Enzymatic Oxidation of Linolenic Acid in Aqueous Wheat Flour Suspensions

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ABSTRACT

Linolenic acid oxidation by the enzyme lipoxygenase in an aqueous wheat flour suspension does not lead to accumulation of linolenic acid hydroperoxides but immediately to secondary oxidation products. The 3 most important products among these were identified as 9-hydroxy-*trans*-10,*cis*-12,*cis*-15-octadecatrienoic acid, 9-hydroxy-10-oxo,*cis*-12,*cis*-15-octadecadienoic acid, and 9,12,13-trihydroxy-*trans*-10,*cis*-15-octadecadienoic acid.

INTRODUCTION

Lipoxygenase has been shown to attack a number of polyunsaturated fatty acids. The enzyme attack is highly specific: it reacts with acids which have a *cis,cis*-1,4-pentadiene system, such as linoleic, linolenic, and arachidonic acid, to produce hydroperoxides with a *cis,trans* conjugated diene system (1).

In a previous publication (2) it was reported that lipoxygenase from wheat flour forms mainly the 9-hydroperoxide (or maybe 9-peroxy-radical) from linoleic acid. This 9-hydroperoxide (or 9-peroxy-radical) is converted immediately into various secondary oxidation products (3).

The free fatty acids of wheat flour include up to 4-5% linolenic acid, i.e. ca. 0.05 mg/g flour. This paper describes the identification of 3 oxidation products from linolenic acid by IR, NMR, and mass spectrometry.

MATERIALS AND METHODS

Flour and Substrate

A commercial wheat flour was used, which contained no bleaching nor improving agent and which had a protein content (N x 5.7) of 10.1% (on 14.0% moisture basis). Linolenic acid was obtained from Fluka, Switzerland.

Enzymatic Oxidation of Linolenic Acid

To study the oxidation of linolenic acid in wheat flour suspensions, 1 g wheat flour defatted with petroleum ether was suspended in 10 ml water, 2 mg linolenic acid added, and the mixture incubated under continuous magnetic stirring at 30 C. After methylation and silylation, the products were determined by gas chromatography (3). The linolenic acid remaining in the flour after extraction, amounting to some 0.01 mg/g flour, is not considered.

For preparative isolation of the oxidation products, a suspension of 20 g defatted flour and 40 mg linolenic acid in 100 ml was incubated.

Extraction and Isolation of Oxidation Products

After incubation of the reaction mixture, the oxidation products were extracted with a chloroform-isopropanol mixture (3). For preparative isolation of the oxidation products, preparative thin layer chromatography (TLC) was used. After applying the extract on the plate with a Camag applicator, the plate was first developed in a solvent mixture of diethylether-benzene-ethanol-acetic acid (40:50:2:0.2) up to 6 cm. After drying for 5 min, the plate was developed in the same direction (15 cm) in the following solvent mixture: isooctane-diethylether-acetic acid (50:50:2). After chromatography the components were made visible on the TLC plate by means of iodine vapor, scraped off, and transferred into a column. The components were eluted from the silica gel with ether.

IR, NMR, and Mass Spectra Analysis

IR spectra were obtained with a Hitachi EPG-3. The analyses were carried out in either solution of CCl_4 or KBr pellet form. NMR spectra were obtained with a JNM-4H-60 spectrometer at 25 and 45 C. The analyses were carried out in $CDCl_3$ solution. Mass spectra were obtained with an Atlas mass spectrometer, type CH-4, equipped with a TO 4 ion chamber. Mass measurements were carried out with an Atlas double focusing mass spectrometer SM-1.

Methylation and Silylation of the Oxidation Products

For analysis, the isolated oxidation products were methylated with diazomethane and silylated with hexamethyldisilazane and trimethylchlorosilane (3).

Gas Chromatography

The methylated and silylated products were determined by gas chromatography. Chroma-

tography was carried out using 1 m stainless steel columns of 0.2 cm internal diameter. The packing used was 3% JXR on Gas Chrom Q 100-120 mesh. Temperature: 170-230 C, Prog. 1.5 C/min.

RESULTS

Figure 1 shows the gas chromatogram of methyl esters derived from an extract of a suspension containing linolenic acid and defatted wheat flour after 30 min shaking. The first peak A is the methyl ester of palmitic acid, which is not extracted by petroleum ether. The second peak B is the methyl ester of the internal standard (heptadecanoic acid), while the third peak C represents a mixture of methyl stearate, oleate, linoleate, and linolenate, which are not extracted by petroleum ether, plus the methyl ester of linolenic acid which was added to the suspension and not oxidized. Further, there are 3 large peaks to be attributed to oxidation products, E, G, and K. These products have been isolated by preparative TLC and identified.

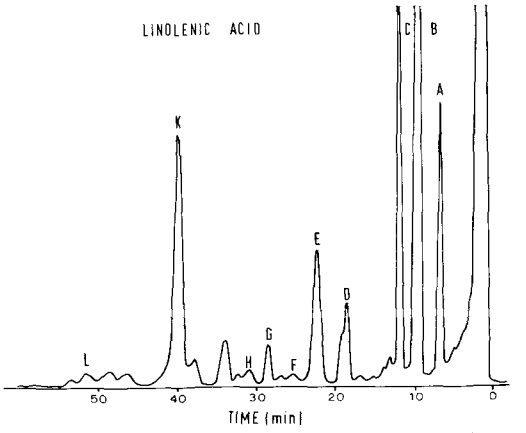


FIG. 1. Gas chromatogram of oxidation products from linolenic acid. A = palmitic acid, B = heptadecanoic acid (standard), C = linolenic acid, D = unknown, E = monohydroxy isomer of linolenic acid, F = unknown, G = α-ketol isomer of linoleic acid, H = unknown, and K = trihydroxy isomer of linolenic acid. These products were all methylated and oxidation products methylated and silylated.

9-Hydroxy-*trans*-10,*cis*-12,*cis*-15-octadecatrienoic Acid (E)

The IR spectrum (Fig. 2A) of the methylated product indicates the presence of a methyl ester (1735 and 1170 cm^{-1}), a *cis,trans* conjugated double bond (3010, 982, and 950 cm^{-1}), and a hydroxy group (3600 cm^{-1} sharp peak and a broad band with a top at 3450 cm^{-1}). The small peak at 1070 cm^{-1} indicates a secondary hydroxy group.

The mass spectrum (Fig. 3) of this methylated and silylated compound shows a small parent (p) peak (m/e 380). Furthermore, the spectrum contains the peaks with m/e values of p15 and p31, attributable to a loss of CH_3 and CH_3 + CH_4 of the trimethylsilyl (TMS) group (4). The p31 also may be attributed to a loss of

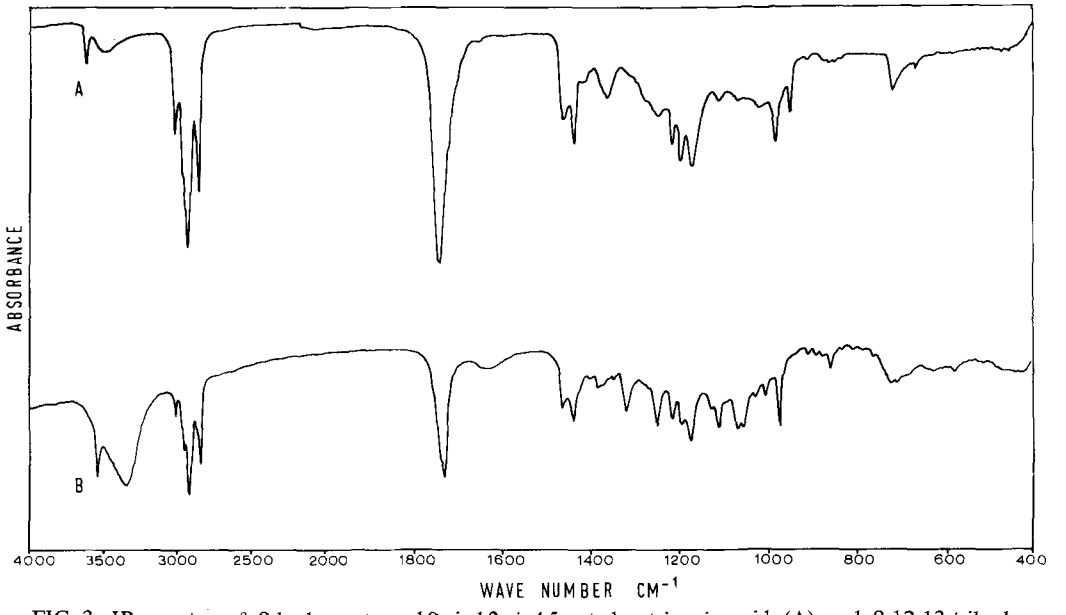


FIG. 2. IR spectra of 9-hydroxy-*trans*-10,*cis*-12,*cis*-15-octadecatrienoic acid (A) and 9,12,13-trihydroxy-*trans*-10,*cis*-15-octadecadienoic acid (B). A in CCl_4 using 0.1 mm KBr cell; B in KBr pellet form.

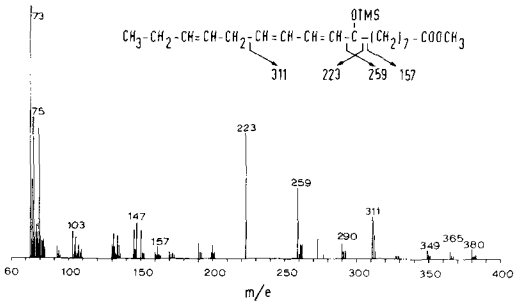


FIG. 3. Mass spectrum of 9-hydroxy-*trans*-10,*cis*-12,*cis*-15-octadecatrienoic acid after methylation and silylation.

the OCH_3 group. Peaks 157, 223, and 259 correspond to the fragments indicated in the formula in Figure 3. These peaks show the location of the TMS group on carbon-9. Peak 311 defines the location of the conjugated dienol system and locates the double bonds in the 10 and 12 positions (5). In this spectrum, there are no peaks which indicate an isomer of this compound. Also with TLC of the methyl hydroxy stearate derivative, it was shown that only the 9-hydroxy isomer was present.

Further, this mass spectrum, like the other mass spectra in this paper, contains a number of peaks which are characteristic of silylated compounds, 73 ($Si[CH_3]_3^+$); 75 ($HO^+ = Si[CH_3]_2$); 103 and 147.

9-Hydroxy-10-oxo,*cis*-12,*cis*-15-octadecadienoic Acid (G)

After reduction with $NaBH_4$ peak G shifts to the place of peak H. This change of retention time indicates the presence of a keto group (3).

The IR spectrum provides no indication of a *cis,trans* conjugation, as no peaks are found at 950 cm^{-1} or at 982 cm^{-1}. Also there is no indication of an isolated *trans* double bond at 970 cm^{-1}. The IR spectrum of the methylated compound indicates the presence of a methyl ester (1735 and 1170 cm^{-1}), a ketone group (1720 cm^{-1}), a hydroxyl group (3490 cm^{-1} and 1075 cm^{-1}), and *cis* double bonds (3010 cm^{-1}). From these results and from the location of this compound in the gas chromatogram, it may be inferred that this compound is a keto-hydroxy acid.

The tentatively established structure of this keto-hydroxy acid was confirmed by the mass spectrum (Fig. 4). It shows a p peak (m/e 396) and 381 (p15), 365 (p31), and 349 (p47). The 2 peaks 239 and 259 correspond to the fragments shown in the formula in Figure 4. Further, there is a large peak 169 (259-TMS). The peaks 259 and 169 establish the location of the TMS group on C-9.

After reduction with $NaBH_4$ followed by oxidative chain cleavage with $NaJO_4$ (2), a reaction product was obtained which was identified by gas chromatography as $OCH(CH_2)_7COOCH_3$. This leads to the conclusion that the keto group is located on C-10. There are no other peaks in the mass spectrum which may indicate any isomer. So it may be inferred that peak G in the gas chromatogram (Fig. 1) is to be attributed only to 9-hydroxy-10-oxo,*cis*-12,*cis*-15-octadecadienoic acid.

9,12,13-Trihydroxy-*trans*-10,*cis*-15-octadecadienoic Acid (K)

The IR spectrum (Fig. 2B) of the methylated product indicates the presence of a methyl ester (1730 and 1173 cm^{-1}), a *cis* double bond (3000 cm^{-1}), and an isolated *trans* double bond (970 cm^{-1}). Hydroxy groups are indicated by a sharp peak at 3520 cm^{-1} and a broad band with a top at 3350 cm^{-1}. The peaks

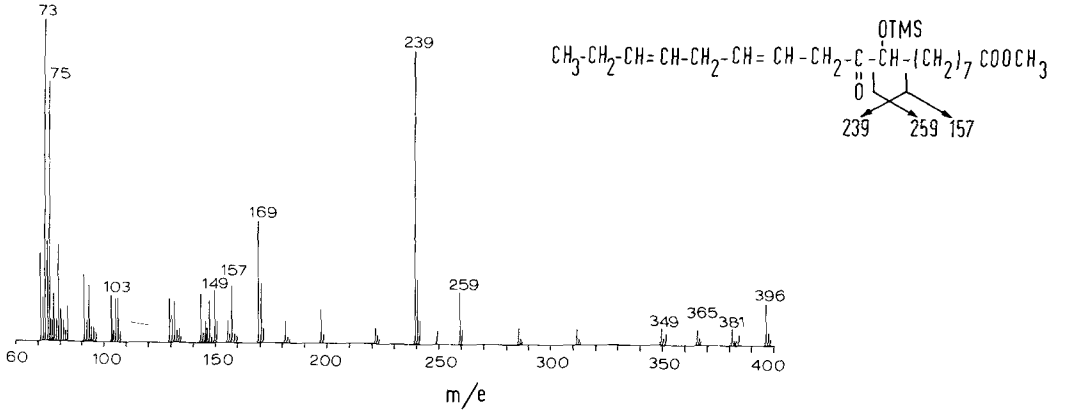


FIG. 4. Mass spectrum of 9-hydroxy-10-oxo,*cis*-12,*cis*-15-octadecadienoic acid after methylation and silylation.

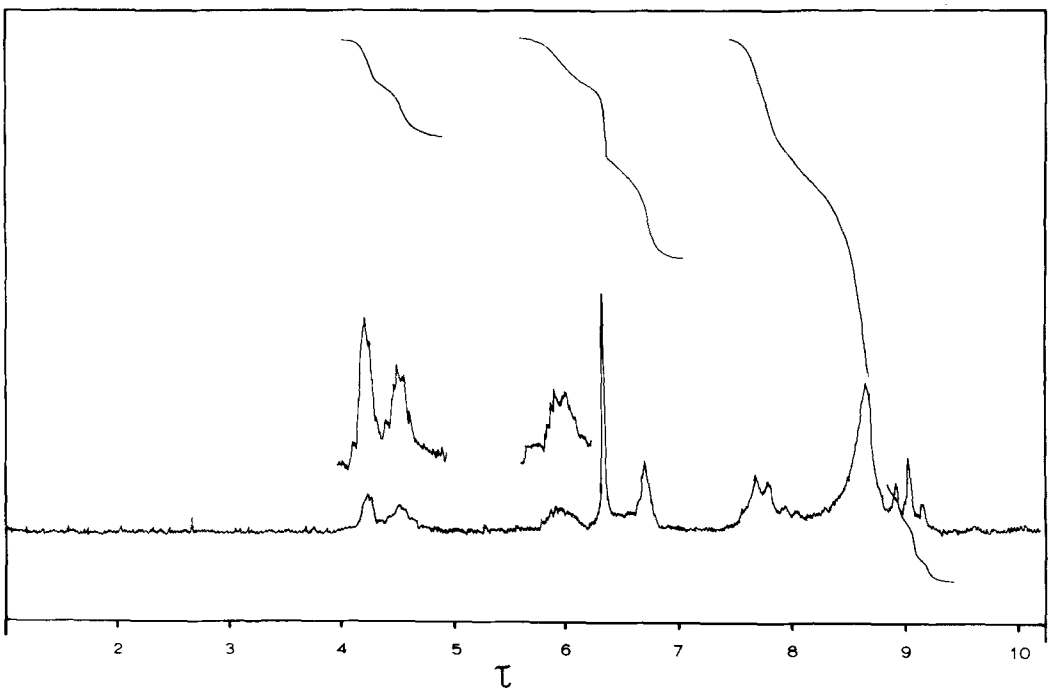


FIG. 5. NMR spectrum of 9,12,13-trihydroxy-*trans*-10,*cis*-15-octadecadienoic acid after methylation.

at 1050 and 1070 cm^{-1} indicate more than 1 secondary hydroxy group.

With the aid of the NMR spectrum (Fig. 5) the number of hydroxy groups could be detected. The NMR spectrum of the methylated compound is made up as follows: a triplet at 9.1 τ (3H; terminal methyl), a broad peak with top at 8.7 τ (12H, $[CH_2]_6$-), a multiplet between 7.6 and 8.6 τ (6H CH_2-$COOCH_3$ and 2 x CH_2-C-OH), a broad peak with top at 6.7 τ (3H HO-C), a broad peak between 6.4-6.8 τ (1H H-COH), a singlet at 6.3 τ (3H OCH_3), a broad peak between 5.8 and 6.3 τ (2H H-COH), a multiplet with top at 4.5 τ (2H HC=CH), and a multiplet at 4.2 τ (2H HC=CH).

This spectrum was photographed in $CDCl_3$ at 25 C. When the spectrum was photographed at 45 C, the OH peak shifted from 6.7 τ to 7.4 τ; and, when the sample was shaken with D_2O, the peak disappeared. From these results it may be inferred that there are 3 hydroxy groups and 2 double bonds. From comparison with the NMR spectrum of 9,12,13-trihydroxy-10(*trans*)-octadecenoic acid (6), it may be concluded that there is 1 *trans* double bond 4.2 τ and 1 *cis* double bond 4.5 τ.

The locations of the 3 hydroxy groups were determined by mass spectrometry. In the mass spectrum (Fig. 6), the p peak 558 is small. The characteristic TMS peaks 73, 75, 103, and 147 are present. Further striking features are 543 (p15) and 527 (p31). The peaks 259, 387, and 171 correspond with the fragments shown in Figure 6. Peak 399 ($C_{20}H_{39}O_4Si_2$) is 489-90. Peak 489 (not occurring in the spectrum) is p-(CH_3-CH_2-CH=CH-CH_2-). The structure of the substance represented by peak 460 is shown in Figure 6 (5).

It has been assumed that the *trans* double bond in the components E and K is *trans*-10, though this has not been proved exactly. By comparison, however, with the products obtained from linoleic acid using the same enzyme system (2), it may be assumed that in the above named products the *trans* double bond is located on C-10.

DISCUSSION

In an aqueous wheat flour suspension linolenic acid is enzymatically oxidized, which results in the formation of 3 major compounds E, G, and K; these have been identified. Smaller quantities of D, F, and H also are formed; the structure of these compounds has not been identified.

From the structures of the compounds E, G, and K, it appears that the positional specificity in the oxygenation of polyunsaturated fatty acids by lipoxygenase from wheat flour increases when the acid is more unsaturated. Lipoxygenase oxidizes linoleic acid primarily at

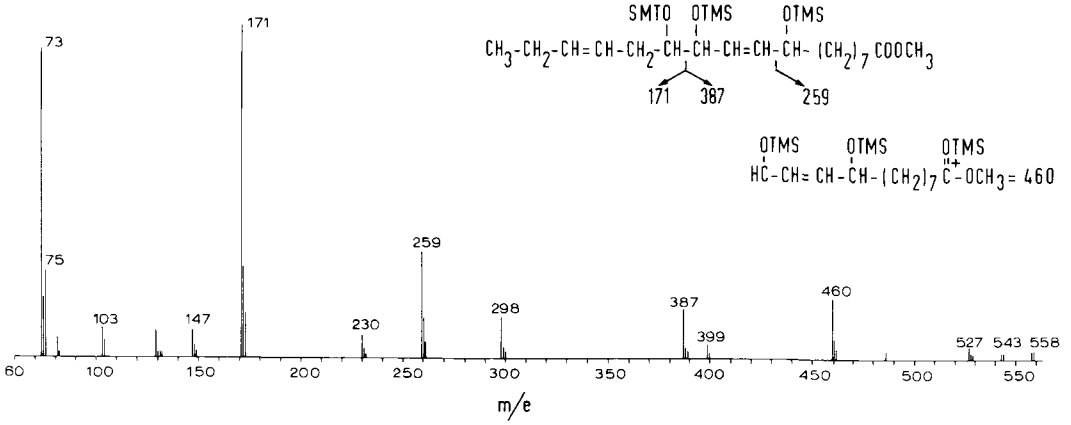
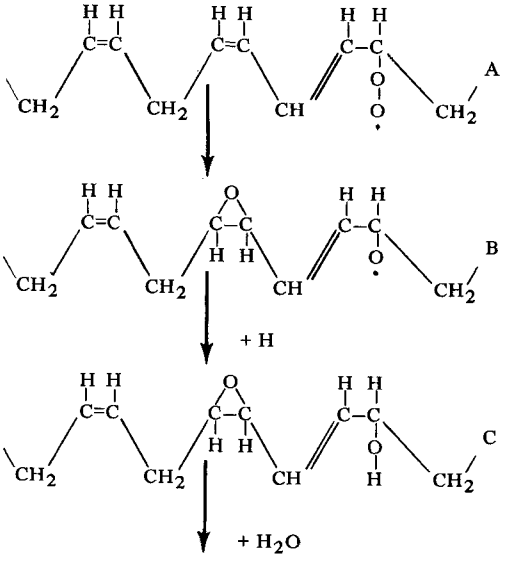


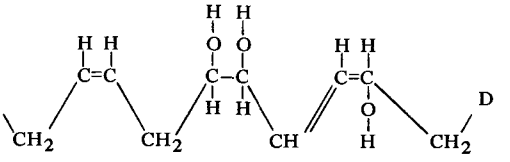
FIG. 6. Mass spectrum of 9,12,13-trihydroxy-*trans*-10,*cis*-15-octadecadienoic acid after methylation and silylation.

carbon-9 (85%) with a small amount (15%) of oxidation at carbon-13 (2), whereas in linolenic acid only oxidation at carbon-9 occurs, while no oxidation takes place at carbon-16, -12, and -13.

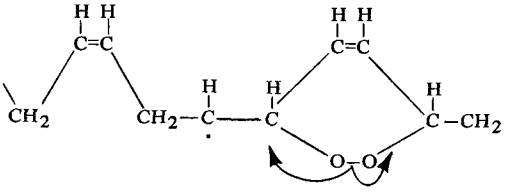
The location of the 3 hydroxy groups in the molecule of trihydroxy acid (K), carbons-9, -12, and -13 is the same as in the quantitatively predominant trihydroxy acid isomer of linoleic acid. From this it may be inferred that the formation of this trihydroxy acid takes place in the same way as it does in linoleic acid, via a hydroxy-epoxy compound. In previous experiments (6), it was found that the hydroxy-epoxy acids are formed from the hydroperoxy acids or peroxy radicals.

The conversion of the peroxy radical of linolenic acid hydroperoxide into a trihydroxy acid may be represented by the scheme below:





The peroxy radical (A) probably is transformed by rearrangement into an epoxy-alkoxy radical (B). The exact mechanism of this rearrangement is still obscure. In the event of a direct formation of an epoxy-alkoxy radical from a peroxy radical via a radical mechanism, a six membered ring with 1 double bond as an intermediate would be required:



However, this double bond in the ring system necessarily has the *cis*-configuration, in contrast to what has been demonstrated, viz. that this double bond has a *trans*-configuration (6). There may be another possible pathway, the formation of a hydroxyl radical in step 1 and its subsequent addition to the double bond.

The possibility should not be excluded that the epoxy-alkoxy radical is formed indirectly; the conversion of the peroxy radical into the epoxy-alkoxy radical may occur via a number of intermediate states.

The hydroxy-epoxy acid (C) was not determined because only the oxidation in suspension was studied. In a suspension hydrolysis takes place so rapidly that it is impossible to detect it.

The formation of an α-ketol isomer (G) is indicative of the presence of the enzyme isomerase of the flax seed type, which was isolated by Zimmerman (8). In all probability, it is one and the same enzyme forming the α-ketol isomer(s) both from linoleic acid (3) and from linolenic acid in aqueous wheat flour suspensions.

The mechanism of the conversion of the linolenic acid hydroperoxide into the monohydroxy isomer (E) is not yet elucidated, as is the oxidation of linoleic acid (3).

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