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HYDROPEROXIDE ISOMERS AND KETOHYDROXY PRODUCT FROM OXIDATION OF LINOLEIC ACID BY EGGPLANT LIPOXYGENASE

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Key Word Index—*Solanum malongena;* Solanaceae; eggplant lipoxygenase; linoleic acid; hydroperoxides; methyl hydroxystearate; *cis–trans* conjugation; α-ketohydroxy compound.

Abstract—Hydroperoxides produced by oxidation of linoleic acid with purified eggplant lipoxygenase were separated by TLC and analysed by IR spectroscopy. The methyl hydroxystearates from the enzymatically produced hydroperoxides were analysed by MS and GLC. Both analyses indicated that the eggplant enzyme converted linoleic acid almost exclusively (96%) into the 13-hydroperoxy isomer whereas the 9-hydroperoxy isomer was only a minor product (4%). HPLC of the methyl ester of the isolated hydroperoxides showed three components. Each component was collected, reduced to methyl hydroxystearate and characterized by GLC, MS and IR analysis. The components were identified as 13-hydroperoxy *cis–trans* isomer (92.8%), 13-hydroperoxy *trans–trans* isomer (2.6%) and 9-hydroperoxy *cis–trans* isomer (4.6%). A polar by-product present in the reaction mixture was identified by IR, ^{1}H NMR, and MS (of the toluene-*p*-sulphonyl derivative) as 13-hydroxy-12-oxo-octadec-*cis*-9-enoic acid.

INTRODUCTION

Lipoxygenase (LOX) [linoleate:oxygen oxidoreductase, EC 1.13.11.12] specifically catalyses the oxidation of methylene-interrupted unsaturated fatty acids and their esters to respective hydroperoxides. LOX has long been considered as a typical plant enzyme and shown to be widely distributed in various plant sources [1–3]. However, a few reports in the literature also recently established the existence of this enzyme in mammalian tissues [4–6].

The enzymatic production of the positional isomers, 9-hydroperoxy-10,12-octadecadienoic acid (9-LOOH) and 13-hydroperoxy-9,11-octadecadienoic acid (13-LOOH), with linoleic acid as substrate varies with enzyme source, pH, temperature and oxygen level [2, 3, 7]. Most LOX are usually not completely specific for production of one positional isomer, but one nearly always predominates. Although Dolev *et al.* [8] reported production of 100% 13-LOOH by soybean LOX, most other investigators reported an isomeric mixture. With soybean LOX the ratio of the 13-LOOH to 9-LOOH usually varies from 93:7 to7:3 [2, 9, 10] and even pure isoenzymes do not yield pure isomeric hydroperoxides [11]. Selective formation of the 9-LOOH over the 13-LOOH was described in LOX studies from corn germ [12], potato [13] and a number of cereals [14, 15]. Gardner *et al.* [16] emphasized that they have never observed the exclusive formation of either 9-LOOH or 13-LOOH, except for the formation of 13-LOOH by *Dimorphotheca sinuata* LOX at pH 6.9.

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The identification of hydroperoxide isomers produced during the LOX reaction is of importance in understanding the involvement of these products in lipid metabolism. The literature on enzymatic and non-enzymatic reaction of lipid hydroperoxides has been reviewed by Gardner [17] and enzymes capable of catalysing the decomposition of these products have been reported. The type of components produced by the enzymatic reactions depends upon the hydroperoxide isomer, which emphasizes the need for identification of the hydroperoxide isomers produced by the LOX from the sources being studied.

In a previous report we described the presence of a LOX enzyme in eggplant, its purification and partial characterization [18]. The present report deals with the characterization by IR, ^{1}H NMR and MS of the hydroperoxide isomers and α-ketohydroxy compound produced by this enzyme from linoleic acid.

RESULTS AND DISCUSSION

Three major types of compounds were generally observed by TLC in a reaction of the purified eggplant LOX on linoleic acid: substrate fatty acids, polar by-products and hydroperoxides. The hydroperoxide compounds ($R_f = 0.3$) absorbed strongly in the UV at 234 nm, and IR showed that the spectrum (CCl_4) has absorption bands at 952 and 990 cm^{-1}, corresponding to *cis–trans* conjugated dienes.

The Me hydroxystearates prepared as previously described [6] were analysed by MS, for the determination of isomers. This revealed fragments at *m/e* 211, 214 and 243 characteristic of Me 13-hydroxystearates,

Table 1. Quantitative analysis of products from eggplant LOX action on linoleic acid

Methyl stearate	MS determination*	GLC* determination†	HPLC determination
13-*cis–trans*	96‡	97‡	92.8
9-*cis–trans*	4	3	4.6
13-*trans–trans*	—	—	2.6

* Relative intensities of MS peaks.
† Relative per cent area.
‡ Part of this is 13-*trans–trans*.

as well as fragments with *m/e* 155, 158 and 187 which correspond to Me 9-hydroxystearate. A quantitative ratio of 96% 13-hydroxy isomer to 4% 9-hydroxy isomer in the hydroperoxide fraction was obtained from the relative intensities of the corresponding fragments (Table 1).

A more precise quantitation was performed by analysing the Me hydroxystearate by GLC [19]. The molar ratio of the 13- and 9-isomers was estimated from the measured peak areas and found to be 97% 13- and 3% 9-hydroxy isomers.

When the $NaBH_4$-reduced hydroperoxide was analysed, it exhibited a UV maximum at 234 nm in EtOH. The IR in CS_2 solution showed absorbance at 3600 and also at 950 and 987 cm^{-1} [20, 21]. Similar absorbance was found when this hydroxydiene was methylated with CH_2N_2 to the appropriate Me ester. The heights of the peaks at 950 and 987 cm^{-1} were in the ratio 2:3, rather than 1:1, which could indicate the presence of a small amount of the *trans–trans* isomer [20].

To verify this possibility, HPLC of the Me ester of the isolated hydroperoxide was performed. Three peaks, A, B and C, which absorbed at 234 nm, were eluted. Each component was collected separately, reduced to the corresponding Me hydroxyoctadecadienoate with $NaBH_4$, and hydrogenated with H_2 and palladium as catalyst to yield the Me hydroxystearate. The separated components were analysed by GLC and identified as Me monohydroxystearates based on the R_t compared to known Me monohydroxystearates [19].

The final identification of the isolated hydroperoxide isomers was established by MS of the Me hydroxystearates. Peak A showed peaks at *m/e* 211, 214 and 243, whereas the corresponding values for peak C were 155, 158 and 187. Thus, peaks A and C are 13-LOOH and 9-LOOH, respectively. Peak B yielded a mixed MS, but the predominant peaks were *m/e* 211, 214 and 243, which identify this component as 13-LOOH.

The geometrical configuration of the isolated hydroperoxides was studied using IR analysis. Based on its absorption bands at 945 and 980 cm^{-1}, component A was identified as the 13-LOOH *cis–trans* isomer. Compound B exhibited a single intense band at 985 cm^{-1} indicating a *trans–trans* configuration; thus, component B is the 13-LOOH *trans–trans* isomer. The absorption bands at 943 and 977 cm^{-1} present in the IR of C characterize this component as the 9-LOOH *cis–trans* conjugated isomer.

Thus, based on the IR data described above and the MS fragmentation pattern [12, 22–24], it can be concluded that components A and B are geometrical isomers. Integration of the separated 13-LOOH (*cis–trans*), 13-LOOH (*trans–trans*) and 9-LOOH (*cis–trans*) peaks indicated the formation of these compounds in the percentages 92.8, 9.6 and 2.6%, respectively (Table 1), under the conditions of the reaction.

The chromatogram published by Pattee and Singleton [22] for peanut LOX appears to be similar to ours, with the exception that they identified component C as the 9-LOOH *trans–trans* isomer. In the present study with eggplant LOX, it is obvious from the IR that C is the 9-LOOH *cis–trans* isomer. Moreover, the percentages of the isomers are significantly different.

The results summarized in Table 1 show that eggplant LOX has a high degree of specificity for the 13-position. A high yield of this isomer—3.08 μmol/mg enzyme/min—was obtained with this enzyme under the reaction conditions described in the present study. It is not yet clear whether the low proportion of the 9-LOOH isomer produced in this system is due to the positional specificity of the eggplant LOX, or to the presence in the eggplant of a hydroperoxide cleavage enzyme, highly specific for the 9-LOOH. Nevertheless, the fact that variations in the incubation time of the enzyme with its substrate had no significant effect upon the ratio of the isomers formed, supports the first possibility. It is also possible that the small amount of 9-LOOH in our preparations is due to auto-oxidation and isomerization [25] processes during the incubation and extraction procedures.

As was stated above, the TLC pattern of the products from linoleic acid oxidation by eggplant LOX revealed the presence of polar by-products. One of these products (R_f 0.27) was extracted, methylated and analysed by IR, ^1H NMR and MS.

The IR (in CS_2 solution) indicates that the product contains a keto group (1720 cm^{-1}), C—H of a *cis*-double bond (3015 cm^{-1}), secondary —OH (3500 cm^{-1}), secondary —OH with α-unsaturation (1070 cm^{-1}) and an ester C=O group (1740 cm^{-1}). Moreover, the typical absorbance for the *cis–trans* conjugation, described above for the hydroperoxide isomers, was absent. The Me ester of the polar by-product was also examined by ^1H NMR and all the groups indicated by the IR data were confirmed. These data support the identification of the polar by-product as a monoenoic ketohydroxy fatty acid. Based on the MS of the toluene-*p*-sulphonyl derivative of this methylated ketohydroxy compound (*m/e* peaks at 155, 197, 225 and 255), the location of the characteristic groups was at position 12 for the keto,

and 13 for the OH group. These results confirm that the OH function was present at C-13 in the original ketohydroxy acid. The spectroscopic results indicate that the isolated compound of the polar by-products is 13-hydroxy-12-oxo-*cis*-9-octadecenoic acid.

The IR, ^{1}H NMR and MS data described here for the ketohydroxy compound separated from the eggplant LOX reaction mixture on linoleic acid are in agreement with those described by Gardner *et al.* [26–28], Zimmerman and Vick [29] and Veldink *et al.* [30] for the α-ketol compound formed by plant hydroperoxide isomerases. They also concur with those we described for a similar compound for the rat testis enzyme [19]. The presence of such a compound raises the question of its origin. The most likely possibility is that a specific enzyme converts the 13-LOOH isomer formed by eggplant LOX to 13-hydroxy-12-oxo-*cis*-9-octadec-enoic acid. However, since the existence of such an enzyme in eggplant has not yet been elucidated, the possibility that the α-ketol could be formed by non-enzymatic means cannot be dismissed. Further investigation is needed in order to determine the origin of this compound.

EXPERIMENTAL

Enzyme preparation. Eggplant cut into samll pieces was homogenized in the presence of Triton X-100, centrifuged and chromatographed on Ecteola cellulose as described in ref. [18]. Fraction B, eluted with 0.1 M Na-Pi buffer (pH 6.5) was precipitated with 60% $(NH_4)_2SO_4$ and the pellet dissolved in 0.1 M Na-Pi buffer; pH 6.5 was used as purified eggplant lipoxygenase in this study.

Enzyme assay. LOX activity, with linoleate as substrate, was assayed by two different techniques. (a) O_2 absorption, measured polarographically according to ref. [31] and (b) spectrophotometric measurement of conjugated diene formation according to ref. [32].

Isolation of products. The products of linoleic acid and the purified eggplant LOX were prepared according to ref. [6]. Prepn of derivatives by $NaBH_4$ reduction, CH_2N_2 methylation, catalytic hydrogenation and product analysis by IR and MS were as described in ref. [6].

HPLC was carried out as described in ref. [22].

GLC was done according to ref. [19].

Spectral analyses. ^{1}H NMR spectra were recorded at 100 MHz dissolved in $CDCl_3$ using 1% TMS as an int. ref. IR were determined with samples placed in 0.1 mm thick NaCl cells containing 10% solns in CCl_4 or CS_2. MS of Me hydroxystearates were determined essentially as described in ref. [8].

Preparation of the toluene-p-sulphonyl derivative was carried out according to [30].

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