Conversion of Linoleic Acid Hydroperoxide by Soybean Lipoxygenase in the Presence of Guaiacol: Identification of the Reaction Products[1]

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ABSTRACT

Linoleic acid hydroperoxide formed by soybean lipoxygenase was metabolized by the same enzyme in the presence of guaiacol. The products of this reaction included trihydroxyoctadecenoic acids, hydroperoxydihydroxyoctadecenoic acids, hydroxyepoxyoctadecenoic acids, dihydroxyoctadecenoic acids, hydroxyoctadecadienoic acids, and oxooctadecadienoic acids.

INTRODUCTION

Lipoxygenase (E.C. 1.13.11.12.) is an enzyme which has been found in many plant sources. It is present in high concentrations, especially in cereals and legumes (1,2). The enzyme is known to catalyze the oxidation of essential unsaturated fatty acids with oxygen from air forming hydroperoxides with *trans-cis* conjugated double bonds. In 1966, Zimmerman (3) found an isomerase in linseed which converted linoleic acid hydroperoxide (LOOH) to an unsaturated α-ketol. Similar enzymes were later detected in barley, wheat, soybeans, corn, and mung beans by Zimmerman and Vick (4). Gardner (5) investigated this reaction in corn, and Graveland (6-8) reported the formation of an abundance of compounds arising after enzymatic LOOH formation during the incubation of linoleic acid with flour suspensions from wheat, rye, barley, oats, and corn. The enzymes participating in the courses of these reactions are unknown, but in wheat, Graveland (9) found that when lipoxygenase was adsorbed on glutenin, the formation of trihydroxy derivatives was favored. Heimann, et al., (10-12) described a lipoperoxydase in oats, which reduced LOOH to the corresponding hydroxyoctadecadienoic acids.

As early as 1943, Balls, et al., (13) reported that crude preparations of soybean lipoxygenase destroyed the previously formed LOOH slowly. It was shown later (14,15) that purified enzyme preparations destroyed LOOH only in the presence of a cofactor, guaiacol, and that it was impossible to separate lipoxygenase activity from the LOOH destroying factor.

The present report describes the formation and structures of compounds formed from LOOH in the presence of soybean lipoxygenase and guaiacol.

MATERIALS AND METHODS

Enzyme and Substrate

Soybean lipoxygenase was prepared from soybeans as reported earlier (15). We used enzyme L-1 according to the nomenclature of Christopher, et al., (16). The substrate was either [1-^{14}C] linoleic acid (Amersham Buchler, Braunschweig, West Germany) diluted with unlabeled material (Roth, Karlsruhe, West Germany) to 30 μCi/mol or [1-^{14}C] linoleic acid hydroperoxide. These substrates did not show any detectable chemical or radiochemical impurities in our chromatographic systems.

LOOH was prepared by incubation of 0.4 g linoleic acid with soybean lipoxygenase (2 portions of 2 mg, the second portion being added 15 min later) at 3 C in sodium borate buffer (0.05 M, pH 8.5). Oxygen was bubbled through the solution during the time of incubation. After 30-40 min, the mixture was acidified with 2N HCl to pH 2-3 and extracted twice with diethyl ether. The ether layer was washed with water until neutral and dried over anhydrous sodium sulfate. The solvent was evaporated in vacuo, and the residue subjected to preparative thin layer chromatography (TLC) using diethyl ether:heptane:acetic acid (50:50:1, v/v/v) as developing solvent mixture. After detection under ultraviolet (UV) light, the band containing the LOOH was scraped off, eluted with diethyl ether, and finally dissolved in ethanol. The concentration of the LOOH was determined photometrically at 234 nm using 25,000 $mol^{-1} cm^{-1}$ as the extinction coefficient.

Conversion of LOOH and Isolation of Products

For preparative isolation of the degradation products, 4 x 10^{-5} moles LOOH or linoleic acid were incubated with 2 mg lipoxygenase in 2

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liters sodium borate buffer (0.05 M, pH 8.5) containing guaiacol (20 mM). To follow the reaction, aliquots of 3 ml were removed and the change in absorbance at 234 nm was recorded.

When no further change in absorbance occurred, another portion of LOOH was added. When the reaction was complete, an additional 1-2 mg lipoxygenase was added and the process was repeated 3-4 times. The total process took ca. 4-5 hr. The reaction mixture was subsequently acidified to pH 2-3 with 2N HCl and extracted twice with diethyl ether. The ether phases were washed with water until neutral, dried over anhydrous sodium sulfate, and evaporated under reduced pressure. The residue was treated with diazomethane and subjected to TLC using double development with acetone:heptane (1:2, v/v).

Chromatographic Methods

TLC was carried out on glass plates (40 x 20 cm or 20 x 20 cm) coated with 0.5 or 0.25 mm Silica Gel HF_{254} (Merck, Darmstadt, West Germany). Methyl esters of the reaction products were separated by double development with acetone:heptane (1:2, v/v) as solvent as shown in Figure 1a. For further purification of single fractions of methyl esters, solvent mixtures of diethyl ether and heptane were used.

Spots and bands were located by spraying with 50% H_2SO_4 and heating to 120 C for 10 min. Carbonyl compounds were seen as yellow spots after spraying with 0.05% 2,4-dinitrophenylhydrazine (DNPH) in 30% $HClO_4$. Peroxides were detected as violet spots by spraying a KI solution followed by 1% starch solution. The KI solution had been freshly prepared from 40 mg KI in 10 ml water mixed with 5 ml acetic acid and a small amount of Zn powder. This solution was filtered immediately before use.

Radioactivity assay of TLC plates was performed with a thin layer scanner LB 2720 (Berthold, Wildbad, West Germany).

GLC was performed with a Hewlett Packard gas chromatograph 7620 A and stainless steel columns (diameter 1/8 in., length 6 ft) packed with 3% JXR on Gaschrom Q 100/120 mesh at 210 C isothermally. Carrier gas was helium at a flow rate of 35 ml/min. All compounds were chromatographed as methyl esters and as trimethylsilyl ethers if hydroxyl groups were present in the molecule.

Diazomethane in diethyl ether containing 10% methanol was used for methylation of carboxylic acids (17). Silylation prior to GLC analyses was carried out on 10-50 μg of the methyl esters with 40 μl bis(trimethylsilyl)trifluoroacetamide (BSTFA) in 20 μl pyridine at 70 C for 10 min. The resulting mixture of silylated compounds was injected directly.

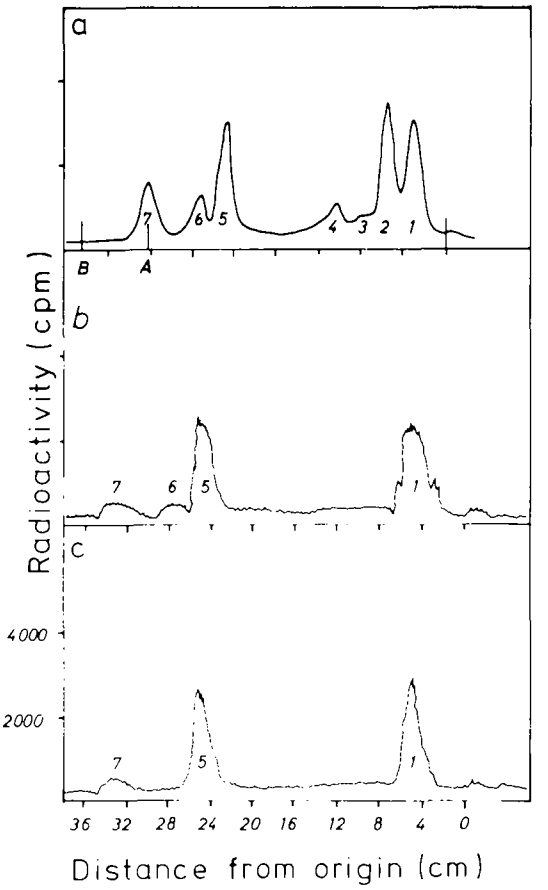


FIG. 1. Thin layer chromatograms of the methyl esters of the reaction mixture on Silica Gel HF_{254}, double development with acetone:heptane (1:2, v/v). A = Front of first development; B = front of second development. a = Untreated mixture, b = mixture after treatment with $SnCl_2$; c = mixture after treatment with $NaBH_4$. The numbers correspond to the compounds shown in Figure 4.

Infrared, Ultraviolet, and Mass Spectral Analyses

Infrared (IR) spectra were obtained with a Perkin Elmer Infracord Spectrophotometer 157E. Spectra were taken mostly from liquid films. UV measurements were obtained with a Zeiss PMQ-2 spectrophotometer. Mass spectra were recorded with the double focusing mass spectrometers Varian MAT CH5-D and Varian MAT 311 coupled with a gas chromatograph Varian 2700. For the coupling, a dual stage Helium separator (Watson-Biemann type) was used. The temperature was 210 C in the gas liquid chromatography (GLC) column as well as

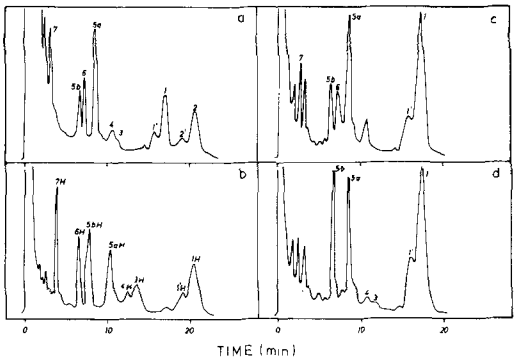


FIG. 2. Gas liquid chromatograms of the silylated methyl esters of the reaction mixture on 3% JXR. a = Untreated mixture; b = mixture after hydrogenation; c = mixture after treatment with $SnCl_2$; d = mixture after treatment with $NaBH_4$. The numbers correspond to the compounds shown in Figure 4.

in the separator and the ion source of the mass spectrometer. Ionization was performed by electron impact with an electron energy of 70 eV. The mass spectra were scanned magnetically with a scan rate of 100 m/e per second. A Packard 3375 liquid scintillation counter was used for counting the radioactivity of liquid samples.

Microchemical Methods

Hydrogenation of small samples of unsaturated hydroxy fatty acid methyl esters was performed in 10 ml ethanol with 10 mg PtO_2 as catalyst. Reduction of hydroperoxides and ketones was carried out with an excess of $NaBH_4$ in methanol at room temperature for 30 min.

Reduction of hydroperoxide with an excess of $SnCl_2$ was carried out in methanol.

Periodic acid oxidation was performed on a microscale with isolated fractions from TLC separations. The substance (0.1-0.5 mg) was dissolved in 1 ml acetic acid and mixed with 0.2 ml of 0.02 M KIO_4 solution. The mixture was stirred 60 min at room temperature. After 30 min of the reaction time, one drop of ethylene glycol was added. The oxidation products were converted to 2,4-dinitrophenylhydrazones (DNPs) by adding 2 ml 0.4% DNPH solution in 4N CHl (18). The DNPs were separated by TLC on Silica Gel G with cyclohexane:diethylether: methanol (100:10:2, v/v/v) as solvent. Ozonolysis of linoleic acid was performed with an apparatus constructed as described by Bonner (19). Linoleic acid was dissolved in methyl acetate and cooled to -78 C for ozonolysis. After 5 min, the reaction was stopped and triphenylphosphine was added. The aldehydes formed were converted to 2,4-dinitrophenyldrazones.

Quantitative determination of peroxides was performed in 5 ml 60% methanol. The solution was acidified with 10 µl concentrated HCl, and after 1 min 10 µl 3.6% $FeSO_4$ solution in 3.6% HCl was added. Exactly 30 sec later 0.5 ml of 20% KSCN solution was added; 150 sec after the addition of the KSCN solution, the absorption was measured at 505 nm against a blank. Peroxide values were calculated from a calibration curve obtained with purified LOOH of the same specific radioactivity as the formed reaction products.

RESULTS

Formation and Separation of Reaction Products

Because of strong irreversible substrate inhibition with LOOH concentrations higher than 6×10^{-5} M (15), the reaction had to be carried out in relatively large volumes to obtain sufficient quantities of products. The substrate ([1-^{14}C]LOOH or [1-^{14}C] linoleic acid) was added in 8-10 portions, each subsequent portion being added when the substrate of the previous addition had been fully converted. In this way, the products of the reaction were pooled without causing substrate inhibition. Radio-TLC showed that at least 6 different compounds were formed (Fig. 1a). TLC fraction 7 consisted of linoleic acid and was present only when LOOH was produced from linoleic acid in the same reaction mixture without preceding purification. The main TLC fractions (1,2, and 5) contained ca. equal amounts of radioactivity with little variation in different incubations. A difficult problem in the purification of the radioactively labeled products was the large number of mainly yellow compounds, which were formed from guaiacol during the course of the reaction. These compounds were distributed from TLC fraction 1 to fraction 5, making it difficult to use IR, nuclear magnetic resonance (NMR), or spray reagents and other chemical methods for the structure elucidation of the radioactively labeled compounds. GLC of the whole reaction mixture (Fig. 2a) was carried out after esterification with diazomethane and silylation. To compare the TLC and GLC peaks, the fractions from TLC were gas chromatographed separately. Each of the TLC fractions 1, 2, and 5 were split into two GLC peaks.

Influence of Different Reaction Conditions

Lipoxygenase isoenzyme L-1 according to Christopher, et al., (16) was used in most of the experiments described here. This isoenzyme, which has a pH optimum of 8.5 for the formation and degradation of LOOH corresponds to

that described by Theorell (20). It was purified and shown to be homogeneous by disk electrophoresis. The reaction of the pure enzyme was compared with that of commercially available soybean lipoxygenase (Fluka, Neu-Ulm, West Germany). Both enzyme preparations showed identical pH optima and product formation. The same reaction product pattern could be obtained by using purified LOOH as well as linoleic acid as substrate. When LOOH was prepared in situ, the only difference found was that some unchanged linoleic acid was left in the reaction mixture (Fig. 1a).

The reaction took place even with very low oxygen concentrations. In preliminary experiments, we found that when the reaction was carried out under nitrogen the usual products were formed. Experiments with $^{18}O_2$, however, showed clearly that atmospheric oxygen was taken up during the reaction (Streckert and Stans, unpublished data).

Lipoxygenase isoenzyme L-2 (16) with a pH optimum for the formation of LOOH at 6.5 also catalyzed the degradation of LOOH in the presence of guaiacol. The pH optimum for the degradation was found to be 8.5 as was the case for isoenzyme L-1.

In earlier reports (15), it was shown that lipoxygenase was irreversibly destroyed during the course of the reaction. The enzyme is destroyed only when both LOOH and guaiacol are present, because preincubation with either LOOH or guaiacol separately did not lead to significant changes in enzyme activity.

Although the physiological role of this reaction is unknown, it is of interest that when the reaction is carried out in soybean meal suspensions the enzyme is active for several hr (Streckert and Stan, unpublished data). Under these conditions, most of the products were identical to those produced in the reactions described above.

Identification of Reaction Products

Determination of functional groups. Fluorescence quenching on developed TLC plates showed 3-6 distinct bands indicating compounds with conjugated double bonds. Because of interference with products from guaiacol, this indication was unambiguous only for TLC fraction 6. Spraying with I_2:starch gave a strong reaction with TLC fraction 2 indicating the presence of peroxy groups, and weaker reaction with fraction 5, possibly due to some unconverted LOOH. Spraying with 2,4-dinitrophenylhydrazine showed a broad band due to products from guaiacol and distinct coloring of TLC fraction 6. Upon reduction of the product mixture with $NaBH_4$, TLC fractions 2 and 6

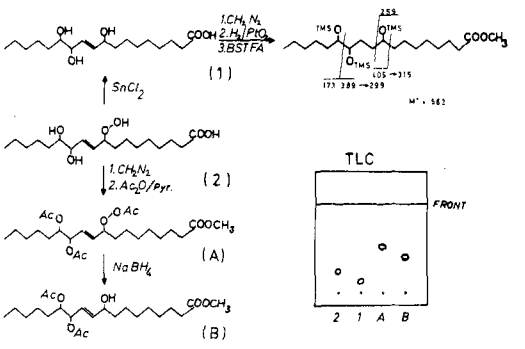


FIG. 3. Reactions carried out on thin layer chromatography fraction 2 on Silica Gel HF_{254} developed with acetone:heptane (2:3, v/v).

disappeared (Fig. 1c) and were not replaced by any new fractions. With the isolated fractions 2 and 6, it could be shown that fraction 2 was converted into fraction 1 and fraction 6 into fraction 5. With $SnCl_2$ TLC fraction 2 again was converted to fraction 1 (Fig. 1b), but fraction 6 remained unchanged.

When the reaction mixture was not silylated, GLC analysis indicated that peaks 6 and 7 remained unchanged, but peaks 1 and 5 were absent. When acetylation was used instead of silylation peaks 1, 1′, 2, 2′, 5a and 5b shifted to slightly higher Rf values (peaks corresponding to TLC fractions 3 and 4 were not clearly detectable because of the low concentrations), whereas, peaks 6 and 7 again remained unchanged. Retention times of hydrogenated products were only slightly different from those of unsaturated compounds except that peaks 2 and 2′ did not appear and peak 6 changed place with peak 5b (Fig. 2b). When TLC fraction 2 was isolated, hydrogenated, and subjected to GLC, it was identical with hydrogenated fraction 1 (Fig. 2b, peaks 1H and 1′H). Reduction with $NaBH_4$ prior to GLC showed that peak 6 was converted to peak 5b, whereas, with $SnCl_2$ peak 6 remained unchanged. Peaks 2 and 2′ disappeared after reduction with either $NaBH_4$ or $SnCl_2$ and were replaced by peaks 1 and 1′ (Fig. 2c and 2d).

The results of these peak shift experiments can be summarized as follows. Peak 7 remained unchanged in all reactions except hydrogenation; it was identified as linoleic acid (Cochromatography and GC-MS). Peak 6 seemed to contain a keto group. All peaks from 1 to 5b contained hydroxyl groups; they all remained unchanged after reduction except for TLC fraction 2. The latter seemed to contain a functional group which could easily be reduced to a hydroxyl group and, therefore, was probably a hydroperoxy or a peroxy group. When TLC

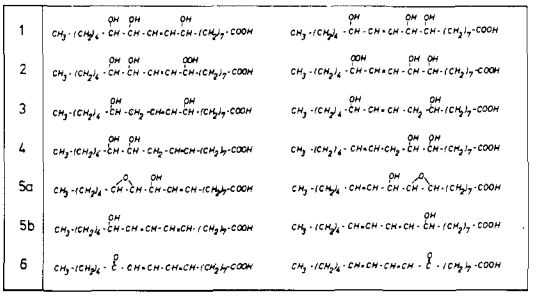


FIG. 4. Structures of reaction products formed from linoleic acid hydroperoxide on incubation with lipoxygenase and guaiacol. **1.** 9,12,13-Trihydroxy-10-*trans*-octadecenoic acid and 9,10,13-trihydroxy-11-*trans*-octadecenoic acid. **2.** 9-Hydroperoxy-12,13-dihydroxy-10-octadecenoic acid and 9,10-dihydroxy-13-hydroperoxy-11-octadecenoic acid. **3.** 9,13-Dihydroxy-10-octadecenoic acid and 9,13-dihydroxy-11-octadecenoic acid. **4.** 12,13-Dihydroxy-9-octadecenoic acid and 9,10-dihydroxy-12-octadecenoic acid. **5a.** 11-Hydroxy-12,13 epoxy-9-*cis*-octadecenoic acid and 11-hydroxy-9,10-epoxy-12-*cis*-octadecenoic acid. **5b.** 13-Hydroxy-9-*cis*-11-*trans*-octadecadienoic acid and 9-hydroxy-10-*trans*-12-*cis*-octadecadienoic acid. **6.** 13-Oxo-9,11-octadecadienoic acid and 9-oxo-10,12-octadecadienoic acid.

fraction 2 was isolated and subjected to GLC, four peaks were found (1, 1′, 2, 2′), showing the high thermal lability of this fraction.

Structures of products. Figure 4 contains all the structures determined for the reaction products. The basis for the assignments of these structures will be discussed below.

TLC fraction 1 consisted of a mixture of 9,12,13-trihydroxy-10-*trans*-octadecenoic acid and 9,10,13-trihydroxy-11-*trans*-octadecenoic acid. Mass spectra of the silylated methyl esters in the GLC peaks 1 and 1′ were identical and showed ions at m/e 545 ($M-CH_3$), 460 (M-hexanal), 387, 301, 297, 259, 211, 173, 155 and ions at m/e 147, 129, 103, 75, and 73, which are typical for trimethylsilyl ether derivatives. The mass spectra are in accordance with those obtained by Graveland (6), Heimann and Dresen (12), Arens and Grosch (21), and Tsuchida, et al., (22) for the same compounds. The hydrogenated compounds showed significant ions at m/e 389, 315, 299, 259, 213, and 173 together with the usual ions for silylated compounds, which is in accordance with spectra reported by Graveland (6). Comparison of the mass spectra of compound 1 before and after hydrogenation showed a double bond between C-9 and C-13. The IR spectrum of TLC fraction 1 showed a strong absorption band at 970 cm^{-1} (*trans* double bond). Several repeated chromatographies of fraction 1 with different mixtures of acetone:heptane gave 0.5 mg of a substance which had a sharp mp at 94 C after three recrystallizations from heptane:acetone. The crystalline substance gave only one peak on GLC (peak 1). The mass spectrum of this substance measured with a direct inlet system was identical with that of peak 1 or 1′ obtained using GC-MS. Periodic acid oxidation carried out on TLC fraction 1 followed by formation of DNP of the cleavage products gave four compounds on TLC. Two of the compounds were ^{14}C-labeled as could be expected from the following cleavage reactions:

$$CH_3\text{-}(CH_2)_4\text{-}CHOH\text{-}CH{=}CH\text{-}CHOH\text{-}CHOH\text{-}(CH_2)_7\text{-}{}^{14}CO_2CH_3 \longrightarrow \quad (I)$$
$$CH_3\text{-}(CH_2)_4\text{-}CHOH\text{-}CH{=}CH\text{-}CHO + \text{(A)}$$
$$OHC\text{-}(CH_2)_7\text{-}{}^{14}CO_2CH_3 \text{ (B)}$$

$$CH_3\text{-}(CH_2)_4\text{-}CHOH\text{-}CHOH\text{-}CH{=}CH\text{-}CHOH\text{-}(CH_2)_7\text{-}{}^{14}CO_2CH_3 \longrightarrow \quad (II)$$
$$CH_3\text{-}(CH_2)_4\text{-}CHO + \text{(C)}$$
$$OHC\text{-}CH{=}CH\text{-}CHOH\text{-}(CH_2)_7\text{-}{}^{14}CO_2CH_3 \text{ (D)}$$

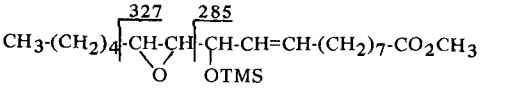
The DNPs of compounds B and C were synthesized by reductive ozonolysis of linoleic acid and used as references.

The DNPs of compounds B (Rf = 0.33) and C (Rf = 0.56) had absorption maxima at 358 nm (DNPs of saturated aldehydes), whereas those of compounds A (Rf = 0.26) and D (Rf = 0.15) had absorption maxima at 371 nm (DNPs of α,β-unsaturated aldehydes). With the crystalline compound (peak 1 on GLC), all four cleavage products were found again, which showed that the separation of TLC fraction 1 on GLC into two peaks (1 and 1′) was not due to a separation of positional isomers, but might be explained by a separation of stereoisomers.

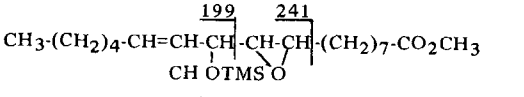
TLC fraction 2 was completely identical (TLC, GLC, GC-MS) with fraction 1 after reduction with $NaBH_4$, $SnCl_2$, or after hydrogenation of both fractions (Fig. 3). Reduction with NaB^2H_4 showed no uptake of deuterium. Quantitative determination of the peroxide content of fraction 2 gave a ratio of 1:1.1 compared with the same amount of LOOH, showing that one hydroperoxy group was present in compound 2. The position of the hydroperoxy group has not yet been definitely determined, but it is most probably attached to carbon atom 9 or 13 as shown in Figure 4. Acetylation followed by reduction with $NaBH_4$ (Fig. 3) and subsequent analysis by GC-MS of the silylated compound gave a significant ion at m/e 259, which is typical for a trimethylsilyl ether group at position 9. Labeling experiments with $^{18}O_2$ also suggest this position (Streckert and Stan, unpublished data).

TLC fraction 5 could be separated into two TLC fractions when rechromatographed using acetone:heptane (3:7, v/v). It also was separated into two peaks (5a and 5b) by GLC.

Fraction 5a consisted of an isomeric mixture of 11-hydroxy-12,13-epoxy-9-*cis*-octadecenoic acid and 11-hydroxy-9,10-epoxy-12-*cis*-octadecenoic acid. The IR spectrum showed bands at 3300 cm^{-1} (hydroxyl), 1750 cm^{-1} (ester), and 890 cm^{-1} (epoxy), but no band at 970 cm^{-1} (*trans* double bond). Mass spectra taken on the ascending GLC peak showed significant ions at m/e 398 (M^+), 383 (M-CH_3), 367 (M-OCH_3), 327 and 285 (base peak), whereas, on the descending peak in addition to the above peaks, ions at m/e 199 (base peak), 241, 211, and 225 were found. The main ions can be explained by the following fragmentations:

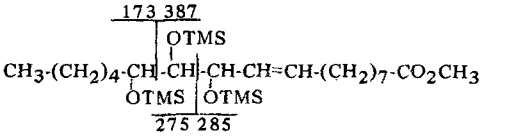


(5a-1) (III)

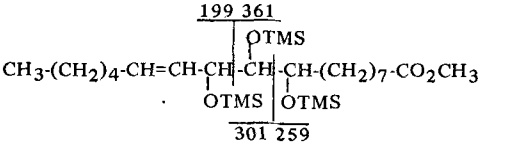


(5a-2) (IV)

Compound 5a-1 was recently described by Hamberg and Gotthammar (23). The mass spectrum reported by these authors is very similar to that obtained on the ascending peak of fraction 5a. When TLC fraction 5a was hydrogenated, significant ions were obtained at m/e 385 (M-CH_3), 369 (M-OCH_3), 287 (base peak), 271, 215, 201, and 183 together with the ions for silylated compounds. The strong ions at m/e 287 and 201 are due to cleavage between the epoxy and the trimethylsilyl ether groups. Comparison of the mass fragmentation pattern of both original and hydrogenated compounds 5a indicated that one double bond is present, which is most probably located between C-12 and C-13 or C-9 and C-10. After hydrolysis with acetic acid (23), TLC fraction 5a gave a more polar compound. On TLC this compound was a little less polar than those in fraction 1, whereas, on GLC it behaved in a similar manner to the compounds in fraction 1. Mass spectra showed significant ions at m/e 460 (M-100, loss of hexanal), 387, 361, 297 (387-90), 285, 275, 271 (361-90), 259, 185 (275-90), 173, and 155. The main ions can be explained by the following fragmentations:



(V)

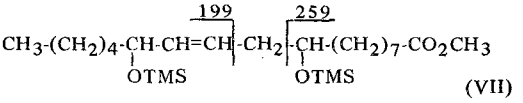


(VI)

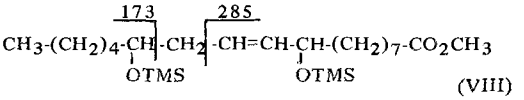
The ions at m/e 199, 301, and 211 (301-90) deriving from the minor compound are weak compared to those of the major compound. For reference we prepared a sample of compound D previously described by Hamberg and Gotthammar (23). This compound was found to be identical (IR, TLC, GLC, and GC-MS) with fraction 5a, but in disagreement with the findings of Hamberg and Gotthammar, the mass spectra showed additional ions at m/e 199 and 241. This might be due to a higher content of 9-LOOH in our preparation. The exact ratio of positional isomers was not determined in this preparation.

Fraction 5b consisted of an isomeric mixture of 13-hydroxy-9-*cis*-11-*trans*-octadecadienoic acid and 9-hydroxy-10-*trans*-12-*cis*-octadecadienoic acid. A sample prepared by reduction of LOOH with $NaBH_4$ was identical to fraction 5b according to UV, TLC, GLC, and GC-MS data. Mass spectra showed significant ions at m/e 382 (M^+), 367 (M-CH_3), 351 (M-OCH_3), 311, 225, 155. Mass spectra recorded on the hydrogenated fraction 5b showed significant ions at m/e 371 (M-CH_3), 355 (M-OCH_3), 339, 315, 259, 229, and 173. The appearance of ions at m/e 173 and 315, as well as 259 and 229, showed that fraction 5b was a mixture of isomers.

TLC fraction 3 behaved on TLC and GLC as expected for a dihydroxy acid. The small amount of material present in fraction 3 was not sufficient for detailed studies. Mass spectra showed significant ions at m/e 472 (M^+), 457 (M-CH_3), 441 (M-OCH_3), 429, 285, 259, 199, 173, and 155. The ions are in accordance with the following scheme of fragmentations:



(VII)



(VIII)

Mass spectra recorded on fraction 3 after hydrogenation showed ions at m/e 459 (M-CH_3), 443 (M-OCH_3), 427, 403, 374 (M-hexanal), 317, 313 (403-90), 259, 227 (317-90), 173, and 155. This clearly established the position of the hydroxyl groups at carbons 9 and 13 and the position of the double bonds.

TLC fraction 4 behaved on TLC and GLC as expected for a dihydroxy acid. The small amount of material again was not sufficient for detailed studies. Mass spectra showed significant ions at m/e 457 (M-CH_3), 441 (M-OCH_3), 382 (M-90), 401, 362 (M-hexanal), 361, 311 (401-90), 299, 275, 271 (361-90), 259, 213, 185 (275-90), and 173. This is in accordance with mass spectra obtained by Graveland (9) for the compounds 4 in Figure 4. The positions of the hydroxyl groups were confirmed by mass spectra of fraction 4 after hydrogenation. They showed ions at m/e 459 (M-CH_3), 443 (M-OCH_3), 374 (M-hexanal), 301, 259, 215, 173, and 155.

TLC fraction 6 was less polar on TLC than fraction 5b, but was eluted later on GLC than 5b. Silylation did not change the retention time on GLC. Reduction with $NaBH_4$ gave a compound, which was identical with fraction 5b according to TLC, GLC, and GC-MS. Treatment with $SnCl_2$ did not have any effect on fraction 6. Analysis of an aliquot by UV spectrophotometry showed an absorption band at 278 nm typical for unsaturated ketones (24). Bands in the IR spectrum (inter alia: 1730 cm^{-1} [methyl ester], 1680 cm^{-1} [unsaturated ketone], 3050, 990, and 960 cm^{-1} [*cis-trans*-conjugated double bond]) and the mass spectrum of fraction 6 was in accordance with that reported by Arens and Grosch (21) for the oxooctadecadienoic acids shown in Figure 4.

The mass spectrum of the fraction after hydrogenation showed ions at m/e 312 (M^+), 281 (M-OCH_3), 256, 241, 200, 185, 170, 150, 114, and 99. This indicated clearly the presence of an isomeric mixture of 9-oxo- and 13-oxo-octadecanoic acid methyl esters (25).

DISCUSSION

Our investigations show that purified soybean lipoxygenase catalyzes a reaction between LOOH and guaiacol, which leads to a number of products, none of which is in predominance. Some of the compounds have been described earlier and are formed enzymatically in plant material. The unsaturated trihydroxy compounds (Fig. 4:1) for example arise from linoleic acid in flours of different cereals. In wheat flour they are the main products, whereas, in flours of rye, oats, and barley they are formed to a lesser extent (8).

The hydroperoxydihydroxyoctadecenoic acid (Fig. 4:2) is an interesting compound which has not been described previously. The proposed structure is based on the ease of reduction to trihydroxyoctadecenoic acid and the occurrence of one hydroperoxy group in the molecule. Because the position of the hydroperoxy group has not yet been determined unequivocally, it is difficult to discuss the mechanism of its formation. It is clear, however, that it cannot arise by isomerization or decomposition, but rather by oxidation of LOOH. Experiments with $^{18}O_2$ confirm that this compound is formed by the incorporation of molecular oxygen into LOOH (Streckert and Stan, unpublished data). Possibly hydroperoxydihydroxyoctadecenoic acid is the precursor of the trihydroxyoctadecenoic acids which can be formed easily by reduction of the hydroperoxy group. In soybean meal suspensions, fraction 2 could not be detected, and the trihydroxyoctadecenoic acids were the main products (Streckert and Stan, unpublished data). This suggests that the hydroperoxy group is rapidly reduced in natural media. Further investigations on the mechanism of formation of the trihydroxy compounds are in progress.

The third group of products, which are present in larger amounts, consisted of hydroxyepoxyoctadecenoic acids (Fig. 4:5a). These compounds have not been detected previously in plant material. Hamberg and Gotthammar (23) have recently described their formation by heating LOOH in aqueous ethanol solution. Obviously, these compounds are quite stable and are hydrolyzed to vicinal trihydroxyoctadecenoic acids only by acetic acid at elevated temperatures. This explains the fact that the vicinal trihydroxy acids were not present in our incubation mixtures.

Monohydroxyoctadecadienoic acids are the main products in oats, where they are formed by a specific lipoperoxidase, which requires reducing agents (10-12). The same compounds were reported to be formed in suspensions of flour from wheat and other cereals by Graveland (8). He suggested, however, that the reduction of the hydroperoxy group was due mainly to nonenzymatic reductions by sulfhydryl groups present in the proteins of cereals. In our system only small amounts of monohydroxyoctadecadienoic acids were formed (Fig. 4:5a). Oxooctadecadienoic acids are known to be by-products in the lipoxygenase reaction (24). Arens and Grosch (21) have found these compounds in peas. Garssen, et al., (26) have reported that 13-oxo-9,11-octadecadienoic acid is formed during the anaerobic

reaction of 13-LOOH in the presence of lipoxygenase and linoleic acid. In the reaction reported here, LOOH was converted to a mixture of 9- and 13-oxooctadecadienoic acids in the absence of linoleic acid as well as in the presence of oxygen. Therefore, the mechanism suggested by Garssen, et al., (26) can be excluded.

Dihydroxyoctadecenoic acids (Fig. 4:3 and 4) were formed only in low concentrations in the reaction reported here, and therefore, could only be studied by GS-MS analysis. The formation of a mixture of vicinal dihydroxy acids in cereals was reported earlier by Graveland, et al., (7) and Arens and Grosch (21), whereas, the 9,13-dihydroxyoctadecenoic acids have not been reported previously.

Remarkably, some of the compounds formed in the reaction with guaiacol and lipoxygenase were found recently in several model reactions with LOOH, i.e., heat treatment in ethanol (23), Fe-ions in 80% ethanol (27), hemoglobin in phosphate buffer (28), and lipoxygenase L-2 from peas at pH 6.5 (21). This suggests that these compounds could be formed in plant material not only by enzymatic reactions, but also by a more general mechanism initiated by the formation of peroxy or oxy radicals from LOOH.

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