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Hydroperoxide Lyase and Other Hydroperoxide-Metabolizing Activity in Tissues of Soybean, *Glycine max*

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ABSTRACT

Hydroperoxide lyase (HPLS) activity in soybean (*Glycine max*) seed/seedlings, leaves, and chloroplasts of leaves required detergent solubilization for maximum *in vitro* activity. On a per milligram of protein basis, more HPLS activity was found in leaves, especially chloroplasts, than in seeds or seedlings. The total yield of hexanal from 13(*S*)-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid (13S-HPOD) from leaf or chloroplast preparations was 58 and 66 to 85%, respectively. Because of significant competing hydroperoxide-metabolizing activities from other enzymes in seed/seedling preparations, the hexanal yields from this source were lower (36–56%). Some of the products identified from the seed or seedling preparations indicated that the competing activity was mainly due to both a hydroperoxide peroxygenase and reactions catalyzed by lipoxygenase. Different HPLS isozyme compositions in the seed/seedling *versus* the leaf/chloroplast preparations were indicated by differences in the activity as a function of pH, the K_m values, relative V_{max} with 13S-HPOD and 13(*S*)-hydroperoxy-*cis*-9,*trans*-11,*cis*-15-octadecatrienoic acid (13S-HPOT), and the specificity with different substrates. With regard to the latter, both seed/seedling and chloroplast HPLS utilized the 13S-HPOD and 13S-HPOT substrates, but only seeds/seedlings were capable of metabolizing 9(*S*)-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic acid into 9-oxononanoic acid, isomeric nonenals, and 4-hydroxynonenal. From 13S-HPOD and 13S-HPOT, the products were identified as 12-oxo-*cis*-9-dodecenoic acid, as well as hexanal from 13S-HPOD and *cis*-3-hexenal from 13S-HPOT. In seed preparations, there was partial isomerization of the *cis*-3 or *cis*-9 into *trans*-2 or *trans*-10 double bonds, respectively.

HPLS[1], an enzyme that cleaves hydroperoxides of polyunsaturated fatty acids into both aldehyde and ω-oxoacid fragments, occurs widely in plants. Depending on substrate specificity of HPLS isoenzymes for isomeric hydroperoxides of linoleic or linolenic acids, the enzyme produces either green/beany/grassy odors (hexanal or *cis*-3-hexenal) from 13-hydroperoxides or cucumber/pear odors (*cis*-3-nonenal or *cis*-3,*cis*-6 nonadienal) from 9-hydroperoxides (reviewed in refs. 4 and 15). The corresponding ω-oxoacid fragments, 12-oxo-*cis*-9-dodecenoic acid and 9-oxononanoic acid, have no known function, except the former isomerizes into 12-oxo-*trans*-10-dodecenoic acid, which has wound healing effects (29).

HPLS activity has been reported in soybean leaves (23), and from soybean seeds and seedlings the enzyme has been partially characterized (17, 20) and purified (20). During development of the soybean seed, HPLS activity continues to decrease and lipoxygenase levels increase (24).

In this paper we report the occurrence and characteristics of HPLS for soybean seeds, seedlings, leaves, and chloroplasts isolated from leaves. HPLS was a significant portion of the hydroperoxide-metabolizing activity in all the tissues studied. In addition, we show that characteristics of the enzyme system in seed/seedlings differ from the leaf/chloroplast.

MATERIALS AND METHODS

Materials

Soybeans, *Glycine max* cv Century, obtained locally, were grown in 1987 and 1988 and were certified by the Illinois Crop Improvement Association.

The following reagents or chemicals were used: hexanal (99%), *trans*-2-hexenal (99%), *cis*-3-hexenol (98%), and pyridinium chlorochromate from Aldrich Chemical Co., Milwaukee, WI; Hepes, *cis*-3-nonenol (95%), *dl*-α-tocopherol (95%), Triton X-100R, hexamethyldisilazane, and soybean lipoxidase (lipoxygenase) type 1 from Sigma Chemical Co., St. Louis, MO; Reacti-flasks and bicinchoninic acid protein assay reagent from Pierce Chemical Co., Rockford, IL; oleic, linoleic, and α-linolenic acids (all >99%) from NuChek Prep, Elysian, MN; chlorotrimethylsilane from Applied Sciences, State College, PA. All other chemicals and solvents not specified were reagent or HPLC grade.

Enzyme Preparation

Unless otherwise specified, either 30 soybean seeds (about 4.9 g), 30 seedlings, or 10 g of leaves were homogenized in a total of 50 mL Hepes buffer (50 mM, pH 7.5) containing 0.5% Triton X-100R at 0°C. Before the seeds or seedlings were homogenized, they were crushed with a mortar and pestle and a small quantity of buffer; a water imbibition of dry seeds for 1.5 h facilitated crushing. Tissues were homogenized with

[1] Abbreviations: HPLS, hydroperoxide lyase; CP-HPLC, chiral-phase HPLC; EI, electron impact; OTMS, trimethylsilyloxy; rac-HPOD, racemic *cis,trans*- and *trans,trans*-diene linoleic acid hydroperoxides; rac-HPOT, racemic *cis,cis,trans*-triene linolenic acid hydroperoxides; 9S-HPOD, 9(*S*)-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic acid; 13S-HPOD, 13(*S*)-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid; 12S-HPOT, 12(*S*)-hydroperoxy-*cis*-9,*trans*-13,*cis*-15-octadecatrienoic acid; 13S-HPOT, 13(*S*)-hydroperoxy-*cis*-9,*trans*-11,*cis*-15-octadecatrienoic acid; SP-HPLC, straight-phase HPLC; Triton X-100R, Triton X-100 reduced; m/z, mass to charge ratio.

a Virtis homogenizer at medium speed for 90 s, and subsequently the homogenate was filtered through several layers of cheesecloth. The resultant brei was centrifuged at 10,000*g* for 30 min to obtain a supernatant containing enzymes of interest. Generally, the supernatant from seeds and seedlings afforded a range of 21 to 32 mg protein/mL and, from leaves, 13 to 15 mg protein/mL.

A membrane fraction was prepared from seedlings germinated for 2 d. Thirty-four seedlings were homogenized in 100 mL of a buffer commonly used for isolation of membrane fractions, containing 0.35 M sucrose, 0.2 mM $CaCl_2$, 0.5 mM dithiothreitol, 0.05% BSA, and 25 mM Hepes (pH 7.5), and this homogenate was strained through cheesecloth. The crude extract was centrifuged, and the material that pelleted between 1,500*g* for 15 min and 110,000*g* for 2 h was collected. The pellet was resuspended in a total volume of 50 mL reaction mixture comprised of the reagents described above for the HPLS reaction.

Chloroplast-enriched fractions were prepared by rate sedimentation of leaf homogenates. Soybean leaves (100–150 g) were homogenized (ice-cold) with a Waring blender at full speed for 15 to 20 s in 1 L of a typical buffer used to isolate chloroplasts (0.4 M sucrose, 50 mM Tes [pH 7.5], 10 mM NaCl, and 5 mM $MgCl_2$). Whole and broken chloroplasts were obtained by centrifugation of the cheesecloth-filtered homogenate, and the pellet obtained between 1,000*g* for 1.5 min and 5,800*g* for 5 min was collected. The chloroplasts were suspended and disrupted further by a Potter-Elvehjum homogenizer in a minimum of buffer (5–6 mL) for subsequent storage in a −80°C freezer. Enzyme extracts were prepared from the frozen chloroplast preparations by mixing the thawed chloroplasts with an equal volume of 1% Triton X-100R in water followed by centrifugation at 10,000*g* for 30 min. The supernatant, used as the enzyme, normally contained 3 to 10 mg protein/mL. Protein was determined by the bicinchoninic acid method (25).

Substrates

Either 13*S*-HPOD or 13*S*-HPOT was prepared by soybean lipoxygenase oxidation of 0.8 g of linoleic or α-linolenic acid, respectively, essentially as described for oxidation of linoleic acid (6), except the lipoxygenase reaction mixture was kept ice-cold to improve the O_2 solubility. The 13*S*-HPOD from oxygenation of linoleic acid was isolated by hexane-acetone elution from a silicic acid column (30 × 2.5 cm i.d.) (9). Collection of the early eluting half by weight of total hydroperoxides resulted in isolation of 13*S*-HPOD with 97 to 98% chiral purity. The same chromatographic method was used to isolate the other hydroperoxides described below, except the volume of solvent in the last of the stepwise elutions with 10% acetone in hexane had to be increased to account for the increased absorption of 13*S*-HPOT, 9*S*-HPOD, and smaller quantities of hydroperoxide applied to the column. By this method 13*S*-HPOT with 99% chiral purity from the oxidation mixture of α-linolenic acid was isolated by collection of the first half of the hydroperoxide peak.

Tomato lipoxygenase was utilized to prepare 9*S*-HPOD from linoleic acid (19). The oxidation mixture from action of tomato lipoxygenase was purified by column chromatography, and the middle of the hydroperoxide peak (0.71–0.9 L) was collected from the total hydroperoxides eluting between 0.59 and 1.05 L. This fraction containing 67 mg of product was purified by preparative TLC (20 × 20 × 0.2 cm, precoated Silica gel 60, Merck) developed by solvent A (see "TLC Separations") affording 9*S*-HPOD of 95% chiral purity.

Racemic linoleic acid hydroperoxides containing both conjugated *cis,trans* and *trans,trans*-dienes (rac-HPOD) were prepared by bubbling pure O_2 into 0.7 g of linoleic acid in mineral oil (linoleic acid:mineral oil, 1:2 [v/v]) for 30 h. In accordance with known theoretical principles (22), mineral oil provided a solvent of poor hydrogen-donating ability to promote the increased percentage of *trans,trans*-dienes. The rac-HPOD was isolated by column chromatography, and virtually the entire hydroperoxide peak between 1.06 and 1.55 L was collected from a total eluting between 1.04 and 1.63 L. The rac-HPOD (30 mg) was composed of approximately equivalent amounts of eight isomers (9- and 13-hydroperoxides, *R* and *S* stereoisomers, and *cis,trans*- and *trans,trans*-conjugated dienes). Racemic α-linolenic acid hydroperoxides (rac-HPOT) were prepared as described by Peers *et al.* (21). Essentially pure O_2 was bubbled into 1.2 g of α-linolenic acid containing 5% *dl*-α-tocopherol for 26 h. The product hydroperoxide was isolated by silicic acid column chromatography, and the entire hydroperoxide peak was collected. This procedure provided 57 mg of rac-HPOT having approximately equivalent amounts of 9-, 12-, 13-, and 16-hydroperoxides with *R* and *S* stereoconfiguration. All eight isomers were only *cis,cis,trans*-trienes with *cis,trans*-diene conjugation.

The hydroperoxides were stable if stored at −20°C as methanolic solutions of about 20 to 30 mg/mL. These methanolic solutions were used to routinly prepare 2 mM aqueous solutions of the hydroperoxides as K salts of the fatty acids. The pH of the substrate was adjusted to between 8 and 8.5, and 0.032% Tween 20 was added to ensure thorough dispersal of the hydroperoxide.

Activity Assays

Hexanal was analyzed by GLC of 1-mL headspace samples which afforded a direct measurement of an HPLS product. Because of the relatively long incubation time required for this measurement, it was most useful when assessing the overall yield from HPLS activity rather than initial rates. The reaction mixture (2–2.25 mL), comprised of <1.0 mM 13*S*-HPOD, 25 mM Hepes (pH 7.5), 0.015% (w/v) Tween 20, and various amounts of enzyme preparations, was sealed in 6-mL Reacti-flasks for incubation at 25°C for 20 min before 1-mL headspace gas was sampled by a Pressure-Lok gas syringe (Precision Sampling Corp., Baton Rouge, LA). One minute before sampling, the flask was agitated vigorously, and the headspace was sampled while the Reacti-flask was submerged in the 25°C bath. Headspace gases were analyzed by GLC. A standard curve was determined at 25°C using various known concentrations of hexanal suspended by sonication in the Hepes-detergent solution used in the assay. Hexanal concentration (mM) in the assay was determined from the standard curve. Because HPLS was inhibited by the Triton X-100R used to solubilize the enzyme, this detergent concentration was kept constant when enzyme quantities varied within an

experiment. In most cases, Triton X-100R was <0.05% in the assay.

Initial rates (initial 15 s) of hydroperoxide-decomposing activity were monitored by the decrease in absorbance of substrate-conjugated diene (λ_{max} 234 nm, ϵ = 26,800) using a Beckman DU-8B kinetic spectrophotometer with a spectrophotometer cell of only 1 mm path length. The reaction mixtures were the same as described for headspace analyses. To obtain reliable measurements, the pH was maintained at >pH 7.4 to ensure substrate solubility. Because this method measures loss of conjugated diene, HPLS and other hydroperoxydiene-destroying activities were assayed together.

For product isolation, the procedure used to assay was scaled up to a total reaction volume between 21 and 68 mL at a substrate concentration of 0.9 mM. The enzyme concentrations used in these reactions were about 0.3 or 3 mg/mL for extracts of chloroplasts or seeds/seedlings, respectively. After 20 min reaction at 25°C, the solution was adjusted to pH 4.5 with citric acid, and the products were extracted into chloroform by addition of 2 volumes of chloroform and 1 volume of methanol. The chloroform layer was washed with water.

Lipoxygenase activity was measured with an O_2 electrode (model 5/6H, Gilson Oxygraph, Middleton, WI) using various concentrations of either linoleic or linolenic acids suspended in the buffer described above.

HPLC Separations

Isocratic elution on silica SP-HPLC separated various isomers of rac-HPOD and rac-HPOT as their $NaBH_4$-reduced and methyl-esterified derivatives (5). Individual isomers, separated by SP-HPLC according to position of oxidation and diene configuration, were subsequently analyzed for *R* and *S* isomeric composition by CP-HPLC as described by Kühn *et al.* (16).

The SP-HPLC system described above was also utilized to isolate metabolites of the hydroperoxides (as methyl esters) with elution by various percentages of acetone in hexane.

TLC Separations

Analytical separations were completed with 20- × 20- × 0.025-cm plates composed of either Silica Gel 60 F-254 or Silica Gel 60 (Merck), and preparative separations required either the plates described above or 20- × 20- × 0.2-cm plates composed of Silica Gel 60 (Merck). The plates were developed without a filter paper tank liner using the following solvents: A, hexane:ethyl ether:acetic acid (50:50:1, v/v); B, hexane:ethyl ether (3:2, v/v); C, ethyl ether:hexane (3:2, v/v); D, chloroform:methanol (99:1, v/v); E, chloroform:methanol (39:1, v/v); F, hexane:acetone (7:1, v/v); G, hexane:acetone (4:1, v/v); H, chloroform:methanol (95:5, v/v).

Various spray reagents were used to detect compounds separated by TLC. A nondestructive spray was useful for isolation of products (13); after the separated bands were sprayed, they were visualized by short UV and long UV with Silica Gel 60 and Silica Gel 60 F-254, respectively. Silica scraped from the bands was extracted with ethyl ether. Ketones and aldehydes were visualized by spraying with 0.4% 2,4-dinitrophenylhydrazine in 2 N HCl. Total composition of product mixtures was assessed by a heat char (160°C) after spraying with vanillin:ethanol:H_2SO_4 (1:40:160, w/v).

GLC Separations

HPLS products were separated and analyzed by a Spectra-Physics, model SP-7100, gas chromatograph using an open tubular capillary column (25 m × 0.25 mm) coated with a 0.25-μm film of 007 CPS-2 from J and S Scientific (Crystal Lake, IL). Carrier gas flow (He) was about 1 mL/min. The temperature was programmed from 45 to 55°C at 5°C/min and then held at 55°C for 1 min to elute hexanal, *cis*-3-hexenal, and *trans*-2-hexenal at 1.7, 1.9, and 2.7 min, respectively. A temperature program from 100 to 140°C at 5°C/min eluted *trans*-2-nonenal and methyl 9-oxononanoate at 2.2 and 7.6 min, respectively. Aldehydes were injected either as 1-mL headspace samples with a Pressure-Lok syringe or by the conventional method of solution in organic solvent.

Chemical and Spectral Methods

Ketones, aldehydes, or hydroperoxides were reduced with an excess of $NaBH_4$ in methanol at 0°C for 30 min. Methyl esters were synthesized from fatty acids with diazomethane at 0°C in ethyl ether:methanol (9:1), v/v); care was taken to prevent long-term exposure of compounds to excess diazomethane when aldehydes or ketones were present. Primary alcohols were oxidized to the corresponding aldehydes with pyridinium chlorochromate (3). OTMS derivatives were produced from alcohols with hexamethyldisilazane: trimethylchlorosilane:pyridine (2:1:1, v/v).

9-Oxononanoic acid was synthesized from 9,10-dihydroxystearic acid by periodic acid oxidation. The 9,10-dihydroxystearic acid was produced by permanganate oxidation of oleic acid (26). The 9,10-dihydroxystearic acid (8 g) was oxidized for 1 h at 25°C with 6.2 g of periodic acid in 650 mL acetone:water (8:5, v/v). The products were extracted with chloroform, and the chloroform layer was washed twice with aqueous sodium dithionite to decolorize the by-product, I_2. The mixture of aldehyde monomers and dimers and/or trimers were hydrolyzed to monomers in acetone:aqueous 1 N HCl (3:2, v/v) for 1.5 h at 58°C under N_2. The hydrolyzed products were extracted with chloroform, and the chloroform layer was washed twice with water. A 100-mg portion of the chloroform-extracted material was separated into nonanal and 9-oxononanoic acid by preparative TLC on a 0.2-cm thick plate with solvent A; R_F of the latter was 0.35. ^{1}H-NMR and ^{13}C-NMR verified that the isolate was 9-oxononanoic acid.

Spectra were obtained by ^{1}H-NMR, ^{13}C-NMR, and MS with EI and chemical ionization as described previously (7).

RESULTS AND DISCUSSION

Activity Assays

Transformation of fatty acid hydroperoxides was assayed by a decrease in A_{234} attributed to decomposition of the conjugated hydroperoxydiene moiety and by headspace analysis of the HPLS product of 13*S*-HPOD, hexanal. Meas-

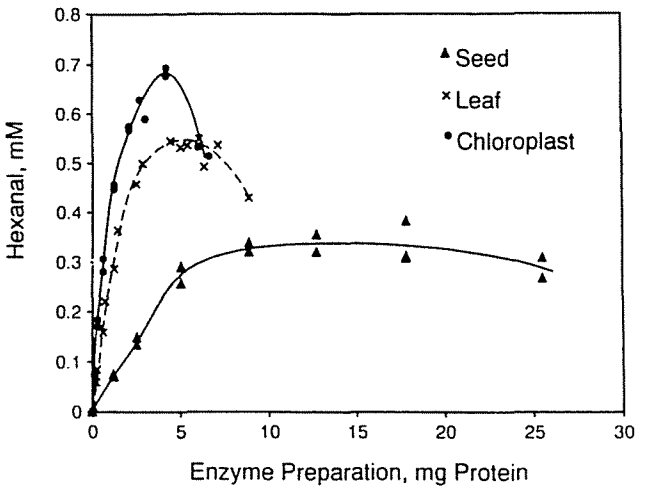

Figure 1. The production of hexanal (headspace analysis) from 13*S*-HPOD as a function of increasing amounts of either seed, leaf, or chloroplast preparations. Reagent concentrations were 0.95 mM 13*S*-HPOD, 26 mM Hepes (pH 7.5), 0.24% Triton X-100R, and 0.015% Tween 20 in a total volume of 2.1 mL.

urement of activity by A_{234} readily assessed initial rates, but this assay was not specific for HPLS alone because other enzyme(s) also destroy the hydroperoxydiene moiety. Headspace analysis of hexanal was a direct measurement of HPLS activity; however, obtaining initial rates by this method was not possible. Reasonably good evaluation of HPLS activity could be obtained by judicious use of both methods. To simplify discussion, the UV absorbance and headspace methods will be referred to as hydroperoxide-metabolizing activity and HPLS activity, respectively.

Enzyme Solubilization and General Stability

According to the literature (4) HPLS from various sources, including soybean (20), is bound to membranes. The characteristics of the activity from seeds or seedlings did not differ significantly in our hands; thus, the possibility of a membrane-bound HPLS was investigated with seedlings. Accordingly, a seedling preparation was differentially sedimented between 1,500*g* for 15 min and 110,000*g* for 2 h. A major portion (70–75%) of the activity with 13*S*-HPOD substrate was found in the sedimented pellet, and the formation of hexanal and 12-oxo-*trans*-10-dodecenoic acid was demonstrated. In accordance with the observed membrane association, HPLS activity was enhanced about twofold by homogenization of seedlings in buffer containing 0.5% Triton X-100R compared with buffer alone. Activity enhancement occurred in spite of an observed inhibition by detergents. For example, 50% inhibition of hydroperoxide-metabolizing activity in the reaction mixtures was obtained with 0.28% Tween 20 (soybean leaf preparation) and with 0.39% Triton X-100R (chloroplast). However, in most assays the Triton X-100R used to solubilize the enzyme was diluted in the final reaction mixture to <0.05%. Because of this inhibition, all comparative measurements were completed with constant detergent concentrations.

Hydroperoxide-metabolizing activity in soybean leaves was also primarily membrane bound. As expected, activity of leaf homogenates increased about twofold with corresponding increases in Tween 20 up to 0.4% in the homogenizing medium. A chloroplast-enriched fraction isolated by rate sedimentation had HPLS activity with higher specific activity than a comparable preparation from leaves (Fig. 1). Accordingly, chloroplasts stored at −80°C served as a convenient source of HPLS activity. As a routine procedure, HPLS was recovered from thawed frozen chloroplasts by solubilization with detergent. As shown in Table I, the hydroperoxide-metabolizing activity in water-suspended chloroplasts could be almost completely sedimented by centrifugation. A concentration of 0.5% Triton X-100R was required for optimal solubilization of activity. On average, Triton X-100R performed better than Tween 20 about 1.7-fold in solubilizing activity from chloroplasts; thus, 0.5% Triton X-100R was adopted as a standard condition for all preparations.

The half-life of Triton X-100R-solubilized hydroperoxide-metabolizing activity from leaf or chloroplasts was approximately 1.5 d stored on ice, which is slightly less than found for the enzyme from seedling (20). The solubilized enzyme from chloroplasts was essentially stable when stored at −80°C.

Tissue-Specific Differences

Hexanal Yield

HLPS activity of leaf and chloroplast preparations was directly assessed by hexanal formation from 13*S*-HPOD substrate as a function of the amount of preparation added (Fig. 1). On a per mg protein basis, more hexanal was produced with less chloroplast preparation than that from leaves. Because the 20-min incubation utilized in headspace analysis resulted in complete reaction at higher enzyme concentrations, the maximal hexanal values reflect total yield rather than rates of reaction. With the leaf preparation, about 4.5 to 5 mg of protein was required to obtain a maximum of 0.55

Table I. *Effect of Various Concentrations of Triton X-100R on the Solubilization of Hydroperoxide Activity of Chloroplasts*

A thawed chloroplast suspension (100 μL) was mixed with 0.5 mL of water or various percentages of Triton X-100R in water (w/v). The mixture was centrifuged at 10,000*g* for 30 min to obtain the supernatant and pellet. The residues were resuspended in 0.5 mL of 0.5% Triton X-100R for assay. Activity was measured by rate of decrease in A_{234}. The buffered substrate contained 1.0 mM 13*S*-HPOD, 25 mM Hepes (pH 7.5), and 0.016% Tween 20. The reaction was initiated by addition of 25 μL of various chloroplast treatments to 500 μL of buffered substrate at 25°C.

Triton X-100R Concentration	Relative Activity	
	Supernatant	Pellet
%	%	
0	3	100
0.25	63	46
0.5	79	53
1.0	81	46
1.5	79	46
2.0	73	38

mM hexanal from 0.95 mM 13*S*-HPOD, giving a substrate to product conversion of 58%. The chloroplast preparation at about 4 mg of protein afforded 0.68 mM hexanal, giving a calculated yield of 72%. Other experiments (not shown) consistently gave maximum hexanal yields from chloroplasts in the range of 66 to 85%. Decreased yields of hexanal from use of levels of enzyme higher than optimum was due to an unknown factor. One plausible explanation is the reduction of hexanal to hexanol by alcohol dehydrogenase (18). In summary, it can be concluded that HPLS activity is a major proportion of the total hydroperoxide-metabolizing activity in leaf and, especially, chloroplast preparations.

In terms of protein, much larger quantities of seed preparation were required to obtain a maximal molar yield of 36% hexanal, compared with leaves and chloroplasts (Fig. 1). The total yield of hexanal was slightly higher at lower substrate concentrations, *i.e.* 55% from 0.12 mM 13*S*-HPOD (data not shown). Results from seedlings germinated 3 d were comparable (data not shown). The lower hexanal yield from seed and seedling preparations was undoubtedly due to diversion of 13*S*-HPOD into other pathways of hydroperoxide conversion, a possibility corroborated by isolation of products that could not be attributed to HPLS activity.

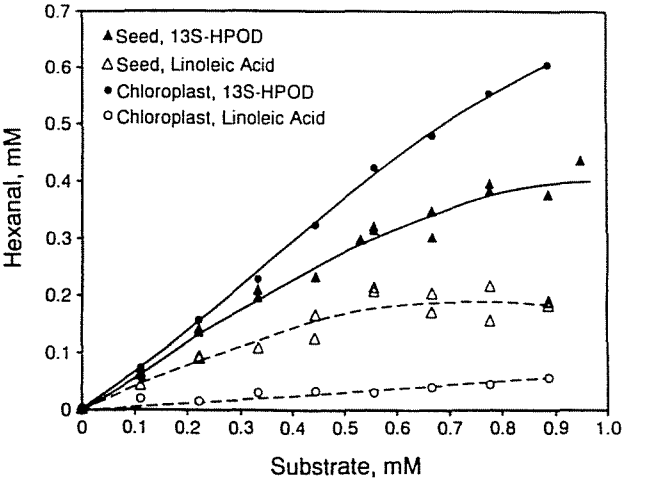

Figure 2. The formation of hexanal (headspace analysis) by either a chloroplast or a seed preparation as a function of either 13S-HPOD or linoleic acid concentration. Other than substrate, the composition of the reaction was 28 mM Hepes (pH 7.5), 0.055% Triton X-100R, 0.014% Tween 20, and enzyme at 3.4 (seed) or 0.67 mg of protein/mL (chloroplast) in a total volume of 2.25 mL.

Hexanal from an "Enzymic Cascade"

In the absence of substrate, the production of hexanal would be dependent on the sequential conversion of glyceride linoleate into 13*S*-HPOD by lipases/hydrolases and lipoxygenases. The use of 0.5% Triton X-100R to homogenize seeds or seedlings ensured the dispersal of relatively large amounts of triacylglycerols which was confirmed by TLC with solvent A of chloroform:methanol (2:1, v/v) extracts of the enzyme preparations. Despite the presence of triacylglycerols, only traces of hexanal were detected in the absence of added substrate. This result did not change when incubation occurred at other pH values (pH 4.5–9.5). Addition of more triacylglycerol by dispersal of the centrifugal flotation pellet composed of oil bodies also did not affect hexanal production significantly. On the other hand, addition of linoleic acid to seed preparations was at least half as effective as 13*S*-HPOD in formation of hexanal (Fig. 2). Thus, a sequence involving a lipoxygenase specific for producing 13(*S*)-hydroperoxides, such as lipoxygenase-1 (5), and HPLS was intact, but triacylglycerol hydrolysis was blocked under these conditions. Because it has been reported that the lipoxygenase-2 isoenzyme from soybean oxidizes triacylglycerol polyunsaturated fatty acids (2), the present data also implied that triacylglycerol hydroperoxide is not a substrate for HPLS. Results from experiments with preparations from seedlings germinated 1 or 3 d were similar to the data obtained from seeds (data not shown).

Similar to the results with seed/seedling preparations, leaf and chloroplast preparations gave only traces of hexanal in the absence of added substrate. By contrast, addition of increasing amounts of 13*S*-HPOD to chloroplast preparations resulted in corresponding increases of hexanal, but the response to linoleic acid was poor (Fig. 2). The result with 13*S*-HPOD and linoleic acid substrates using leaf preparations was similar to the chloroplast result, except that the hexanal yields with 13*S*-HPOD were somewhat lower even though 2.4-fold more leaf preparation on a per mg protein basis was used (data not shown). As shown in Figure 1, this result reflected the lower hexanal yields obtained with leaf preparations compared with chloroplast preparations. The poor response with linoleic acid with both leaf and chloroplast preparations indicated that a lipoxygenase specific for 13*S*-HPOD formation is very low in these preparations. This was corroborated by the measurement of low lipoxygenase activity in chloroplasts compared to seed/seedling preparations using an O_2 electrode assay (data not shown). The O_2 uptake by the chloroplast preparation was comparably low using either linoleic or linolenic acid substrates. According to David Hildebrand (personal communication), soybean leaf lipoxygenase can be relatively unstable, and its activity is often dependent on the method of extraction and the condition of the leaves.

Effect of pH and Substrate Concentration

As a function of pH, the activity of HPLS was quite different when seedling preparations were compared with those from chloroplasts (Fig. 3). The pH profile of a leaf preparation was essentially indistinguishable from that of chloroplasts (not shown). Similarly, the pH curve of seed preparations was comparable to that of seedlings. Matoba *et al.* (17) and Olias *et al.* (20) reported that seed and seedling preparations had a pH optimum between 6 and 7, which is slightly lower than the pH optimum of 7 to 7.5 that we observed (Fig. 3).

K_m values were calculated from double reciprocal plots of velocity *versus* either 13*S*-HPOD or 13*S*-HPOT substrate concentrations. The spectrophotometric method of assay had to be utilized to obtain initial velocities; therefore, K_m values were based on hydroperoxide-metabolizing activity rather than HPLS activity *per se*. Other data, *e.g.* Figure 1, indicated that hydroperoxide-metabolizing activity of chloroplasts was

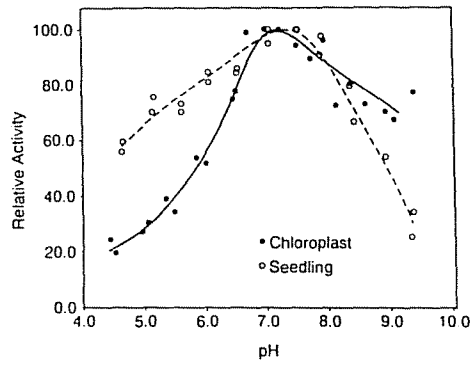

Figure 3. Relative HPLS activity (by hexanal headspace analysis) dependent on pH for both a seedling (germinated for 2 d) and a chloroplast preparation. The composition of the assays were for chloroplast and seedling preparations, respectively, 0.98 and 0.91 mM 13*S*-HPOD, 24 and 23 mM buffer, 0.012 and 0.045% Triton X-100R, 0.18 and 1.3 mg of protein/mL, and 0.015 and 0.014% Tween 20 in a reaction volume of 2.05 and 2.2 mL. Buffers (K salts) were acetate/acetic acid (pH 4–5.3), Mes (pH 5.4–6.7), Hepes (6.8–8.2), and borate/boric acid (pH 8.3–9.5).

primarily due to HPLS, but in seeds/seedlings activities other than HPLS were also indicated. With this caveat, K_m values were obtained for seedling and chloroplast preparations using 13*S*-HPOD and 13*S*-HPOT substrates (Fig. 4). Although there were no significant differences in K_m values between the 13*S*-HPOD and 13*S*-HPOT substrates, there was a difference between the chloroplast and seedling preparations. According to others (17, 20), the K_m of seed and seedling HPLS with 13*S*-HPOD was much lower, in the range of 0.04 to 0.06 mM; however, their method (headspace analysis) utilized a 1- (20) or 5-min (17) sampling time compared to initial rates in the present spectrophotometric method. On the other hand, the K_m value of 0.16 mM reported for seedling HPLS with 13*S*-HPOT (20) compared favorably with our findings.

The most striking difference between seedling and chloroplast preparations was the disparity in the V_{max} obtained with 13*S*-HPOT *versus* 13*S*-HPOD which is reported as a ratio of the two values in Figure 4. The V_{max} of chloroplast hydroperoxide activity was much greater with 13*S*-HPOT than with 13*S*-HPOD, whereas the opposite was true of seedlings. Olias *et al.* (20) found the ratio of V_{max} values to be 0.73 for seedlings compared to the ratio of 0.37 found in this study. It is interesting that this preference for substrate reflects the predominance of endogenous glyceride fatty acids in chloroplasts or seedlings, namely, linolenic and linoleic acids, respectively.

Specificity for rac-HPOD Isomers

The rac-HPOD used in this study was obtained by autoxidation of linoleic acid and was analyzed by SP-HPLC and CP-HPLC to be an approximately equivalent mixture of eight isomers comprised of 9- and 13-hydroperoxides with *R* and *S* stereoconfiguration and conjugated dienes with *cis,trans* and *trans,trans* configuration (Fig. 5, Table II). Exposure of rac-HPOD to the various enzyme preparations would reveal by difference which isomers were preferred substrates. Because the depleted substrate was examined as a $NaBH_4$ reduction product, any preferential utilization of rac-HPOD in the enzymic production of hydroxyoctadecadienoic acid would not be detected; *i.e.* these products would be synonymous with reduced hydroperoxides giving no net effect. Also, any oxooctadecadienoic acid products would be reduced by $NaBH_4$ to equivalent *R* and *S* mixtures of the corresponding hydroxyoctadecadienoic acid, thus tending to obscure any preferential utilization by this reaction. The net effect of the above is to somewhat increase the detection of HPLS specificity. This would also apply to the examination of rac-HPOT specificity discussed later.

Exposure of rac-HPOD to the chloroplast preparation showed that only one isomer, 13*S*-HPOD, was utilized from the eight (Fig. 5, Table II). It was demonstrated that the depleted isomer was 13*S*-HPOD by cochromatography with the corresponding derivative obtained from standard 13*S*-HPOD derived from soybean lipoxygenase oxidation of linoleic acid (5, 6). That 9*S*-HPOD was not a substrate was confirmed by spectrophotometric assay which afforded only a trace of activity compared with 13*S*-HPOD.

In contrast to the high degree of specificity exhibited by the chloroplast enzyme(s), the seed preparation utilized two of the eight isomers, 9*S*-HPOD and 13*S*-HPOD (Table II). The 9*S*-HPOD isomer was identified by cochromatography with a standard derived from 9*S*-HPOD formed through oxidation of linoleic acid by tomato lipoxygenase (19). The utilization of both 9*S*-HPOD and 13*S*-HPOD by the seed preparation was corroborated by about equivalent activity with both 9*S*-

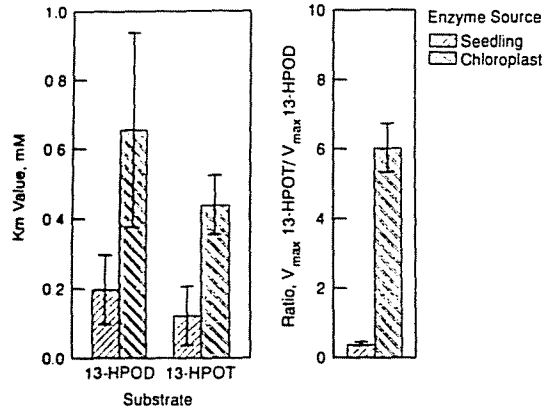

Figure 4. Left, K_m values are reported for both a seedling (2 d germinated) and a chloroplast preparation in the presence of either 13*S*-HPOD or 13*S*-HPOT substrate; right, V_{max} for the seedling and chloroplast systems shown as a ratio of the values obtained using either the 13*S*-HPOT or 13*S*-HPOD substrate. Initial rates were obtained by spectral analysis of loss of substrate-conjugated diene at λ_{max} 232 nm. K_m and V_{max} values were determined by linear regression of reciprocal plots of velocity *versus* substrate concentration (20 data points per determination). For the K_m values, the mean and SD shown represent duplicate determinations for chloroplasts and four determinations for seedlings. The V_{max} ratios were calculated from V_{max} values obtained using either 13*S*-HPOD or 13*S*-HPOT substrates analyzed at the same time with the same HPLS preparation. The mean and SD were calculated from duplicate V_{max} ratios for chloroplasts and four V_{max} ratios for seedlings.

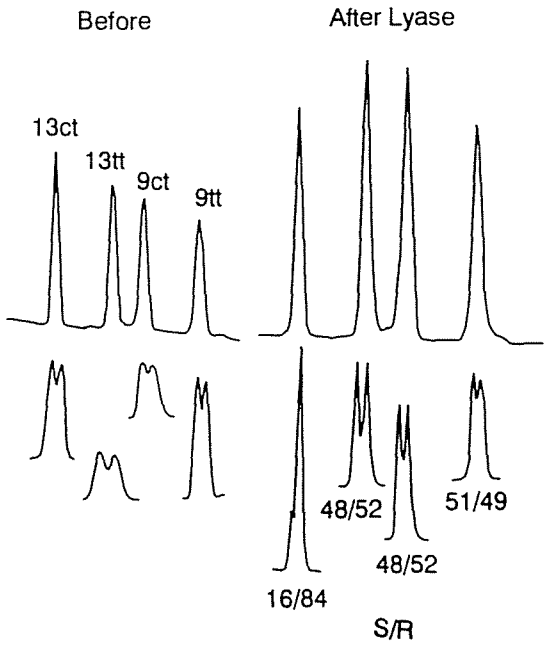

Figure 5. A racemic mixture of eight linoleic acid hydroperoxides (9- and 13-hydroperoxides; *cis,trans* and *trans,trans* dienes; and *R* and *S* stereoisomers) was exposed to the chloroplast preparation to determine substrate specificity. Isomeric linoleic acid hydroperoxides were analyzed by straight-phase silica HPLC separation of 9- and 13-hydroperoxides with either *cis,trans* or *trans,trans* diene conjugation (top) and by CP-HPLC separation of *R* and *S* isomers (bottom) after methyl esterification and $NaBH_4$ reduction.

HPOD and 13*S*-HPOD by spectrophotometric assay. Product analysis described below also proved that seed HPLS was specific for both 9*S*-HPOD and 13*S*-HPOD. In most respects, we confirmed the work of others (17, 20) who also examined the substrate specificity of various HPOD isomers with the seed or seedling enzyme. The current work differs in one important aspect: the other workers reported that 9*S*-HPOD was not a substrate. Because one group of workers (17) used headspace analysis of nonenal to assay for activity, this discrepancy possibly can be explained. Compared to the GLC headspace response of hexanal, the response of the nonenals are notoriously small because of reduced vapor pressure, and our recent data indicate that *cis*-3-nonenal is further converted into 4-hydroxy-*trans*-2-nonenal (unpublished data).

Specificity for rac-HPOT Isomers

Autoxidation of linolenic acid in the presence of 5% α-tocopherol afforded rac-HPOT composed of eight isomers in about equivalent amounts. This mixture contained 9-, 12-, 13-, and 16-hydroperoxides with *R* and *S* stereoconfiguration. All isomers contained only *cis,trans*-diene conjugation with a methylene interrupted *cis*-monoene. The identification of the peaks separated by SP-HPLC was checked by GC-MS of their methyl OTMS-stearate derivatives and showed that the positional isomers of rac-HPOT eluted in the same sequence as previously reported (21). In the presence of a chloroplast preparation, two of the eight rac-HPOT isomers were preferentially utilized (Fig. 6, Table II). One of these isomers, 13*S*-HPOT, was identical with the product of soybean lipoxygenase oxidation of linolenic acid. The other isomer consumed, one stereoisomer of the 12-hydroperoxide, was surmised to be 12*S*-HPOT based on the fact that the *S* isomer elutes before the *R* isomer with all hydroperoxides thus far examined by the CP-HPLC system used in this study (16). It is intriguing that the 12*S*-HPOT was utilized even though it has not been reported as a product of linolenic acid oxidation by any of the soybean lipoxygenase isoenzymes. The products of 12*S*-HPOT metabolism were not investigated because of the difficulty of obtaining this substrate. Application of the proposed mechanism of HPLS action (4, 15) to cleavage of 12*S*-HPOT should result in the same products as those obtained from 13*S*-HPOT, but this hypothesis remains to be tested.

With rac-HPOT as the substrate, the seed preparation exhibited a complex pattern of utilization (Table II). It is readily apparent that the *S* stereoisomers of all the isomers were preferentially depleted compared with the *R* isomer. Unlike the other results shown in Table II, the comparative abundance of the positional isomers was shifted more than could be accounted for by a simple depletion of *S* isomers. Evidently, at least some of the *R* isomers had been utilized as well. A similar result was obtained with half as much enzyme on a per mg protein basis, illustrating that experimental error was not the cause of this result.

Product Formation

13S-HPOD

Hexanal was identified as a product of HPLS action on 13*S*-HPOD; however, a number of other products, primarily fatty acid derivatives, were identified. These compounds were methyl esterified to facilitate isolation and identification.

The 12-carbon fragment from HPLS action, methyl 12-oxo-*trans*-10-dodecenoate, was isolated as a product of 13*S*-HPOD from both chloroplast and seedling preparations (Table III). The seedling preparation was a membrane fraction prepared by rate sedimentation of homogenates directly demonstrating the presence of HPLS in seedling membranes, as reported by others (20).

Other compounds were noted as products of 13*S*-HPOD metabolism. The seedling membrane fraction afforded methyl 13-hydroxy-*cis*-9,*trans*-11-octadecadienoate, as well as a compound tentatively identified as methyl oxooctadecadienoate by its TLC migration, UV absorbance, and reaction with 2,4-dinitrophenylhydrazine spray. On the other hand, hexanal, methyl 12-oxo-*trans*-10-dodecenoate, and unreacted hydroperoxide were the only compounds obtained from the chloroplast reaction.

During the SP-HPLC isolations, two separate peaks were often isolated which gave a ^{1}H-NMR spectrum consistent with methyl 12-oxo-*trans*-10-dodecenoate. This indicated that isomerization was occurring after separation, and indeed, it is well known that the initial product of HPLS, 12-oxo-*cis*-9-dodecenoic acid, readily isomerizes to the *trans*-10-monoene (4, 15). $NaBH_4$ trapping of the products immediately after extraction from the reaction stabilized the compounds from further isomerization.

Table II. *Isomeric Analyses of rac-HPOD and rac-HPOT Substrates before and after Exposure to Seed or Chloroplast Preparations*

Either rac-HPOD or rac-HPOT substrates were incubated with either a chloroplast or seed preparation for 10 min at 25°C and acidified, and the unreacted substrate was extracted into $CHCl_3$ as described for product isolation. The hydroperoxide fatty acids were converted into their corresponding hydroxy fatty esters by esterification and $NaBH_4$ reduction, and these esters were then separated by TLC (solvent B), collecting the broad band of slightly separated isomers of hydroxy-diene or -triene fatty esters as detected by UV absorbance. This partially purified isolate was subjected to analyses by SP-HPLC and CP-HPLC as shown in Figures 5 and 6. The reaction mixtures were comprised of essentially the same reagents used in activity assays. Substrate concentrations were set at 0.2 to 0.24 mM, and enzyme concentrations were 0.48 to 0.65 (chloroplast) and 3.1 to 5.5 mg of protein/mL (seed).

Enzyme Source	Substrate	% Isomeric Composition[a]							
		C-9				C-13			
		cis,trans		*trans,trans*		*cis,trans*		*trans,trans*	
		S	R	S	R	S	R	S	R
Control[b]	rac-HPOD	12.2	12.6	12.4	12.4	12.8	13.3	11.9	12.3
Chloroplast		13.9	15.0	13.5	13.0	2.9	15.5	12.5	13.6
Seed		3.6	12.9	15.4	16.6	3.9	15.5	14.4	17.7
		C-9		C-12		C-13		C-16	
		S	R	S	R	S	R	S	R
Control[b]	rac-HPOT	11.6	10.8	12.0	12.0	14.2	14.2	12.9	12.4
Chloroplast		14.6	14.5	2.8	13.5	3.7	17.9	16.2	16.8
Seed		11.1	14.7	6.0	10.6	15.1	30.7	3.4	8.3

[a] Regio-isomers of hydroperoxides are identified by the carbon number of their position. The *cis*, *trans*- and *trans,trans*-diene isomers were found only in the rac-HPOD sample; the rac-HPOT isomers were all *cis,cis,trans*-trienes with *cis,trans*-diene conjugation. The enantiomeric composition of the hydroperoxide is represented by *S* and *R*. [b] Composition of the substrate before treatment with HPLS.

As shown in Table III, $NaBH_4$ reduction not only trapped the *cis*-9-monoene but also seemed to result in increased yields in the isolation of the 12-carbon product of HPLS, possibly because of its increased stability or reduced volatility. From the action of the chloroplast enzyme on 13*S*-HPOD, the reduction products were mainly methyl 12-hydroxy-*cis*-9-dodecenoate and methyl 12-hydroxy-*trans*-10-dodecenoate in a 88:12 ratio. Another minor product, methyl 13-hydroxy-*cis*-9,*trans*-11-octadecadienoate, was surmised to originate from $NaBH_4$ reduction and esterification of unreacted 13*S*-HPOD. Thus, it can be seen that chloroplast preparations could catalyze a significant conversion of 13*S*-HPOD to HPLS products with little isomerization of the initially formed 12-oxo-*cis*-9-dodecenoic acid.

On the other hand, the mixture of $NaBH_4$-reduced products from reaction of 13*S*-HPOD with a preparation from seedlings (germinated 1 d) was more complex (Table III). The HPLS product was isolated as an approximately equimolar mixture of methyl 12-hydroxy-*cis*-9-dodecenoate and methyl 12-hydroxy-*trans*-10-dodecenoate, indicating that either enzymic or nonenzymic factors in the seedling preparation causes partial isomerization of the double bond. This isomerization was also observed by Olias *et al.* (20) in preparations from soybean seedlings. Two other products were identified as methyl 13-hydroxyoctadecadienoate and the *threo* isomer of methyl *trans*-12,13-epoxy-11-hydroxy-*cis*-9-octadecenoate. With regard to this latter $NaBH_4$-reduced product, the original moiety at carbon-11 may have been either a keto, hydroperoxy, or hydroxy group. The product with an 11-hydroxy group has been specifically identified from a reaction of 13*S*-HPOD with soybean lipoxygenase-1 (11), and the compound with an 11-hydroperoxy moiety has been found as a result of reacting 13*S*-HPOD with a soybean seed homogenate (10). Methyl *threo trans*-12,13-epoxy-11-hydroxy-*cis*-9-octadecenoate was not readily detected as a product of the seedling membrane preparation (see above), indicating the involvement of a soluble enzyme, such as lipoxygenase (11). The isolation of the hydroxyepoxyene fatty acid provided direct evidence for at least one competing pathway of hydroperoxide activity that could provide a compelling reason for the lower yields of hexanal with seed/seedling preparations (Fig. 1).

13S-HPOT

As determined by headspace analysis, there were smaller apparent yields of *cis*-3-hexenal and/or *trans*-2-hexenal from 13*S*-HPOT in reactions catalyzed by both chloroplast and seed/seedling preparations when compared with hexanal detection with 13*S*-HPOD substrate. The first of several reasons was the apparent utilization of *cis*-3-hexenal by another reaction, especially at higher enzyme concentrations (>0.25 mg of protein/mL), which resulted in optimum *cis*-3-hexenal levels at only 2 min reaction time with rapid depletion at longer times. With seed preparations, only trace amounts of

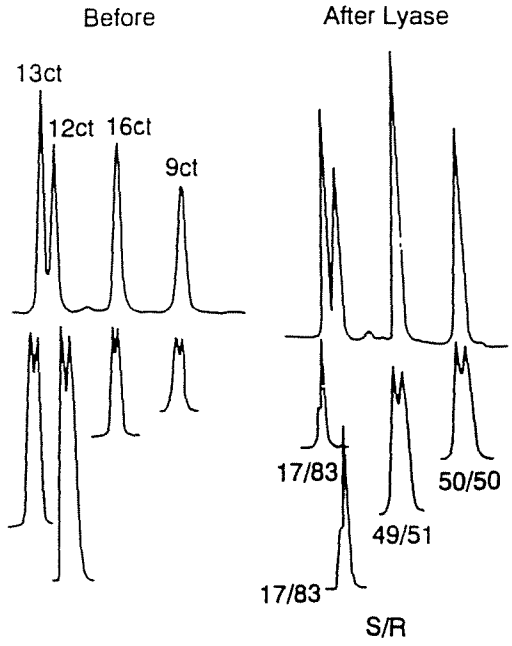

Figure 6. A racemic mixture of eight linolenic acid hydroperoxides (9-, 12-, 13-, and 16-hydroperoxides with *R* and *S* stereoconfiguration; all isomers contained only the *cis,trans*-diene conjugation) was exposed to a chloroplast preparation to determine substrate specificity. Positional isomers were separated by straight-phase silica HPLC (top), and *R* and *S* isomers were separated by CP-HPLC (bottom) after methyl esterification and $NaBH_4$ reduction of hydroperoxides.

the hexenals were detected at any time. Second, there appeared to be a comparatively lower partition of *cis*-3-hexenal and *trans*-2-hexenal from aqueous solution into headspace. A standard solution of *trans*-2-hexenal in buffer gave a response only 12.5% as great as a comparable solution of hexanal, which was in contrast to an almost equivalent response from a neat liquid of the two mixed in equimolar amounts. Third, there often was some isomerization of *cis*-3-hexenal to *trans*-2-hexenal. This isomerization in chloroplast preparations was only slight and became apparent as a trace *trans*-2-hexenal peak after 20 min. In seed preparations, a trace of *trans*-2-hexenal could be detected at sampling times >3 min. The largest yield of *cis*-3-hexenal observed in terms of peak area compared with a similar 20-min reaction producing hexanal was about one-third; this occurred after 2-min reaction with 13*S*-HPOT (0.95 mM) in a chloroplast reaction containing 0.6 mg of protein/mL.

The nonvolatile products of 13*S*-HPOT reaction were primarily examined as $NaBH_4$ reduction compounds. However, one reaction with a chloroplast preparation was not reduced, which permitted the isolation of methyl 12-oxo-*trans*-10-dodecenoate and methyl 13-hydroxyoctadecatrienoate (Table III). After $NaBH_4$ reduction, the chloroplast products were identified as only methyl 12-hydroxy-*cis*-9-dodecenoate and methyl 13-hydroxyoctadecatrienoate (Table III). Again, chloroplast preparations did not catalyze significant isomerization of the *cis*-9-monoene HPLS product and had little tendency to form many other products.

The products obtained after $NaBH_4$ reduction of a reaction of 13*S*-HPOT with a seed extract was more complex (Table III). The HPLS products, methyl 12-hydroxy-*cis*-9-dodecenoate and methyl 12-hydroxy-*trans*-10-dodecenoate, were isolated as a molar ratio of 68:32, again demonstrating the presence of isomerizing factors in seeds and seedlings. Additionally, methyl *cis*-15,16-epoxy-13-hydroxy-*cis*-9,*trans*-11-octadecadienoate and methyl 13-hydroxy-*cis*-9,*trans*-11-tridecadienoate were isolated, showing that the seed preparations diverted substrate into other hydroperoxide-degrading activities. Because these latter two compounds were transformed by $NaBH_4$ reduction, possibly the hydroxy groups were originally aldehyde/ketone or hydroperoxide moieties. For example, 13-oxo-*cis*-9,*trans*-11-tridecadienoic acid is known to be formed from 13*S*-HPOD by anaerobic cycling of soybean lipoxygenase in the presence of linoleic acid (12) or from 13*S*-HPOT by a *Chlorella pyrenoidosa* extract (28). Similarly, it was not possible to know whether methyl *cis*-15,16-epoxy-13-hydroxy-*cis*-9,-*trans*-11-octadecadienoate contained a hydroxy group before reduction; however, this product is reminiscent of the kind of transformation of 13*S*-HPOT that could

Table III. *Products, including Molar Percentage Yields, Isolated in Experiments using Different Substrates with Enzyme Preparations*

Substrate	Reduced by $NaBH_4$	Products[a] from Various Preparations		
		Seed	Seedling	Chloroplast
13S-HPOD	No	6	6	6
			1 (12.4)[b]	1 (11)
	Yes		2 (16.4)	2 (41)
			3 (16.4)	3 (5.5)
			15 (11)	15 (13)
			14 (5.7)	
13S-HPOT	No	7		7
		8		1 (9.6)
				9
	Yes	2 (6.4)		2 (25.8)
		3 (3)		9 (9)
		4 (6.7)		
		5 (12.9)		
		9 (16.1)		
9S-HPOD	No	10		
		11 (28.5)		
		12 (13.6)[c]		
		13 (8)		

[a] Numerical identification of methyl-esterified products are as follows: 1, methyl 12-oxo-*trans*-10-dodecenoate; 2, methyl 12-hydroxy-*cis*-9-dodecenoate; 3, methyl 12-hydroxy-*trans*-10-dodecenoate; 4, methyl *cis*-15,16-epoxy-13-hydroxy-*cis*-9,*trans*-11-octadecadienoate; 5, methyl 13-hydroxy-*cis*-9,*trans*-11-tridecadienoate; 6, hexanal; 7, *cis*-3-hexenal; 8, *trans*-2-hexenal; 9, methyl 13-hydroxyoctadecatrienoate; 10, *trans*-2-nonenal; 11, methyl 9-oxononanoate; 12, methyl 9-oxo-*trans*-10,*cis*-12-octadecadienoate; 13, methyl 9-hydroxy-*trans*-10,*cis*-12-octadecadienoate; 14, *threo* isomer of methyl *trans*-12,13-epoxy-11-hydroxy-*cis*-9-octadecenoate; 15, methyl 13-hydroxy-*cis*-9,*trans*-11-octadecadienoate. Molar percentage values of volatile compounds are not given. [b] The molar percentage yields in parentheses are based on weight of isolates obtained after chromatography. Yields of volatile aldehydes were determined separately by headspace analysis. [c] Includes 7.5% of other isomeric methyl oxooctadecadienoates.

occur by action of the hydroperoxide-dependent epoxygenase/peroxygenase enzymes reported in *Vicia faba* (14) and soybeans (1).

9S-HPOD

Experiments with rac-HPOD to determine substrate specificity illustrated that seed preparations utilized 9*S*-HPOD, but chloroplasts did not (Table II). Reaction of 9*S*-HPOD with a seed preparation afforded *trans*-2-nonenal, possibly *cis*-3-nonenal, and methyl 9-oxononanoate, conclusively showing that seed HPLS was capable of metabolizing 9*S*-HPOD (Table III). In contrast to earlier studies (17, 20), HPLS from soybean seed preparations is fully capable of utilizing 9*S*-HPOD. Several other products were isolated, such as isomeric methyl hydroxyoctadecadienoates (methyl 9-hydroxy-*trans*-10,*cis*-12-octadecadienoate, 89% of the total of four isomers) and isomeric methyl oxooctadecadienoates (methyl 9-oxo-*trans*-10,*cis*-12-octadecadienoate, 45% of the total of four isomers). With regard to the latter, soybean lipoxygenase-1 has been shown to react with 9*S*-HPOD to form oxooctadecadienoic acid (27), and this may account for its formation in the present study. Finally, an unknown was isolated that was tentatively identified as hydroxynonenal by its mass spectrum (note added after manuscript review: the unknown has been identified as 4-hydroxy-*trans*-2-nonenal and details will be reported in a future communication).

In conclusion, soybean seed/seedling and chloroplast preparations converted various substrates into the compounds summarized in Table III. The current work may explain the odor profile of various tissues from soybeans but does not give a satisfactory explanation for the physiological significance of these reactions or the need for lipoxygenase and HPLS isoenzymes. Although 12-oxo-*trans*-10-dodecenoic acid has wound-healing effects (29), no physiological significance has been attributed to the other HPLS products other than growth inhibition, especially of parasitic fungi (8).

With regard to odor, it is easy to understand why soybean leaves have an intensely green/grassy odor, because the leaf/chloroplast system is heavily skewed to favor the formation of the intensely green odor compound, *cis*-3-hexenal. Not only are leaves enriched in linolenate-containing galactolipids but 13*S*-HPOT, the immediate precursor of cis-3-hexenal, has a sixfold greater maximal velocity of decomposition than 13*S*-HPOD.

On the other hand, seeds/seedlings afford less intense odors of a green/beany character that probably can be explained by their lipid and enzyme profile. First, the predominant glyceride fatty acid of seed/seedling tissue is linoleate, whereas linolenate only amounts to 3 to 8%. Second, the seed/seedling lipoxygenase-HPLS composition results in a larger variety of aldehydes of which hexanal probably predominates. Also, as observed *in vitro*, the intense odor compound, *cis*-3-hexenal, may become isomerized to *trans*-2-hexenal, which has a less intense odor of grass/spice. However, it is curious that the characteristic cucumber-like odor of the nonenals are not notably expressed by soybeans. The overall green/beany odor of soybeans is undoubtedly controlled more by a predominant specificity for production of 13-hydroperoxides by soybean lipoxygenases; however, 9-hydroperoxides are also produced. For example, lipoxygenase-1, which is well known for its oxidation specificity at carbon-13, produces almost 20% 9*S*-HPOD at neutral pH values (5). It may be a combination of factors, including the relatively low vapor pressure of nonenal, that may cause the suppression of the cucumber note. Also, it seemed plausible that the nonenals may be converted into the unknown now identified as 4-hydroxy-*trans*-2-nonenal. As discussed in the following section, the yield of the latter was determined to be much greater than the nonenals.

Product Isolation and Characterization

Headspace volatiles, hexanal, *trans*-2-hexenal, and *cis*-3-hexenal, were identified by their GLC retention times and by coinjection with standards. The *cis*-3-hexenal standard was generated in a Reacti-flask by *in situ* oxidation of *cis*-3-hexenol by pyridinium chlorochromate. Additionally, hexanal and *cis*-3-hexenal were confirmed by GC-MS analysis of their EI spectra as follows for hexanal (m/z [% relative intensity, ion structure]): 100 (0.2, M^+); 82 (13, M^+-H_2O); 72 (17, M^+-CO); 71 (8, M^+-CHO); 69 (12); 67 (9); 57 (45); 56 (71); 44 (100); 43 (52); and 41 (83) and for *cis*-3-hexenal (m/z [% relative intensity, ion structure]): 98 (7, M^+); 83 (45, M^+-CH_3); 69 (58, M^+-CHO); 57 (38); 55 (73, M^+-CH_2CHO); 42 (41); and 41 (100).

The vapor pressure of *trans*-2-nonenal was too low to identify this compound from the headspace of reactions of 9*S*-HPOD with the seed enzyme. However, an ether extract of the product mixture was subjected to GC-MS, which resulted in identification of a component essentially identical in GC and mass spectral data with standard *trans*-2-nonenal. The following EI mass spectrum was obtained (m/z [% relative intensity, ion structure]): 122 (12, M^+-H_2O); 111 (13, M^+-CHO); 98 (18); 96 (31); 84 (31); 83 (86); 70 (83); 69 (53); 57 (48); 55(100, CHCHCHO); 43 (85); and 41 (85). An earlier eluting peak of approximately equal size furnished a similar, but not identical, mass spectrum compared to *trans*-2-nonenal; this peak was tentatively identified as *cis*-3-nonenal. The yield of the nonenals, estimated from the GC-MS results, was about 10-fold lower than expected. However, a correspondingly larger peak of the unknown, now identified as 4-hydroxy-*trans*-2-nonenal, could actually be a metabolite of one or both of the nonenals, possibly accounting for the lower yields of the latter.

The nonvolatile products of reaction of specific hydroperoxide isomers with the enzyme preparations were isolated by chromatography after methyl esterification. Because the enzyme preparations contained emulsified endogenous lipid, these lipids were extracted along with products of interest. To locate the actual products, a minus-substrate blank was extracted and methyl esterified for comparative analysis by SP-HPLC or two-dimensional TLC. The two-dimensional TLC system was solvent B followed by solvent F. With product mixtures that had been $NaBH_4$ reduced, separation was obtained by two-dimensional TLC with solvent C followed by double development with solvent G.

Methyl 12-oxo-*trans*-10-dodecenoate was isolated by chromatography as described in Table IV. The methyl 12-oxo-*trans*-10-dodecenoate isolates furnished a ^{1}H-NMR spectrum (Table V) and GC-MS data (EI) consistent with its structure as follows (m/z [% relative intensity, ion structure]): 194 (15,

Table IV. *Chromatographic Methods used to Isolate Products of Hydroperoxide Metabolism*

Isolate[a]	Enzyme Source	Substrate	Chromatographic Method (Sequence[b])	Solvent[c]	Retention Time (min) or R^F Value
1	Seedling	13S-HPOD	(1) TLC	B	0.54
			(2) SP-HPLC	95:5, 3 mL/min	17
	Chloroplast	13S-HPOD	(1) Preparative[d]	Ether	
		13S-HPOT	(2) SP-HPLC	92:8, 3 mL/min	10.7
2	Seed	13S-HPOT	(1) TLC	C	0.31
			(2) SP-HPLC	85:15, 2.5 mL/min	17.6
3	Seed	13S-HPOT	(1) TLC	C	0.38
			(2) SP-HPLC	85:15, 2.5 mL/min	17.6
4	Seed	13S-HPOT	(1) TLC	C	0.31
			(2) SP-HPLC	85:15, 2.5 mL/min	18.9
5	Seed	13S-HPOT	(1) TLC	C	0.38
			(2) SP-HPLC	85:15, 2.5 mL/min	18.6
9	All	13S-HPOT	TLC or SP-HPLC	C 92:8, 3 mL/min	0.65 16.7
11	Seed	9S-HPOD	(1) TLC	D	0.47
			(2) TLC	F	0.39
12	Seed	9S-HPOD	(1) TLC	D	0.61
			(2) SP-HPLC	95:5, 2.5 mL/min	23, 25.2[e], 26.3, 28.8
13	Seed	9S-HPOD	TLC	D	0.35
14	Seedling	13S-HPOD	TLC	C	0.45
15	All	13S-HPOD	TLC	C	0.66
Unknown	Seed	9S-HPOD	TLC	D	0.14

[a] The isolated products are identified in a footnote to Table III. [b] The products were usually isolated from two-step chromatographic separations by the sequence indicated. [c] TLC solvents are indicated by letters. The SP-HPLC solvent was hexane: acetone (v/v) composed of the ratios indicated; the flow rate is also indicated. [d] A preparative column (10 g Silic AR CC-7, Mallinckrodt, packed into a 2.5-cm i.d. column) was eluted with 53 mL of ethyl ether to collect methyl esters and retain chloroplast lipids on the column. [e] Four isomers of methyl oxooctadecadienoate eluted at the times indicated (in order of abundance): methyl 9-oxo-*trans*-10,*cis*-12-octadecadienoate, 25.2 min; methyl 13-oxo-*trans*-9,*trans*-11-octadecadienoate, 26.3 min; methyl 9-oxo-*trans*-10,*trans*-12-octadecadienoate, 28.8 min; and methyl 13-oxo-*cis*-9,*trans*-11-octadecadienoate, 23 min.

M^+-CH_3OH); 176 (3, M^+-CH_3OH-H_2O); 166 (10); 149 (10); 134 (13); 124 (10); 112 (20); 98 (100); 83 (47); 74 (46); 70 (35); and 55 (82). The chemical ionization mass spectrum was as follows: 227 (100, MH^+); and 195 (27, MH^+-CH_3OH).

The two methyl 12-hydroxydodecenoate isomers (*cis*-9- and *trans*-10-monoenes) were only separable by TLC with solvent C (R_F 0.31 and 0.38, respectively) permitting an ^{1}H-NMR analysis (Table V) of each isolate after removal of other components by SP-HPLC (Table IV). Usually, these two isomers were isolated together as a pair after a two-step purification (solvent C collecting both bands; solvent E, paper-lined tank, R_F 0.37). The composition of the mixtures was determined by integration of selected ^{1}H-NMR absorbances (protons at C-12 and olefin). ^{1}H-NMR assessment of isomeric mixtures was confirmed by GC-MS which resulted in slight GC separation with the same percentage ratio. The EI spectrum of methyl 12-hydroxy-*cis*-9-dodecenoate was (m/z [% relative intensity, ion structure]): 210 (6, M^+-H_2O); 198 (14, M^+-CH_2O); 178 (23, M^+-H_2O-CH_3OH); 166 (37); 161 (10); 148 (16); 137 (23); 136 (23); 124 (29); 95 (47); 87 (43); 81 (70); 74 (50); 67 (75); 55 (100); and 41 (75), and the EI spectrum of methyl 12-hydroxy-*trans*-10-dodecenoate gave: 211 (2, M^+-OH); 196 (2, M^+-CH_3OH);178 (17, M^+-H_2O-CH_3OH); 172 (4); 167 (3); 161 (12); 153 (12); 149 (16); 143 (16); 137 (21); 135 (22); 129 (26); 121 (18); 112 (90); 98 (97); 87 (84); 83 (52); 81 (53); 74 (54); 67 (57); 57 (58); 55 (100); and 41 (86).

Methyl *cis*-15,16-epoxy-13-hydroxy-*cis*-9,*trans*-11-octadecadienoate and methyl 13-hydroxy-*cis*-9,*trans*-11-tridecadienoate were isolated by the procedures described in Table IV and were characterized by their spectra. The latter product was identified by its ^{1}H-NMR spectrum (Table V) and GC-MS of its OTMS derivative. The EI mass spectrum furnished the following (m/z [% relative intensity, ion structure]): 312 (5, M^+); 297 (3, M^+-CH_3); 265 (4, M^+-CH_3-CH_3OH); 215 (3); 183 (4); 173 (9); 169 (8); 155 (48, M^+-$(CH_2)_7COOCH_3$; 79 (21); 75 (28); 73 (100, $Si^+(CH_3)_3$); 67 (20); 55 (13); and 41 (13). The relative positions of the hydroxydiene moiety was confirmed by correlated NMR spectroscopy ^{1}H-NMR (data not shown). Methyl *cis*-15,16-epoxy-13-hydroxy-*cis*-9,*trans*-11-octadecadienoate was similarly characterized by ^{1}H-NMR (Table V) and GC-MS of the OTMS derivative. The EI mass spectrum was as follows (m/z [% relative intensity, ion structure]): 367 (1, M^+-CH_2CH_3); 349 (1, M^+-CH_3-CH_3OH; 337 (3); 311 (20, M^+-$CH_3CH_2CHOCHCH_2CH$); 239 (6, M^+-$(CH_2)_7COOCH_3$); 207 (10); 181 (15); 171 (13); 157 (11); 143 (10); 131 (17); 91 (15); 79 (20); 75 (29); 73 (100, $Si^+(CH_3)_3$); 67 (22); 55 (16); 41 (16). A correlated NMR spectroscopy spectrum fully established the relative positions of the epoxyhydroxydiene functional groups (data not shown). The *cis*-epoxide and *cis,trans*-diene geometry were assigned on the basis of their coupling constants of 4.2, 10.5, and 15.1 Hz, respectively.

The *threo* isomer of *trans*-12,13-epoxy-11-hydroxy-*cis*-9-

Table V. *^{1}H-HMR Chemical Shifts (δ) and Proton Couplings (J, Hz) for Several Products Obtained from the Substrates, 13S-HPOD or 13S-HPOT*

Proton	Product[a] Isolate				
	1	2	3	4	5
	Chemical Shifts, Multiplicity				
Ester CH_3	3.66s	3.65s	3.66s	3.66s	3.66s
2	2.30t	2.30t	2.29t	2.29t	2.29t
3	1.61m	1.61m	1.61m	1.61m	1.61m
4–7		1.30m		1.30m	1.30m
4–8	1.30m		1.30m		
8		2.05m		2.18m	2.17m
9	2.32dtd	5.55dtt	2.04m	5.45dt	5.44dt
10	6.84dt	5.35dtt	5.61m	5.97dd[b]	5.99dd[b]
11	6.11ddt	2.32m	5.69m	6.56dd	6.53dd[c]
12	9.50d	3.63t[d]	4.08m	5.70dd	5.80dt
13				4.47m	4.20t[e]
14a				1.87ddd[b]	
14b				1.69ddd[b]	
15				3.10ddd[b]	
16				2.89dt	
17				1.54m	
18				1.04t	
	Proton Couplings				
	1	2	3	4	5
2	7.5	7.5	7.5	7.5	7.5
8, 9	α7	7.3		7.7	7.6
8, 10		1.5			
9, 10	6.8	10.8	5.0	10.5	10.7
9, 11	1.5	1.5			
10, 11	15.6	7.4	15.4	11.1	11.1
11, 12	7.9	6.5	5.6	15.1	15.2
12, 13				6.7	5.9
13, 14a				4.4	
13, 14b				7.9	
14a, 14b				14.2	
14a, 15				3.9	
14b, 15				7.9	
15, 16				4.2	
16, 17				6.4	
17, 18				7.5	

[a] Numerical designations of product isolates are given in footnotes of Table III. [b] Overlapping multiplicity. [c] Includes complex long-range coupling (dt) of about 1 to 1.3 Hz probably due to 9, 11 and 11, 13 couplings. [d] Partly obscured. [e] Apparent multiplicity.

octadecadienoate was isolated by the method described in Table IV. Its ^{1}H-NMR spectrum and GC-MS of its OTMS derivative were essentially identical with those described previously for this compound (11).

Methyl 9-oxononanoate, isolated as described in Table IV, was confirmed to be authentic by coelution with methyl 9-oxononanoate standard by both GLC and TLC with solvents B and F. Both the standard and the isolate gave lemon-yellow spots on TLC plates after spraying with 2,4-dinitrophenylhydrazine reagent.

Four isomers of methyl oxooctadecadienoate, derived from 9*S*-HPOD, were isolated by SP-HPLC (Table IV). These isolates were identified by $NaBH_4$ reduction and SP-HPLC analysis of their methyl hydroxyoctadecadienoates. Because the SP-HPLC isolates had partially isomerized before the $NaBH_4$ reduction step, it was concluded that methyl oxooctadecadienoates are particularly susceptible to isomerization; nevertheless, the major isomer was determined to be methyl 9-oxo-*trans*-10,*cis*-12-octadecadienoate.

Methyl 9-hydroxy-*trans*-10,*cis*-12-octadecadienoate was isolated (Table IV) and identified by SP-HPLC using a standard obtained from reduction and esterification of 9*S*-HPOD.

Methyl 13-hydroxy-*cis*-9,*trans*-11-octadecadienoate and methyl 13-hydroxy-*cis*-9,*trans*-11,*cis*-15-octadecatrienoate, isolated as described in Table IV, were characterized by either of two methods. At a minimum, the isolate was subjected to UV analysis (λ_{max} 232 nm) and TLC (solvent B) compared with a standard prepared by $NaBH_4$ reduction and esterifica-

tion of the corresponding hydroperoxide fatty acid. Alternatively, the isolates were analyzed by SP-HPLC using standards for identification by coelution.

CONCLUSIONS

The research reported here compared the HPLS activity of soybean leaves, including chloroplast preparations obtained from leaves, with that of seeds/seedlings.

Previously, the HPLS activity of soybean leaves had been measured by headspace analysis of hexanal using 13*S*-HPOD as a substrate (23); we are aware of no other more detailed prior research concerning soybean leaf HPLS. It is reported here that leaves from soybean contain an HPLS activity that makes up a significant portion (58%) of the total hydroperoxide-metabolizing activity. Furthermore, it was shown that the leaf HPLS was associated with the chloroplasts, and the use of a chloroplast fraction resulted in a further increase in the yield of HPLS products to 66 to 85%. The chloroplast HPLS was highly specific for 13*S*-hydroperoxides of linoleic and linolenic acids affording C-6 aldehydes and 12-oxo-*cis*-9-dodecenoic acid. The 12*S*-hydroperoxide of linolenic acid also may serve as a substrate for the chloroplast HPLS, but this was not directly demonstrated.

Several characteristics of the HPLS activity of leaves/chloroplasts proved to be not identical with that of seeds/seedlings, indicating the presence of different isoenzyme(s). Among these differences were pH optima, K_m values, comparative V_{max} values for 13*S*-HPOD and 13*S*-HPOT substrates, and the specificity for the 9*S*-HPOD substrate.

Previous investigators (17, 20) had characterized the HPLS of soybean seeds/seedlings, and our work, for the most part, confirmed their findings. However, our work differed significantly by the finding that 9*S*-HPOD is a substrate for seed HPLS but not for chloroplast HPLS. Seed HPLS produced a good yield of 9-oxononanoic acid from 9*S*-HPOD. However, the yield of *cis*-3-nonenal was poor, presumably because it was further metabolized into *trans*-2-nonenal and 4-hydroxy-*trans*-2-nonenal (details to appear in a future communication). It was also demonstrated that other hydroperoxide-metabolizing activities were present in seeds/seedlings consistent with previous work (1, 10, 11, 12, 27). Inasmuch as the other activities competed for substrate in the seed, the yield of hexanal from 13*S*-HPOD was low (36–55%).

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