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Positional Specificity of Ketodienes from Linoleic Acid Aerobically Formed by Lipoxygenase Isozymes from Kidney Bean and Pea

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Two lipoxygenase isozymes (LOX-1 and LOX-2) from kidney bean and pea flours were purified by a combination of ammonium sulfate fractionation, gel filtration, and ion-exchange chromatography. Besides the production of hydroperoxides, LOX-1 catalyzed the aerobic formation of ketodienes. The positional and geometrical isomers were separated and quantified by HPLC. LOX-1 yielded approximately equal proportions of 9- and 13-hydroperoxides and the 9- and 13-ketodienes; LOX-2 formed mainly the 13-hydroperoxide isomer.

INTRODUCTION

Lipoxygenase has been reported to be distributed throughout the plant kingdom. Soybean, considered to be a model, has been the source most thoroughly studied. Four different soybean lipoxygenases have been purified to homogeneity and characterized. Other plants besides soybean have also been shown to express lipoxygenase in multimolecular forms. For example, isozymes have been demonstrated in bush bean (Hurt and Axelrod, 1977), pea (Yoon and Klein, 1979), potato (Mulliez et al., 1987), bengal gram (Borthakur et al., 1988), maize (Poca et al., 1990), and chickpea (Sanz et al., 1992). These all catalyze, as primary reaction, the dioxygenation of fatty acids containing a *cis*-1,*cis*-4-pentadiene system to form hydroperoxides.

Some lipoxygenases, besides the hydroperoxidation reaction, form other secondary products such as ketodienes (Vioque and Holman, 1962). Originally these were shown to be formed only anaerobically (Garssen et al., 1971), but they were later reported to occur also under aerobic condition (Pistorius, 1974; Hurt and Axelrod, 1977; Borthakur and Ramadoss, 1986). However, this reaction was not well characterized with respect to positional specificities.

We recently reported the presence of two forms of lipoxygenase in chickpea (Sanz et al., 1992), one of which formed mainly 13-hydroperoxy-*cis*-9,*trans*-13-octadecadienoic acid from linoleic acid, while the other yielded approximately equal proportions of the 9- and 13-hydroperoxides and the 9- and 13-ketodienes. To the best of our knowledge, previous investigations have not focused on positional specificity of ketodienes; hence, we proposed to gain further information on the proportion of 9- and 13-ketodienes yielded for lipoxygenase isozymes from other plants. This paper deals with the investigations of partially purified lipoxygenase isozymes from kidney beans and peas, which exhibit the ability to produce carbonyl compounds.

EXPERIMENTAL PROCEDURES

Materials and Reagents. Seeds of kidney beans (*Phaseolus vulgaris* cv. Tender Green) and peas (*Pisum sativum* cv. Voluntarioso) were used. Linoleic acid was obtained from Nu Check Prep Inc., Elysian, MN. Sephacryl S-300 and Mono-Q columns were purchased from Pharmacia, and DEAE-cellulose DE-52 was from Whatman Biosystems Ltd. Octadecyl (C_{18}) disposable extraction columns (6 mL) were obtained from J. T. Baker Chemical Co. *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was from Supelco, Bellefonte, PA. A stock solution of 10 mM sodium linoleate in Tween 20 was prepared as described by Axelrod et al. (1981).

Lipoxygenase Isolation. All steps were performed at 0–4 °C. Preparation of crude extract and gel filtration were performed similarly with both seeds. Kidney bean and pea dry seeds were ground to a fine flour (0.4-mm mesh), defatted with hexane, and extracted with 50 mM sodium phosphate buffer, pH 6.8 (1:7 w/v), for 2 h. The extract was filtered through four layers of gauze and the resulting filtrate centrifuged at 12000*g* for 20 min. The supernatant was treated with $(NH_4)_2SO_4$, and the fraction precipitating between 30 and 60% saturation was redissolved in ca. 10 mL of extraction buffer and applied to a Sephacryl S-300 column (2.8 cm × 90 cm), which was equilibrated with the same buffer; flow rate was regulated to 12 mL/h, and 4.3-mL fractions were collected. The fractions containing lipoxygenase activity were pooled and applied to a DEAE-cellulose DE-52 column (3.2 cm × 40 cm).

Kidney bean lipoxygenases were eluted with a linear gradient of 20 mM sodium phosphate (400 mL) to 200 mM (400 mL), pH 6.8; flow rate was 25 mL/h, and fractions of 4 mL were collected. Lipoxygenase activity appeared in two separated peaks. Pooled fractions for each activity peak were finally purified by a fast protein liquid chromatography (FPLC) system, using an analytical anion-exchange column, Mono Q HR 5/5 (Pharmacia LKB Biotechnology, Uppsala, Sweden), equilibrated with 20 mM sodium phosphate (pH 6.8). Before loading, proteins were conditioned to 20 mM sodium phosphate by using a Sephadex G-25 disposable column (PD-10 column, Pharmacia). After loading, the equilibrium buffer was run for 10 min at 10 mL/min flow, and then the proteins were eluted with a gradient from 20 (buffer A) to 200 mM (buffer B) sodium phosphate, pH 6.8. The gradient was developed in two steps: (A) 10–50 min, 0–20% buffer B; (B) 50–70 min, 20–100% buffer B. Protein elution was detected at 280 nm, and fractions were assayed for lipoxygenase activity. The two forms of the enzyme were designated KB-LOX_1 and KB-LOX_2 regarding order of elution.

Active fractions of pea lipoxygenase from the Sephacryl column were pooled and applied to DE-52 column. The protein was eluted with a linear gradient generated from 50 (300 mL) to 200 mM sodium phosphate buffer, pH 6.8 (300 mL); flow rate was 25 mL/h, and fractions of 4 mL were collected. The active pea

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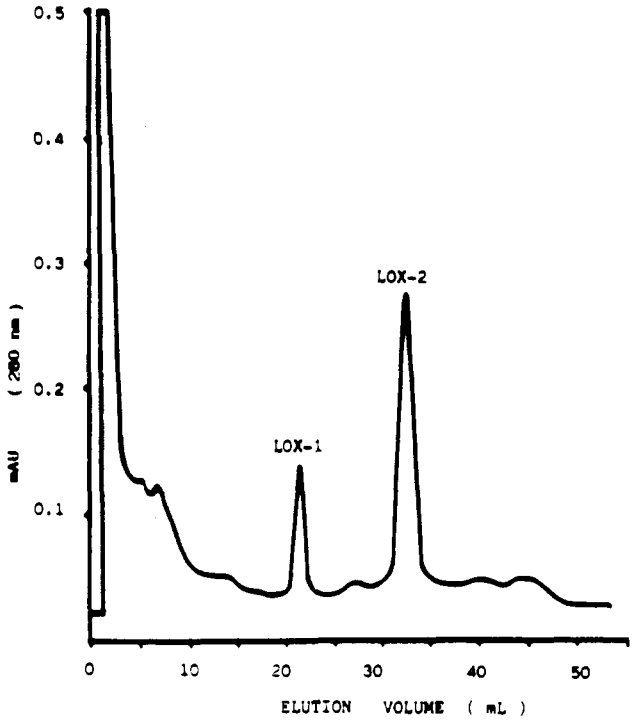

Figure 1. FPLC chromatogram of kidney bean lipoxygenase isozymes LOX-1 (forms keto- and hydroperoxydienes) and LOX-2 (produces exclusively hydroperoxydienes).

lipoxygenase fraction was resolved in two isozymes, designated P-LOX$_1$ and P-LOX$_2$.

Protein Determination. During chromatography, the concentration of protein was measured by absorbance at 280 nm.

Lipoxygenase Assay. Lipoxygenase activities were determined spectrophotometrically. The standard assay mixture consisted of 3 mL of sodium phosphate buffer (50 mM, at appropriate pH), 25 μL of substrate solution (10 mM linoleic acid), and the appropriate volume of the enzyme solution. The reaction was initiated by addition of the enzyme solution, and formation of both hydroperoxides and ketodienes was followed by change in absorbance at 234 ($\epsilon = 2.5 \times 10^4$ M^{-1} cm^{-1}) and 274 nm ($\epsilon = 2.2 \times 10^4$ M^{-1} cm^{-1}), respectively at 25 °C (Axelrod et al., 1981). One unit of activity is defined as the amount of enzyme catalyzing the formation of 1 μmol of the product/min.

Analysis of Reaction Products. Linoleic acid (10 μmol) was incubated with 2 units of lipoxygenase isozyme activities in 30 mL of oxygen-saturated 50 mM sodium phosphate buffer at appropriate pH: 5.7 for KB-LOX-1 and -2, 5.8 for P-LOX-1, and 6.3 for P-LOX-2. Reactions were carried out at 20 °C with a constant flow of oxygen for 10 min and then stopped by adjusting to pH 3 with 2 N HCl. The products were extracted from the incubation mixture on a reversed-phase C_{18} microcolumn and eluted with methanol. The concentrated products were esterified with diazomethane (Cohen, 1984), and the positional and geometrical isomers were analyzed by HPLC with a LiChrosorb Si-60 5-μm (4 mm × 250 mm) column (Merck, Darmstadt, FRG) eluted with *n*-hexane/diethyl ether (92:8 v/v); flow rate 1.25 mL/min, detection at 234 and 270 nm). To identify the reaction products, a large-scale isolation was carried out by using a Lichrosorb Si-60 7-μm (10 mm × 250 mm) column (Merck); flow rate was 2 mL/min. Each separated product was identified by IR and GC/MS. GC/MS analysis (MS-30/70, VG Analytical, Manchester, England) was performed on a 30-m SP-2380 BP capillary column (Supelco), ionization potential 70 eV. Hydroperoxides were reduced with $NaBH_4$ and analyzed, after hydrogenation with H_2/PtO_2, as trimethylsilyl derivatives (BSTFA was used as reagent). The capillary column was operated isothermally at 190 °C. Ketodienes were analyzed, without derivatization, isothermally at 200 °C during 5 min and then programmed at 2 °C/min to 250 °C.

RESULTS

Gel chromatographic separation of the 30–60% ammonium sulfate precipitate of extracts from both kidney beans and peas on Sephacryl S-300 resulted in a single lipoxygenase-active peak. The active fractions, when applied to the DE-52 ion-exchange column, were resolved into two active peaks, LOX-1 and LOX-2. The elution profile with pea lipoxygenases was similar to that obtained by Yoon and Klein (1979). In the case of kidney bean the two isoenzymes were further purified by FPLC on a Mono Q column, the 280-nm absorbance profile from a representative elution is shown in Figure 1. Regarding the FPLC profiles and kinetics of product formation (Figure 2), both isozymes were considered free enough of contamination for our objectives.

With linoleic acid as a substrate the pH optima were as follows: for KB-LOX-1 and KB-LOX-2, pH 5.7; for P-LOX-1, pH 5.8; and for P-LOX-2, pH 6.3. The kinetics of formation of hydroperoxides, 234-nm absorbance (Axelrod et al., 1981), and ketodienes, 274-nm absorbance (Vioque and Holman, 1962) and the absorption spectra of

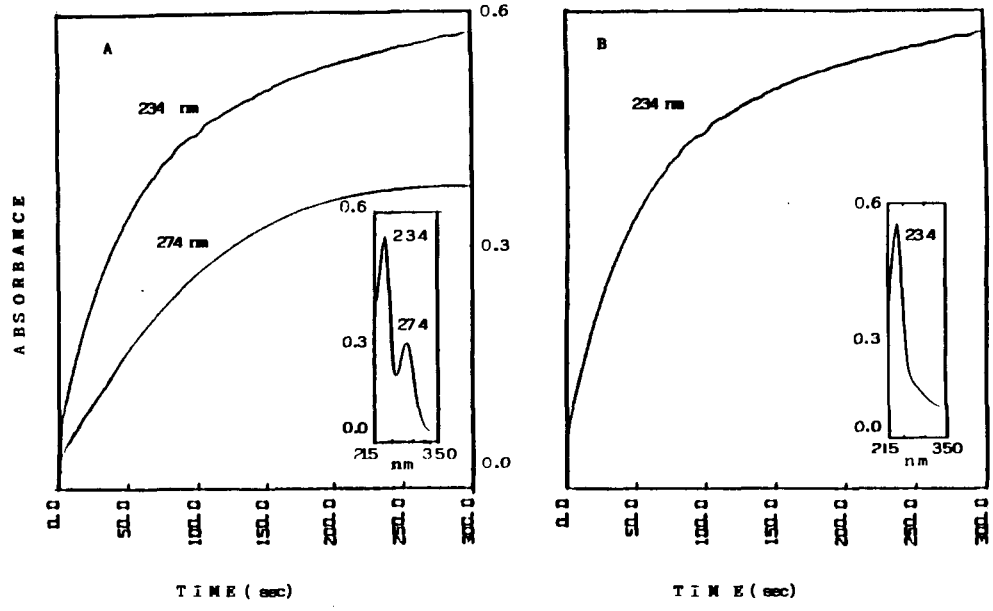

Figure 2. Rate of formation of 234- and 274-nm-absorbing products formed from linoleic acid by KB-LOX-1 (A) and KB-LOX-2 (B). (Inset) Ultraviolet spectra of products.

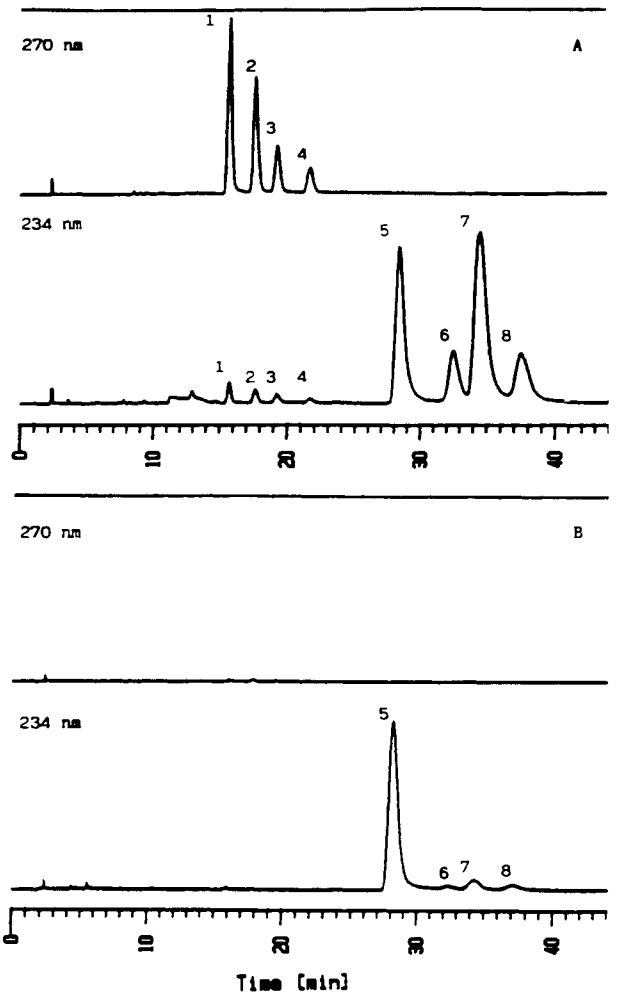

Figure 3. HPLC chromatograms obtained with oxidation products of linoleic acid by pea lipoxygenase isozymes (A) P-LOX-1 and (B) P-LOX-2. Peaks 1–4 were identified as ketodienes and peaks 5–8 as hydroperoxides; see Table I for identification.

the products generated from linoleic acid by kidney bean lipoxygenase isoenzymes are given in Figure 2 (with pea isozymes similar results were obtained). It is evident that LOX-1 produced both compounds while LOX-2 formed exclusively the conjugated hydroperoxydiene and none of the 274-nm-absorbing products. It may be noted that the rate of the reaction falls off rapidly with time. Similar observations had been made with animal and plant lipoxygenases and attributed to inactivation of the enzyme by the hydroperoxides.

The absorption at 278 nm of reaction products was shown to be due to the formation of ketodienes (Vioque and Holman, 1962). To establish that the 274-nm absorption in our study was due to ketodiene formation, reduction with $NaBH_4$ was carried out. Upon reduction, the 274-nm peak disappeared completely and the intensity at 232 nm increased; this is attributed to the formation of the hydroxy conjugated dienes from the ketodienes (Vioque and Holman, 1962).

For characterization of the specificity for the site of O_2 insertion, linoleic acid was used as substrate. To eliminate the interference coming from isomerization, the enzymatic oxidation in oxygen-saturated buffer, extraction, and methylation were carried out swiftly at low concentration and temperature, and the determination of the isomeric ratio was performed immediately by HPLC. Figure 3 shows chromatograms of the oxidation products from linoleic acid catalyzed by P-LOX-1 (A) and P-LOX-2 (B). Peaks 5 and 7 had retention times identical to those of 13- and 9-hydroperoxide isomers prepared according to the methods of Hamberg and Samuelsson (1967) and Galliard and Phillips (1971), respectively. To obtain evidence on chemical structure, the products were collected separately from HPLC.

The compounds from peaks 1–4 gave positive reaction (orange color) with 2,4-dinitrophenylhydrazine; the ultraviolet absorption spectrum had high absorption at 270–274 nm, and IR spectra maxima at 1690 and 1638 cm^{-1} indicated the presence of an unsaturated ketone. Products in peaks 1 and 2 absorbed slightly at 964 and 997 cm^{-1}, suggesting cis,trans geometric isomers, while peaks 3 and 4 absorbed strongly at 1002 cm^{-1}, trans,trans isomers. These isomers were analyzed without derivatization by GC/MS; spectra of peaks 1 and 3 rendered characteristic fragments (McLaffertty rearrangements) at m/z 252 [CH_2=COHCH=CHCH=CH$(CH_2)_7$COOCH_3] for the 13-isomers, while spectra of peaks 2 and 4 showed diagnostic fragments at m/z 166 [$CH_3(CH_2)_4$-CH=CHCH=CHCOH=CH_2] for the 9-isomers.

The products from peaks 5–8 absorbed strongly in UV at 232–234 nm, suggesting the presence of hydroperoxides. After reduction with $NaBH_4$, the maximum absorption was at 230–232 nm; this is attributed to the formation of the hydroxy conjugated diene from the hydroperoxides. The IR spectra of reduced compounds had bands at 3460 and 3250 cm^{-1} associated to hydroxyl absorption in all of them; the diene conjugation was evidenced by the maxima 950 and 990 cm^{-1}. The IR spectra of peaks 5 and 7 had absorption bands at 951 and 986 cm^{-1}, indication of cis,-trans isomers, while for peaks 6 and 8 spectra showed a single high-intensity band at 990 cm^{-1}, suggesting trans,-trans isomers. The final proof of the structures was provided by GC/MS. Reduced and hydrogenated hydroperoxides yield methyl hydroxystearates which were converted into trimethylsilyl ethers. The TMSi derivatives prepared from the presumed 13-isomers, peaks 5 and 6, gave rise to the expected ions, due to α,α'-fragmentation of the TMSi group, m/z 173 [$CH_3(CH_2)_4CHOSi(CH_3)_3$] and 315 [$(CH_3)_3SiOCH(CH_2)_{11}COOCH_3$]; in the spectra of peaks 7 and 8, ions m/z 229 [$(CH_3(CH_2)_8CHOSi(CH_3)_3$] and 259 [$(CH_3)_3SiOCH(CH_2)_7COOCH_3$] proved the presence of 9-isomers.

The percentages of isomers found in incubations of linoleic acid with kidney bean and pea lipoxygenase isoenzymes are shown in Table I. In both seeds LOX-2 produced only hydroperoxides, showing a high regiospecificity since it formed predominantly 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid. From the data shown in this table it is evident that LOX-1 from kidney bean and pea exhibits less regiospecificity and geometric specificity than LOX-2 inasmuch as it produces nearly similar proportions of 9- and 13-isomers of ketodienes and hydroperoxides in both cases with high levels of trans,-trans isomers.

Although the primary products of lipoxygenase isoenzyme reaction are hydroperoxides, the formation of ketodienes raises the question of whether preformed hydroperoxides can serve as substrates for the LOX-1 reaction. To test this possibility, [1-^{14}C]-13-hydroperoxylinoleic acid was prepared from pure soybean lipoxygenase, isozyme L_1 (Hamberg and Samuelsson, 1961). Experiments carried out by incubating radiolabeled hydroperoxide and linoleic acid with LOX-1 isoenzymes, as stated under Experimental Procedures, produced ketodienes, but they are not radiolabeled. TLC radioactive scanning showed that no radioactive band was detected

Table I. Product Specificity of Kidney Bean (KB) and Pea (P) Lipoxygenase Isozymes with Linoleic Acid as Substrate

product	HPLC peak	LOX-1,[a] %		LOX-2,[a] %	
		KB	P	KB	P
13-keto-*cis*-9,*trans*-11-octadecadienoic acid	1	42.26	43.67		
13-keto-*trans*-9,*trans*-11-octadecadienoic acid	3	12.50	7.80		
9-keto-*trans*-10,*cis*-12-octadecadienoic acid	2	33.77	42.46		
9-keto-*trans*-10,*trans*-12-octadecadienoic acid	4	11.46	5.87		
13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid	5	48.46	24.65	83.23	90.64
13-hydroperoxy-*trans*-9,*trans*-11-octadecadienoic acid	6	7.35	9.03	3.72	1.82
9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic acid	7	32.90	50.43	8.02	4.94
9-hydroperoxy-*trans*-10,*trans*-12-octadecadienoic acid	8	10.98	15.88	5.01	2.58

[a] Percentage within each class of products; average of three determinations.

at the R_f of ketodienes, and nearly 80% of radioactivity was counted at the origin. This band could be the reaction products of protein with 13-hydroperoxides which could be involved in the inactivation of the enzyme (Cook and Lands, 1975; Regdel et al., 1985); similar results were found with chickpea LOX-1 (Sanz, 1991).

DISCUSSION

Garssen et al. (1971) observed that soybean lipoxygenase, isoenzyme L-1, acting at pH 9 under anaerobic conditions and in the presence of linoleic acid and 13-hydroperoxide gives 13-ketooctadeca-9,11-dienoic acid and the split products pentane and 13-ketotrideca-9,11-dienoic acid. The appearance of ketodiene absorbance at 270–280 nm in lipoxygenase assays had been considered as a diagnostic of insufficient oxygenation. Because so much of the mechanistic work on lipoxygenase has been carried out with soybean isozyme L-1, formation of secondary products has often been referred to as the anaerobic reaction of lipoxygenase, reflecting the fact that secondary products only appear with lipoxygenase isozymes under conditions of low-oxygen tension.

At the present time there is a reasonable consensus regarding the major features of lipoxygenase reaction mechanism: the initial abstraction of a hydrogen atom from the methylene carbon of the substrate leads to formation of an intermediate fatty acid radical-containing species. At ambient atmospheric oxygen concentrations, the normal catalytic cycle of lipoxygenase (hydroperoxides formation) is apparently rapid and efficient enough that dissociation of the intermediate radical species rarely takes place. Under anaerobic conditions, there will be no oxygen available to react with the bound radical, so eventually some of the radicals will dissociate from the enzyme. Being free in solution, this reactive species can now undergo any of the standard secondary reactions.

In our opinion this is only true for some lipoxygenase isozymes since under aerobic conditions other such lipoxygenase isozymes from soybean (Pistorius, 1974), bush bean (Hurt and Axelrod, 1977), pea (Yoon and Klein, 1979), bengal gram (Borthakur et al., 1986), and chickpea (Sanz et al., 1992) form ketodiene. The ability of some lipoxygenase isozymes to catalyze the formation of secondary products under aerobic conditions suggests that the enzyme-bound radical intermediates formed during its reaction sequence are more readily able to dissociate from the enzyme (Siedow, 1990). The incubation conditions used in this work (oxygen saturation buffer, 20 °C temperature, low concentration of substrate, and continuous flow of oxygen) suggest that the so-called secondary products, 9- and 13-ketodiene isomers, probably arise from true aerobic enzymatic reaction.

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