ON THE DIFFERENT POSITIONAL SPECIFICITIES OF PEROXIDATION OF LINOLEATE SHOWN BY TWO ISOZYMES OF SOYBEAN LIPOXYGENASE*

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SUMMARY

A number of contradictory reports concerning the products of hydroperoxidation of linoleate as catalyzed by soybean lipoxygenase have appeared with claims ranging from 100% 13-hydroperoxyoctadecadienoate to 70% 13-hydroperoxyoctadecadienoate: 30% 9-hydroperoxyoctadecadienoate. Determination of the specificity of two homogeneous isozymes of soybean lipoxygenase offers a resolution of this anomaly. The first of these enzymes, the "classical" lipoxygenase originally crystallized and described by Theorell et al. (1) (lipoxygenase-1 of Christopher et al. (2)) yields only the 13-isomer. The second isozyme, lipoxygenase-2 (2), forms a 50:50 mixture of the 9- and 13-isomers.

INTRODUCTION

Lipoxygenase (EC 1.13.1.13) catalyzes the hydroperoxidation of polyunsaturated fatty acids and esters containing a cis,cis-pentadiene system. Linoleic acid, a naturally occurring example of these compounds, has been extensively studied as a substrate for soybean lipoxygenase. While there is general agreement that the major product is 13-hydroperoxy-cis-9, trans-11-octadecadienoate, disagreement prevails concerning the formation of 9-hydroperoxy-trans-10, cis-12-octadecadienoate. Thus, Hamberg and Samuelsson (3) found the ratio of the 13-to 9-hydroperoxy isomers to be 70:30 while Dolev et al. (4) detected only the 13-isomer. Veldink et al. (5), as well as Chang et al. (6), have also reported a ratio of 70:30. In other instances, Veldink et al. (7) and Hamberg and Samuelsson (8) found ratios of 90:10. Eriksson and Leu (9) obtained a ratio of approximately 95:5. Moreover lipoxygenases from sources other than

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soybeans have been reported to yield mixtures of the 9- and 13-hydroperoxides (6,10,11).

The occurrence of both isomers raises the possibility that lipoxygenase may not be rigidly specific for the position oxygenated in linoleic acid. In addition, the variations seen in the ratio might be attributable to a contaminating enzyme which selectively attacks and destroys one of the two isomers. However, an alternative explanation is that two species of lipoxygenase are present with differing positional specificities. Gardner and Weisleder (12) have found a lipoxygenase in maize which produces only the 9-hydroperoxy isomer.

In much of the previous work, rigorously purified enzymes have not been employed. We have recently isolated a new isozyme of soybean lipoxygenase (lipoxygenase-2) in a high state of purity (2). We have also prepared the classical soybean lipoxygenase of Theorell et al. (1) (lipoxygenase-1). We report here that the product of lipoxygenase-1 is exclusively the 13-isomer, whereas lipoxygenase-2 forms both the 9- and 13-isomers in approximately equal amounts.

MATERIALS

Lipoxygenases-1 and -2 were prepared as previously described (2) and shown to be homogeneous by disc gel electrophoresis. Linoleic acid, having a negligible peroxide value, was purchased from Hormel Institute (U. of Minn.), sodium borohydride from Ventron, Diazald from Aldrich, and platinum oxide (Adam's catalyst) from Matheson Coleman and Bell.

METHODS

Incubation - The buffers used in the reaction mixtures were oxygenated at 0°C for 30 min prior to their use. The reaction mixtures, 18 ml in total volume, contained, in the case of lipoxygenase-1, 155 mM borate buffer, pH 9.0, 1.78 mM linoleate (Surrey (13)), and 0.1 mg of lipoxygenase-1, or in the case of lipoxygenase-2, 155 mM phosphate buffer, pH 6.8, 1.78 mM linoleate (13), and 0.1 mg of lipoxygenase-2. The reactions were run for 30 min at 0°C with

continuous oxygenation. Peroxidation was followed by measuring the absorption of small aliquots at 234 nm.

$NaBH_4$ reduction - In order to convert the hydroperoxides to the corresponding hydroxy compounds, 15 mg of $NaBH_4$ were added to each reaction mixture. The reaction mixture from the lipoxygenase-2 experiment was adjusted to pH 9.0 with 0.2 N NaOH prior to the reduction. The solutions were stirred magnetically for 1 hr at room temperature, while a constant stream of N_2 was maintained over the surface.

Methylation - The above reaction mixtures were acidified to pH 3.0 with concentrated HCl and extracted 4 times with equal volumes of diethyl ether. The ether extracts were treated with anhydrous sodium sulfate and taken to near dryness under N_2. The hydroxy fatty acids and unreacted linoleic acid were then converted to their methyl esters with diazomethane generated from Diazald (14).

Catalytic hydrogenation - The solutions of the methyl esters were evaporated to dryness and the residues were dissolved in 2 ml of absolute ethanol. Platinum oxide (5 mg) was added to each, and hydrogenation performed under 40 PSI of H_2 at room temperature for 3 hrs, with shaking.

Gas-liquid chromatography - GLC was performed on a Varian Aerograph Series 1200 instrument equipped with a 6 ft. x 1/8 in. I.D. glass column packed with 3% Dexsil 300 on Gas-Chrom Q. The temperature was programmed for a linear increase of $6^{\circ}C$/min., and the flow rate of helium gas was 40 ml/min.

RESULTS AND DISCUSSION

The extent of reaction was followed by monitoring the change in absorption at 234 nm. Based on a molar extinction coefficient of $2.5 \times 10^4 M^{-1}\text{-}cm^{-1}$ for the conjugated hydroperoxide, a 76% yield of product was realized with lipoxygenase-1 and a 62% conversion with lipoxygenase-2. After borohydride reduction and methyl esterification, there was approximately a 70% recovery of the initial hydroperoxide products as the hydroxy unsaturated fatty acid esters. Catalytic hydrogenation completely eliminated the absorption at 234 nm.

Gas-liquid chromatography of the unresolved reaction mixtures revealed the presence of two major components. One of these, appearing at 210°C in each mixture, corresponded to methyl stearate formed from unreacted substrate. This was confirmed using a standard solution of methyl stearate. The second peaks, appearing at 230°C in each sample, could not be resolved on gas-liquid chromatography as evidenced by cochromatography of both solutions.

The material from the 230°C peak was analyzed on an LKB type 9000 combined gas chromatograph-mass spectrometer. The lipoxygenase-1 material was shown to contain the 13-hydroxy isomer of methyl stearate almost exclusively (Fig. 1A). The mass spectrum was in agreement with that determined by Dolev et al. (4) for this compound. However, analysis of the product of the lipoxygenase-2 catalyzed reaction clearly indicated the presence of both the 9- and 13-hydroxy isomers (Fig. 1B).

It is reasonable to assume that both isomers show nearly identical probabilities of fragmentation, and indeed, Dr. W. K. Rohwedder, of the Northern Regional Research Laboratory, Peoria has found this to be the case (15). It may therefore be judged from the intensities of the mass peak lines that the relative amounts of the 9- and 13-isomers were 50:50.

It is interesting that lipoxygenase-2 forms the 9- and 13-isomers in equal quantities in contrast to the maize enzyme of Gardner and Weisleder (12), which is specific for the 9 position. Chang et al. (6) however find that a partially purified lipoxygenase obtained from alfalfa seeds gives an equal mixture of 9- and 13-isomers.

Autoxidation of the fatty acids leads to equal amounts of 9- and 13-isomers and could conceivably confuse enzymic studies. However the mass spectrum of the product obtained with lipoxygenase-1 showed a negligible amount of fragmentation particles corresponding to the 9-isomer (Fig. 1). It may therefore be concluded that autoxidation was an insignificant factor, and the same must be true for the reaction products formed by lipoxygenase-2,

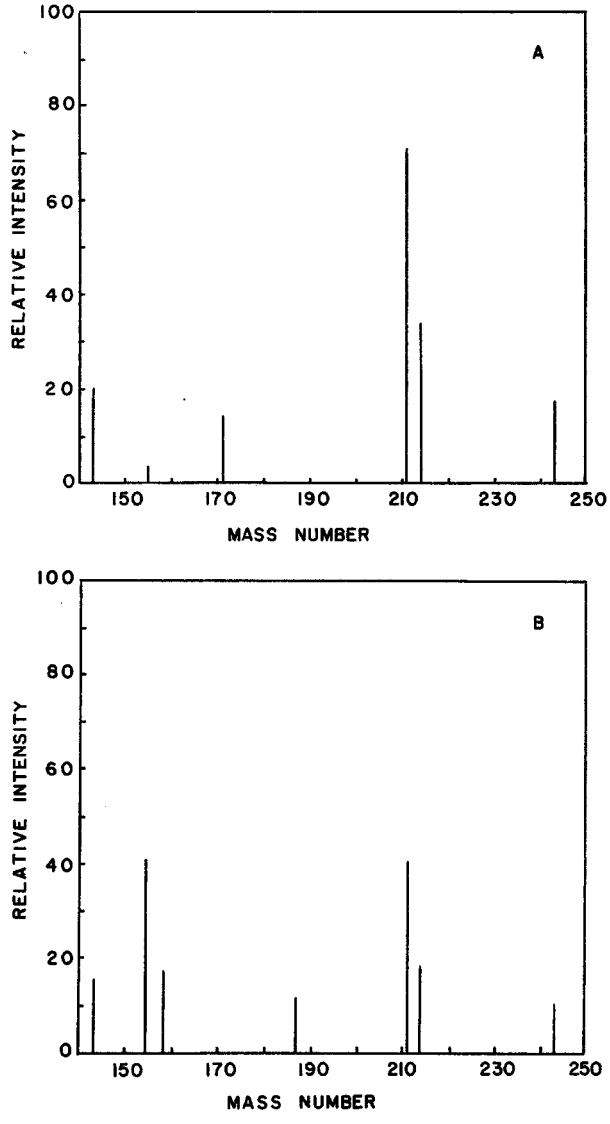

Fig. 1 Mass spectra fragmentation patterns showing only diagnostic lines of each isomer after normalization to mass peak 55. A - material of 230°C peak of lipoxygenase-1 mixture exhibits peaks at mass numbers 211 $[HOC(CH_2)_{11}CO]^+$, 214 and 243 resulting from the 13-isomer. B - material of 230°C peak from lipoxygenase-2 exhibits peaks at 155 $[HOC(CH_2)_7CO]^+$, 158 and 187 resulting from the 9-isomer as well as above peaks of the 13-isomer.

since both experiments were carried out simultaneously with the same batch of linoleic acid. Furthermore, follow-up studies on the effect of pH of incubation mixtures on the resulting products indicate that this factor does not account for the observed isomer ratios.

Soybean lipoxidase preparations might give rise to varying ratios of 9- and 13-isomers depending on the relative contents of the isozymes. Since, as we have observed, lipoxygenase-2 is considerably less stable than lipoxygenase-1, storage, too, will affect the observed ratios of enzymically heterogeneous preparations.

The possibility that still other isozymes of lipoxygenase are present in soybeans cannot be overlooked, for Guss et al. (16) have indicated the existence of 4 isozymes. During our isolation of lipoxygenase-2 we too have seen evidence of additional isozymes (2); albeit in comparatively small amounts. The specificity of the additional enzymes remains to be established.

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