelon, peas, white radish, spinach, and watermelon metabolized the linolenic acid hydroperoxide very rapidly, but the disappearance of the hydroperoxide could not be accounted for stoichiometrically by the increase in 12-oxo-PDA or α-ketol. It is likely that these tissues contained a hydroperoxide lyase enzyme that converted the hydroperoxide to 12-oxo-dodecenoic acid and hexenal (8). These products would have eluted near the solvent during GC and would not have been observed under our experimental conditions. Three of these plants, cucumber, muskmelon, and watermelon, have been shown previously to possess the hydroperoxide lyase enzyme (8). Examples of hydroperoxide-cleaving activity have also been shown in cucumber (3) and tomato fruit (2), and in leaves of *Phaseolus vulgaris* (6) and *Thea sinensis* (4). Hatanaka *et al.* (5) have recently demonstrated the synthesis of C_6

aldehydes from C_{18}-unsaturated fatty acids by 37 plant tissues. The hydroperoxide lyase enzyme was undoubtedly involved in those reactions.

The function of 12-oxo-PDA in plant metabolism has not yet been determined. These investigations are continuing in our laboratory and the results will be reported in future communications.

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