ARTICLE

Formation of Octadecadienoate Dimers by Soybean Lipoxygenases

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The aim of this investigation was to determine whether the regioselectivity found for lipoxygenases in the formation of fatty acid hydroperoxides from linoleic acid is reflected in the formation of dimeric products in secondary reactions involving linoleic acid, product hydroperoxide and lipoxygenase. A method was therefore developed for the separation and identification of dimers formed by fusion of two linoleic acid radicals or a linoleic acid radical and linoleate. The method includes solid-phase extraction, preparative separation of products by thin-layer chromatography, derivatization to the corresponding fully hydrogenated methyl esters and capillary gas chromatography (GC) coupled with electron impact mass spectrometry. We present evidence that the formation of octadecadienoate dimers, during the secondary reaction of soybean lipoxygenase-1 or lipoxygenase-3, is a nonenzymic process that can be envisaged by nonspecific association of intermediate fatty acid radicals (L•) that have dissociated from the enzyme. We could show that the relative amounts of different octadecadienoate dimers formed remain unaltered, regardless of pH and type of soybean isoenzyme used. Quantitative analysis by GC showed that under the reaction conditions used, the formation of dimers branching at the 13-position is preferred.
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Lipoxygenases (LOX; linoleate:oxygen oxidoreductases, EC 1.13.11.12) comprise a class of enzymes that catalyze the regio- and stereoselective dioxygenation of polyunsaturated fatty acids containing one or more (1*Z*,4*Z*)-pentadiene systems. The products are chiral *Z,E*-conjugated fatty acid hydroperoxides. In mammalian tissues, LOX are involved in the initial steps of the biosynthesis from arachidonic acid of physiologically active compounds, including leukotrienes and lipoxins (1). In plants, these enzymes have been suggested to play a role in germination and possibly in the formation of anti-pathogens (2,3). Furthermore, LOX has been shown to be involved in the biosynthesis of 12-oxo-phytodienoic acid (4), a precursor of jasmonic acid (5). Considerable attention has been paid to the plant LOX, and in particular the soybean isoenzymes have been thoroughly investigated.

Three distinct types of LOX isoenzymes can be purified from soybeans, usually designated as LOX-1, LOX-2 and LOX-3. These isoenzymes differ with respect to isoelectric point, reactivity toward substrates, and optimal reaction conditions. The product of the aerobic reaction of LOX-1 with linoleic acid is almost exclusively 13*S*-hydroperoxy-9*Z*, 11*E*-octadecadienoic acid, while with LOX-2 and LOX-3, regio- and stereospecificity are less pronounced. Privett *et al.* (6) have reported that in addition to this primary reaction, lipoxygenases are also capable of performing a secondary reaction during which, among others, polymeric compounds are formed. Garssen *et al.* (7) were the first to identify the mechanism of this secondary reaction in which dimeric compounds, oxodienoic acids and *n*-pentane are formed *via* a coupled enzymic reaction of linoleic acid and product hydroperoxide. LOX-1 shows this secondary reaction only under anaerobic conditions (7). With LOX-3 it can occur both under aerobic and anaerobic conditions whereas LOX-2 shows very little formation of such products under any condition (8). In previous studies only limited information on the structures of the linoleic acid radical-derived dimeric products that are formed during the secondary reaction of lipoxygenases with linoleic acid could be obtained (9,10). This was mainly due to the lack of suitable methods for the separation and analysis of the various positional isomers. The aim of this investigation was to determine whether the regiospecificity found for soybean LOX in the formation of fatty acid hydroperoxides is reflected in the formation of dimeric products. To this end, the positional isomers of the octadecadienoate dimers were completely separated and analyzed.

MATERIALS AND METHODS

LOX-1 and LOX-3 were isolated from soybeans (11,12). Specific activities, as measured polarographically using a thermostated Clark-type oxygen electrode (Hansatec, King's Lynn, United Kingdom) were 230 μmol min^{-1} for LOX-1 (pH 9.0) and 25 μmol min^{-1} mg^{-1} for LOX-3 (pH 6.6). Regiospecificity of the different isoenzymes was determined by performing small-scale (10 mL) incubations with linoleic acid (100 μM) dissolved in oxygen-saturated 0.1 M sodium borate (pH 9.0) or 0.1 M sodium phosphate buffer (pH 6.6) at 25°C. After acidification of the reaction mixtures to pH 3.0 followed by extraction with octadecyl reversed-phase extraction columns (J.T. Baker, Deventer, The Netherlands), the crude hydroperoxides were analyzed by reversed-phase high-performance liquid chromatography on a CP-Spher-C18 column (5 μm, 4.6 × 250 mm) (Chrompack, Middelburg, The Netherlands) with tetrahydrofuran/methanol/water/acetic acid (25:30:45:0.1, by vol) at a flow rate of 1.0 mL min^{-1} as eluent. Detection was by a ultraviolet (UV) detector (Kratos Spectroflow 783, Separations, H.-I. Ambacht, The Netherlands) set at 234 nm. All preparative reactions were carried out at 25°C, by incubation of linoleic acid (500 μM) with either LOX-1 (36 nM) or LOX-3 (200 nM) in 250 mL air-saturated 0.1 M sodium borate (pH 9.0) or 0.1 M sodium phosphate buffer (pH 6.6), using reaction flasks with an airtight seal cap ([O_2] = 240 μM). The appearance of secondary reaction products was monitored by the change in absorbance at 285 nm, corresponding to the absorption maximum of oxodienoic acids, in a Hewlett-Packard model 8450A UV-visible spectrophotometer (Hewlett-Packard, Amstelveen, The Netherlands).

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Abbreviations: GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; LOX, lipoxygenase(s); TLC, thin-layer chromatography; UV, ultraviolet.

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The reaction mixtures were acidified and extracted as described above. The free fatty acids were esterified by reaction with diazomethane and separated by preparative thin-layer chromatography (TLC) on precoated plates (Silicagel 60 F_{254}, 0.5 mm, E. Merck, Darmstadt, Germany) using the solvent system *n*-hexane/diethyl ether (40:60, vol/vol). The fractions, containing dimers were collected and, after extraction with methanol, hydrogenated over a platinum catalyst (PtO_2, MeOH). The hydrogenated fractions were analyzed on a Hewlett-Packard model 5890 series-II gas chromatograph equipped with a split injector. Gas chromatography (GC) was done under isothermal conditions for 2 min at 280°C followed by temperature programming to 320°C at 2°C min^{-1} using an HT-5 fused silica capillary column (25 m × 0.33 mm i.d., 1-μm phase thickness, Siloxane-Carborane, 5% phenyl equivalent; SGE, Ringwood Victoria, Australia) at a column pressure of 5 kPa with He as carrier gas (flow rate, 2 mL min^{-1}). The injector temperature was kept at 300°C. The column outlet was connected directly to the ionization source of a JEOL AX 505-W mass spectrometer (JEOL Ltd., Tokyo, Japan), operated at an ionization energy of 70 eV and kept at a temperature of 230°C. Gas chromatography/mass spectrometry (GC/MS) scanning was from *m/z* 35–700, and the following ions were selected in the mass chromatogram: *m/z* 297, 381, 409, 437, 463, 467, 491 and 594 (scan rate, 1 s^{-1}). The relative amounts of different positional isomers were determined by integration of the mass chromatograms.

RESULTS AND DISCUSSION

By use of a high-temperature capillary column (HT5) for the purification of the octadecadienoate dimers formed during the secondary reaction of soybean LOX, we were able to completely separate and analyze all positional isomers, as can be judged from the mass chromatograms obtained for the reaction of LOX-1 at pH 9.0 (Fig. 1). The mass spectra were collected at the center of each peak. Each fragmentation pattern is indicative of one type of linkage between the C_{18}-chains of the dimeric compounds only (Fig. 2 and Table 1). In addition to the major peaks, a small shoulder eluting at 10.7 min can be observed (Fig. 1, Trace 1). The mass chromatograms (Fig. 1) suggest that this shoulder could stem from a very minor amount of C(11)-C(11′)-linked positional isomer since the mass fragments characteristic for compounds branching at C(11) were more abundant, as compared to the spectra B [C(9)-C(11′)isomer] and D [C(11)-C(13′)-isomer] (Fig. 2). At least five positional isomers are formed during the secondary reaction of LOX-1 at pH 9.0 [C(9)-C(9′); C(9)-C(11′); C(9)-C(13′); C(11)-C(13′); C(13)-C(13′) and possibly a very minor amount of C(11)-C(11′)]. This finding extends the results reported by Garssen *et al.* (9), who detected only four of the positional isomers under identical reaction conditions and did not report any C(9)-C(9′)-linked compounds. The authors suggested that the formation of octadecadienoate dimers involves nonenzymic processes that can be conceived as a coupling of either two enzymically formed linoleic acid radicals or an enzymically formed linoleic acid radical and linoleate in the proximity of the enzyme (9). Studies on the reticulocyte enzyme corroborate the proposal of nonspecific association of linoleic acid radicals or a linoleic acid radical and linoleate (13).

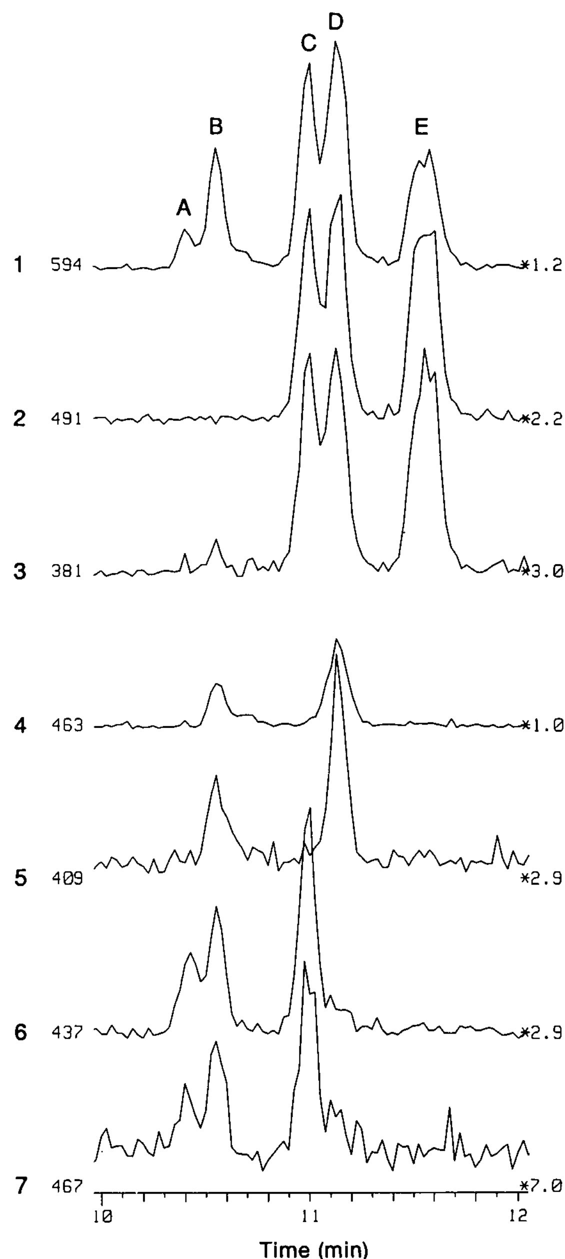

FIG. 1. Single-ion chromatograms from the gas chromatography/mass spectrometry analysis of octadecadienoate dimers from the reaction of lipoxygenase-1 with linoleic acid at pH 9.0. Trace 1: *m/z* 594, indicative of octadecadienoate dimers in general; Trace 2: *m/z* 491, indicative of C(13); Trace 3: *m/z* 381, indicative of C(13); Trace 4: *m/z* 463, indicative of C(11); Trace 5: *m/z* 409, indicative of C(11); Trace 6: *m/z* 437, indicative of C(9); Trace 7: *m/z* 467, indicative of C(9). A: C(9)-C(9′)-isomer; B: C(9)-C(11′)-isomer; C: C(9)-C(13′)-isomer; D: C(11)-C(13′)-isomer; E: C(13)-C(13′)-isomer.

Our results show that the relative amounts of isomeric octadecadienoate dimers formed during the secondary reactions of LOX-1 and LOX-3 (Fig. 3) remain unaltered, regardless of the regiospecificities found under aerobic conditions. This observation, and the fact that all positional isomers that could be expected from the reaction of free linoleic acid radicals in solution are formed, further corroborates the notion of nonenzyme controlled dimer formation. As only a very small amount of the

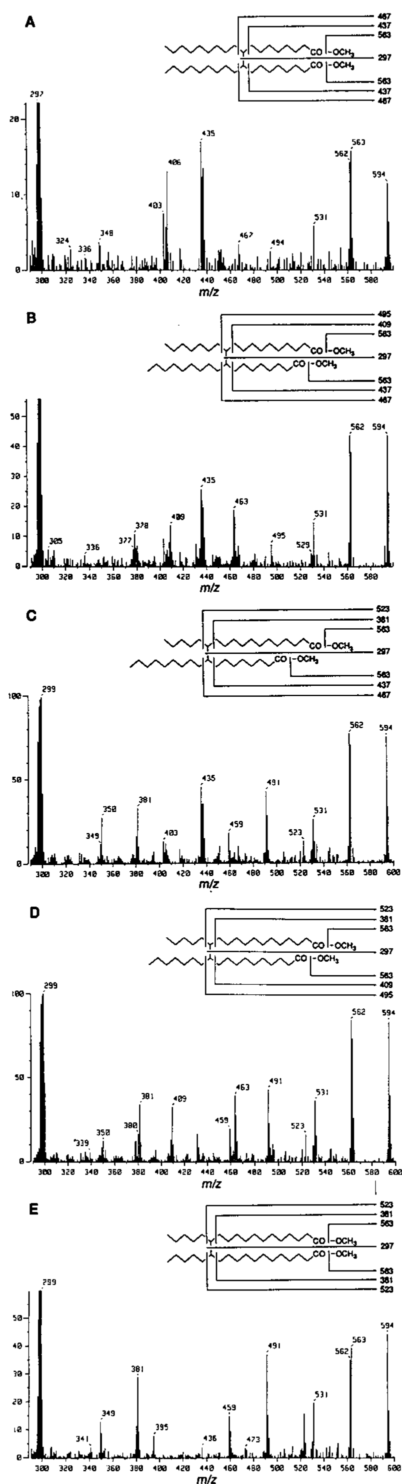

FIG. 2. Mass spectra of the regioisomeric octadecadienoate dimers from the secondary reaction of soybean lipoxygenase-1 with linoleic acid at pH 9.0. Lettering corresponds to that of the peaks in Figure 1 (Trace 1).

C(11)-C(11′)-linked isomer is produced, whereas the C(9)-C(11′) and the C(13)-C(11′)-linked isomers are among the major products, it can be concluded that the latter positional isomers are formed by the reaction of a free linoleic

TABLE 1

Characteristic Fragment Ions in the Mass Spectra of Esterified and Hydrogenated Octadecadienoate Dimers[a]

	m/z	m/z $-CH_3OH$	m/z $-2 \times CH_3OH$
$M^{+\bullet}$	594	562	
$[M - CH_3O]^+$	563	531	
$[M - C_5H_{11}]^{+a}$	523	491	459
$[M - C_7H_{15}]^{+b}$	495	463	431
$[M - C_9H_{19}]^{+c}$	467	435	403
$[M - C_7H_{14}CO_2CH_3]^{+c}$	437	405	
$[M - C_9H_{18}CO_2CH_3]^{+b}$	409	377	
$[M - C_{11}H_{22}CO_2CH_3]^{+a}$	381	349	
$1/2\ M^+$	297	265	

[a]Mass fragments characteristic for: [a]C(13)-branching; [b]C(11)-branching; [c]C(9)-branching.

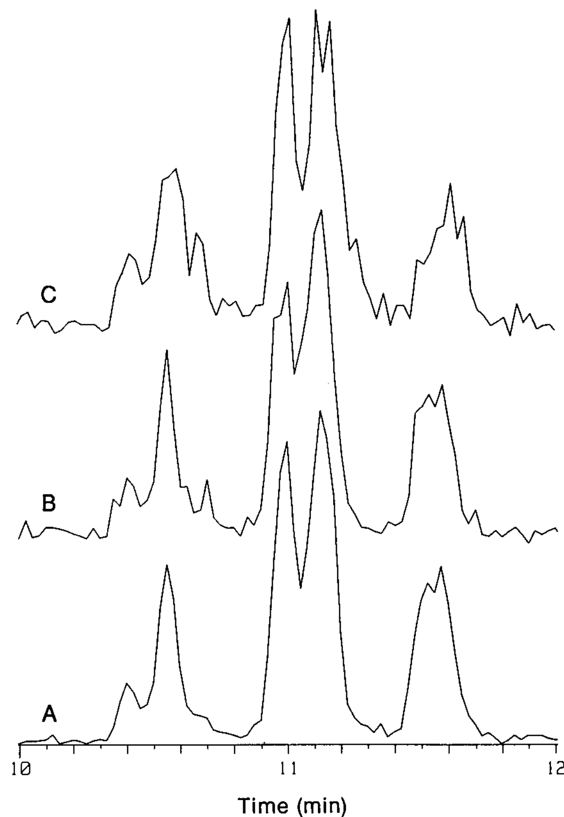

FIG. 3. Single-ion chromatograms (m/z 594) from the gas chromatography/mass spectrometry analysis of octadecadienoate dimers from the secondary reactions of lipoxygenases with linoleic acid under different conditions. Trace A: lipoxygenase-1/pH 9.0; Trace B: lipoxygenase-1/pH 6.6; Trace C: lipoxygenase-3/pH 6.6

acid radical with linoleate in solution. This is in agreement with the observation that only coupling to the C(13) and the C(9) positions of the linoleic acid radicals occurs in experiments in which the water-soluble radical scavenger 2-methyl-2-nitrosopropanol was used (14). Quantitative analyses of the octadecadienoate dimers show that in all cases dimeric compounds linked *via* C(13) are more abundant than those linked *via* C(9)-branched isomers (Table 2). Chan and Newby (15) have reported similar results for hemoprotein or metal-ion catalyzed peroxidations of linoleic acid in aqueous solution. These authors found that under a variety of reaction conditions13-hydroperoxylinoleic

TABLE 2

Percentage of Positional Isomer Branching Under Different Reaction Conditions[a]

	LOX-1/pH 9.0	LOX-1/pH 6.6	LOX-3/pH 6.6
C(9)-branching	36.3	37.3	38.8
C(13)-branching	63.7	62.7	61.2

[a]Values were obtained by integration of mass chromatograms. LOX, lipoxygenase.

acid constitutes 65–72%, and 9-hydroperoxylinoleic acid constitutes 28–35% of the total amount of hydroperoxy compounds formed in nonenzymic oxidations of linoleic acid. This result, and the observation that regioselectivity is greatly diminished when methyl linoleate was used as a substrate instead of the free acid, or when linoleic acid was oxidized in organic solvents, led them to suggest that the relative reactivity of C(9) and C(13) of linoleic acid in aqueous media is determined by the carboxylate group. The results reported here reflect a similarly remarkable deviation from the occurrence of C(13)- and C(9)-derived products in a 1:1 ratio.

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