# Isolation of Isomeric Hydroperoxides from the Peanut Lipoxygenase-Linoleic Acid Reaction[1]

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## ABSTRACT AND SUMMARY

Hydroperoxides were isolated from the peanut lipoxygenase-linoleic acid reaction mixture and were separated as their methyl esters by high performance liquid chromatography. Mass spectrometry and infrared analysis indicated the isolated hydroperoxides to be 13-hydroperoxy-*cis*-9, *trans*-11-octadecadienoic acid; 13-hydroperoxy-*trans*-9, *trans*-11-octadecadienoic acid; and 9-hydroperoxy-*trans*-10, *trans*-12-octadecadienoic acid. The percentages of the hydroperoxides in the reaction mixture were 72.8%, 3.6%, and 23.6% under the conditions used.

## INTRODUCTION

Prior to the development of high performance liquid chromatography methodology (1), positional isomers of linoleic acid hydroperoxide were difficult to separate (2). The enzymatic production of the positional isomers, 9-hydroperoxy-10,12-octadecadienoic acid (9-LOOH) and 13-hydroperoxy-9,11-octadecadienoic acid (13-LOOH) varies with enzyme source pH, temperature, and oxygen level (3,4).

Enzymatic metabolism of lipid hydroperoxides is a new and developing area of research in lipid oxidation research. Free lipid hydroperoxides can damage biomembranes, react with proteins and small metabolites, and initiate autoxidation. Enzymatic metabolism of the lipid hydroperoxides prevents these deleterious effects. The literature on enzymatic and nonenzymatic reactions of lipid hydroperoxides has recently been reviewed by Gardner (5). The type of components produced by the enzymatic reactions depends upon the hydroperoxide isomer, i.e. 9-LOOH or 13-LOOH. Thus it is important to know the hydroperoxide isomers produced by the lipoxygenase (E.C. 13.11.12) from the plant source being studied.

Peanut lipoxygenase has been isolated, purified (6), and separated into at least three isozymes (7). Pentane is the major volatile constituent of the peanut lipoxygenase-linoleic acid reaction (6); and it appears that the lipoxygenase-linoleic acid reaction and subsequent hydroperoxide-enzymatic reaction are partially responsible for raw peanut flavor (8). However, there has been no definitive work on the characterization of hydroperoxide isomers produced by peanut lipoxygenase. We report our results of such a study.

## EXPERIMENTAL PROCEDURES

### Sources of Materials

Peanut lipoxygenase was obtained from acetone powders of peanuts and purified through Sephadex G-150 as described previously (8). High purity, grade III linoleic acid was from Sigma Chemical Co. (St. Louis, MO); N-methyl-N-nitroso-p-toluenesulfonamide from Eastman Organical Chemicals (Rochester, NY); and high purity hexane from Phillips Petroleum Co. (Bartlesville, OK). All other solvents were commercially available reagent grades. All solvents except anhydrous diethyl ether and absolute ethanol were purified by appropriate chemical reactions and distillation for removal of carbonyl contaminates and 234 nm-absorbing materials. Linoleic acid was prepared just prior to use, 18 mmoles/ml 0.1M borate (pH 10.2).

### Hydroperoxide Formation, Isolation, and Methylation

The bulk reaction mixture (190 ml total volume) contained 246.6 nmoles of linoleic acid in 0.1M phosphate buffer (pH 6.0). The mixture was saturated with $O_2$ before addition of the enzyme (35-40 mg) and was so maintained throughout the 30 min, 22 C incubation. Initial and final readings at 234 nm were made in absolute ethanol to determine the amount of hydroperoxide formed.

Immediately after termination of the reaction by the addition of HCl to pH 3, the mixture was extracted three times with 200-ml aliquots of $CHCl_3$-$CH_3$-OH (2:1). The three $CHCl_3$ extraction aliquots were combined and washed with distilled deionized water several times to remove the $CH_3OH$. The volume was reduced under vacuum and a maximum water bath temperature of 30 C. Exposure to elevated temperature was minimized (about 30 min) so that isomerization was avoided. The solvent was changed to diethyl ether and made 0.5% with respect to $CH_3OH$. The hydroperoxides were then methylated with diazomethane (9). The solvent was reduced with a stream of $N_2$, and the sample solvent was changed to hexane for purification by column chromatography. The column (ID 2.5 cm) was packed with 40 g Mallinckrodt silica gel (CC-7, 100-200 mesh), in hexane and the sample was transferred directly onto the column. The elution series was 100 ml of 5% anhydrous ether, 100 ml of 20% ether, and 150 ml of 50% ether in hexane followed finally by 100 ml of methanol. Each fraction of the series was collected in bulk and checked for 234 nm absorption. Total recovery of 234 nm absorbing material was 92.5%. The 20% and 50% ether fractions were combined, concentrated, and stored at -20 C for later analysis.

### High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) was carried out at ambient temperature with a Partisil-10 packed stainless steel column (4.6 mm ID X 500 mm). The liquid chromatography system was a Varian Model 8500 attached to a Varian Model 635D spectrophotometer with an 8 $\mu$l flow-cell. The areas under the absorption peaks at 234 nm were integrated by an Autolab System I Computing Integrator. The eluting solvent was 0.26% absolute ethanol in hexane and was delivered at 4 ml/min.

### Spectral Methods

Infrared (IR) spectra were recorded with a Perkin Elmer Model 521 spectrometer. Spectra of isolated compounds were determined as liquid films on CsBr plates.

The mass spectra of the isolated compounds as methyl hydrostearates were obtained with an AEI Model 902 instrument by direct probe insertion at 130 C.

## RESULTS AND DISCUSSION

### Optimum Reaction Time

To obtain workable quantities of reaction products from the lipoxygenase-linoleic acid mixture, we used bulk reactions (246.6 nmoles linoleic acid). Reported bulk-reaction

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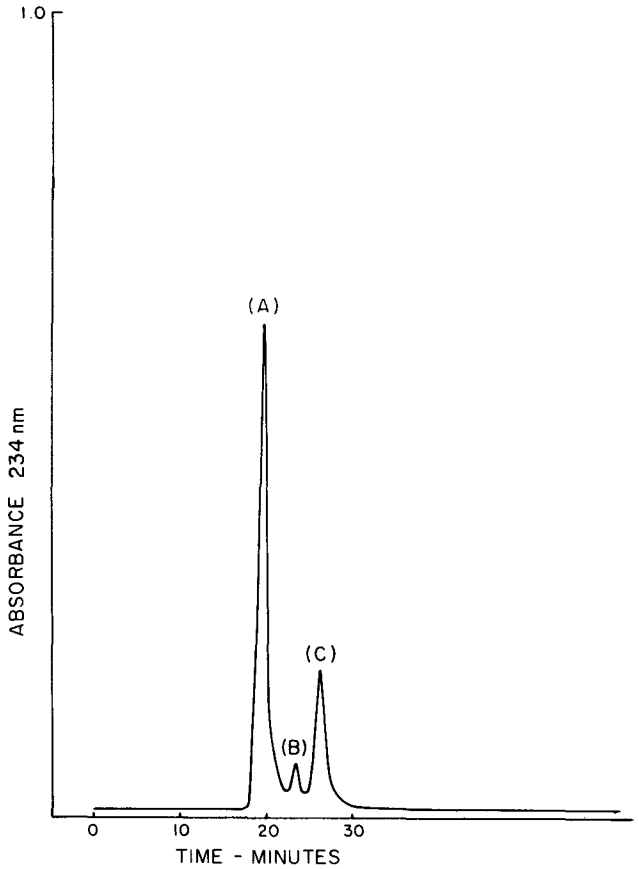


FIG. 1. High performance liquid chromatography of methyl esters of hydroperoxides produced during the incubation of peanut lipoxygenase with linoleic acid. A 20-μl sample of hydroperoxides in hexane was injected. Attentuation of the integrator was 4X. Recorder response was one absorbance unit (full scale). Peak A: 13-hydroperoxy-*cis*-9, *trans*-11-octadecadienoic acid; Peak B: 13-hydroperoxy-*trans*-9, *trans*-11-octadecadienoic acid; Peak C: 9-hydroperoxy-*trans*-10, *trans*-12-octadecadienoic acid.

times vary from 30 min to 48 hr; therefore, we determined the optimum bulk-reaction time for the peanut lipoxygenase-linoleic acid mixture. No significant increase was found in hydroperoxide absorption at 234 nm after 27 min. Thus, a reaction time of 30 min was selected and routinely used throughout this study.

### Isomeric LOOH Separation and Identification

High performance liquid chromatography of the methyl esters of the isolated hydroperoxides separated three major compounds that absorbed at 234 nm (Fig. 1). Each component was collected separately, reduced to the corresponding methyl hydroxyoctadecadienoate with $NaBH_4$, and hydrogenated with $H_2$ and Adams catalyst to yield the methyl hydroxystearate. Gas liquid chromatography on 1/8 in. x 6 ft OV-25 column at 210 C gave identical retention times and cochromatographed with known methyl monohydroxystearates. This indicates that the separated methyl hydroperoxides were converted to the methyl hydroxystearates and confirms that the 234 nm absorbing peaks were indeed methyl monohydroxystearates after reduction and saturation.

Positional specificity of peanut lipoxygenase and identification of the hydroperoxides were accomplished by mass spectra analysis of the methyl hydroxystearates. The mass spectral data showed characteristic fragmentation patterns for methyl hydroxystearates resulting from the scission of carbon-carbon bonds on each side of the carbon with the hydroxyl group. Peak A (Fig. 1) yielded diagnostic mass peaks at 211, 214, and 243; whereas peak C yielded diagnostic peaks at 155, 158, and 187. Thus peaks A and C are 13-LOOH and 9-LOOH, respectively. Peak B yielded a mixed mass spectra, but the predominant mass peaks were 211, 214, and 243. Therefore, peak B is predominantly 13-LOOH. Apparently, a small amount of cross contamination occurred during the collection of fractions B and C. Since peaks A and B were both determined to be the same by mass spectrometry, the two hydroperoxides must be geometrical isomers.

Absolute geometrical configuration of the isolated hydroperoxides was determined by studying the out-of-plane deformation of the attached hydrogen atoms by infrared spectral analysis. Characteristic absorption bands in the 900 to 1000 $cm^{-1}$ region, which are largely independent of the surrounding structure, differentiate geometrical isomers (10). Absorption bands at 943 $cm^{-1}$ and 977 $cm^{-1}$ were present in the IR spectrum of component A (Fig. 1). These absorption bands are characteristic of *cis-trans* conjugation; therefore component A (Fig. 1) is the 13-LOOH *cis-trans* isomer. Component B exhibited a single intense band at 986 $cm^{-1}$ in its IR spectrum. A single absorption band at this wavelength is characteristic of *trans-trans* conjugation; thus component B is the 13-LOOH *trans-trans* isomer. Also, a single intense absorption band at 985 $cm^{-1}$ in the IR spectrum of component C characterizes this component as the 9-LOOH *trans-trans* conjugated isomer. The IR data for geometrical assignment of the isolated isomers agrees with the findings of others applying infrared spectrophotometry to designate geometrical configurations of double bonds (10-12).

The presence of 9-LOOH (*trans,trans*) and 13-LOOH (*trans,trans*) components in the lipoxygenase-linoleic acid reaction mixture has been presumed (2,11,13), but this is the first report of their isolation and identification. By HPLC, mass spectrometry, and IR we successfully isolated and identified the methyl ester of 9-hydroperoxy-*trans*-10, *trans*-12-octadecadienoic acid. The two 13-LOOHs isolated are characterized as 13-hydroperoxy-*cis*-9, *trans*, 11-octadecadienoic acid and 13-hydroperoxy-*trans*-9, *trans*-11-octadecadienoic acid. We identified them on the basis of chromatographic and spectral data and the observation that the double bond $\alpha,\beta$ to the hydroperoxy group in the hydroperoxides from the lipoxygenase-linoleic acid reaction always adopts the *trans* configuration (12). Integration of the separated 13-LOOH (*cis,trans*), 13-LOOH (*trans,trans*) and 9-LOOH (*trans,trans*) peaks indicated the formation of these compounds in the percentages 72.8%, 3.6%, and 23.6%, respectively, under the conditions of the reaction. St. Angelo et al. (14) had previously suggested that 13-LOOH was the only hydroperoxide formed by peanut lipoxygenase. Their conclusion is supported by our results if the current hypothesis is correct that the lipoxygenase-linoleic acid reaction produces only *cis-trans* products. However, the methodology used by them has been questioned previously (6), and further questions regarding their methodology and observations are raised by these results. Through the use of appropriate controls and blank runs we have determined that the 9-LOOH is formed during the reaction time period. Thus St. Angelo et al. should have observed the appropriate methyl esters if their methodology is valid. Our results also raise new questions regarding the hydroperoxide ratios found by other workers; such as, in flax (15) where the 13-LOOH:9-LOOH ratio was 80:20, whether the minor 9-LOOH component is *trans-trans* as in our results or *cis-trans* as inferred in the results of Chan et al. (16). The chromatogram published by Chan et al. (16) appears to be identical to ours; however, they did not identify the geometrical configuration of their center peak which must be *trans,trans* 13-LOOH based upon our results and their data on the peak. No *trans,trans* 9-LOOH appeared to be present since it would have eluated beyond

their peak 3—inferred to be *cis,trans* 9-LOOH—because of the increased polarity resulting from the *trans,trans* configuration. The similarity of the chromatograms and difference in identification of the geometrical configuration of the 9-LOOH indicates the necessity of reevaluating the previous results on hydroperoxide ratios to determine if the minor positional hydroperoxide isomers is a *cis,trans* or *trans,trans* component. Additional work would also appear to be needed to determine if an enzymatic mechanism does exist for the formation of the *trans,trans* geometrical configuration. As previously stated our results do not enable us to ascertain whether all components were formed by enzymatic action on linoleic acid. Future studies involving environmental and chemical variables and optical activity may enable us to determine the path(s) by which these components arise.

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