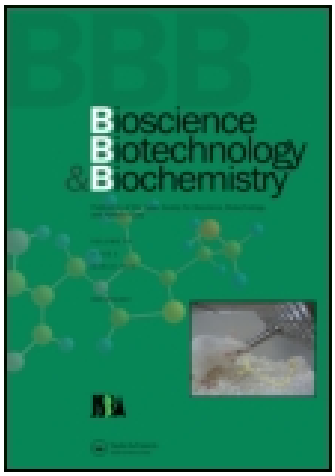

Bioscience, Biotechnology, and Biochemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbbb20

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Published online: 12 Jun 2014.

To cite this article: Kenji Matsui, Tadahiko Kajiwara, Akikazu Hatanaka, Doris Waldmann & Peter Schreier (1994) 5,6-Epoxidation of All-trans-retinoic Acid with Soybean Lipoxygenase-2 and -3, Bioscience, Biotechnology, and Biochemistry, 58:1, 140-145, DOI: 10.1271/bbb.58.140

To link to this article: http://dx.doi.org/10.1271/bbb.58.140

Biosci. Biotech. Biochem., **58** (1), 140–145, 1994

5,6-Epoxidation of All-*trans*-retinoic Acid with Soybean Lipoxygenase-2 and -3

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Received July 15, 1993

Cooxidation activity of highly purified soybean lipoxygenase-2 and -3 was investigated with all-*trans*-retinoic acid as a co-substrate. Both the isoenzymes rapidly degraded retinoic acid in the presence of linoleic acid, but lipoxygenase-2 had more cooxidation activity than lipoxygenase-3. During cooxidation, 5,6-epoxyretinoic acid was formed as a major product. HPLC on a chiral phase (Ceramospher Chiral RU-1) found a racemic mixture of the epoxide, indicating its formation by oxidative attack by peroxyl radicals.

Lipoxygenase (linoleate: oxygen oxidoreductase, EC 1.13.11.12) catalyzes the regio- and enantioselective hydroperoxidation of fatty acids having a (*Z*,*Z*)-1,4-pentadiene system.[1] This enzyme has a crucial role in the formation of biologically active eicosanoids in animal tissues.[2] In plants, it has been recognized not only as an enzyme involved in jasmonic acid synthesis[3] but in some cases also as a storage protein.[4] One of the richest sources of lipoxygenase are soybean seeds, in which at least four isoenzymes are present, *i.e.*, lipoxygenase-1, -2, -3a, and -4b.[5] The reaction mechanism of lipoxygenase-1 has been extensively studied, but reports on those of lipoxygenase-2 and -3 are scarce.[1]

The capacity of lipoxygenase to cooxidize polyenes such as carotenoids or xanthophylls has long been known.[1] Cooxidation is regarded to proceed by radical species formed during the lipoxygenation reaction. During its catalysis, lipoxygenase-1 forms a pentadienyl radical after H-abstraction from the bis-allylic methylene of a fatty acid.[6] This isoenzyme is considered to be a poor catalyst of cooxidation under aerobic conditions, while it has been found to cooxidize polyenes effectively under anaerobic conditions.[7] As the radical would be released from the enzyme-radical intermediate unless oxygen is available, one can assume that the released radical forms oxidants attacking polyene compounds. On the contrary, lipoxygenase-2 and -3 are able to cooxidize polyenes effectively even in the presence of oxygen.[7,8] From this observation, it has been suggested that these two isoenzymes have higher capacities to generate and release radical species even under aerobic conditions. This is consistent with the observation that under aerobic conditions both the isoenzymes can form ketodiene compounds, which are thought to be formed from fatty acid radical species.[5,7] Despite the abundant information on lipoxygenase-catalyzed cooxidation, the mechanisms of the reaction is still indefinite. The characterization of a lipoxygenase-oxidized polyene compound would undoubtedly provide an insight into the mechanism.

Retinoic acid has been shown to be a normal intermediate in the metabolism of retinol and its esters.[9] Its cancer chemopreventive activities have been documented.[10] The use of retinoic acid in many animal models of carcinogenesis has also suggested that its physiological effect may be due to antioxidant activity. In addition, it has been reported that 13-*cis*-retinoic acid inhibits liver microsomal lipid peroxidation.[11] Several oxygenated metabolites have been isolated and characterized both from *in vivo* and *in vitro* studies.[12,13] The mode of the antioxidative activity of retinoids is thought to be almost the same as that of carotenoids which are also known to be powerful antioxidants and effective protectors against the onset of cancer.[14] Thus, retinoic acid is an helpful model substance to examine the cooxidation reaction which is thought to be an oxidative radical reaction.

Most of earlier reports on lipoxygenase catalyzed cooxidation have dealt with C_{40}-polyenes.[7,8,15–17] However, they have many sites susceptible to oxidative attack and it is quite difficult to isolate and analyze the reaction products. Therefore, we selected the simpler molecule of all-*trans*-retinoic acid as a co-substrate for our study of the cooxidation catalyzed by lipoxygenase-2 and -3. In this paper, the isolation and characterization of the primary oxidization product of all-*trans*-retionic acid is described.

Materials and Methods

Chemicals. All-*trans*-retinoic acid was purchased from Sigma and used without purification. Linoleic acid purchased from Wako Pure Chemicals was purified by flash chromatography using a silica gel (Kieselgel 60, Merck) column (eluted with hexane–AcOEt–AcOH, 50 : 2 : 0.5, v/v) before use to remove any hydroperoxides. Linoleic acid 13-hydroperoxide was prepared with purified soybean lipoxygenase-1[18] and purified by flash chromatography on silica gel (eluted with hexane–AcOEt, 9 : 1, v/v) to remove linoleic acid. Other chemicals were of reagent grade, and solvents were glass-distilled before use.

General. NMR spectra were taken on a Fourier transform Bruker AC 200 spectrometer. CD spectra were recorded on an ISA Jobin Tvon CD 6 dichrometer. Capillary GC and capillary GC-MS analyses were done with a Hewlett-Packard 5890 gas chromatograph and a Varian 3300 gas chromatograph combined by direct coupling to a Finnigan MAT 44 mass spectrometer with a PCDS data system, with a J & W fused silica DB-5 capillary column (30 m × 0.25 mm i.d., film thickness 0.25 mm) as described elsewhere.[19]

Purification of soybean lipoxygenase-2 and -3. A mixture of soybean lipoxygenase isozymes-2 and -3 was obtained as described.[5,20] Briefly,

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seeds of *Glycine max* (L. cv. Tamanishiki, 100 g) were swollen for 24 h in 200 ml of 4 mM EDTA at 4°C in the dark and homogenized with 750 ml of 50 mM Na acetate buffer, pH 4.5. The homogenate was brought to 50 mM $CaCl_2$, and the calcium-precipitated material was removed by centrifugation. The supernatant was fractionated with 30–60% sat. of $(NH_4)_2SO_4$, dialyzed against 5 mM Na phosphate, pH 6.8, and resolved on a column of DEAE-Cellulofine A-500 (Seikagaku Kohgyo, 2.7 × 48 cm) equilibrated with 5 mM Na phosphate, pH 6.8. The first peak of lipoxygenase activity contained both lipoxygenase-2 and -3. The active fraction was collected and dialyzed against 5 mM Na phosphate, pH 6.8. A portion (2 ml) of the mixture was further resolved with HPLC (L-6000, Hitachi) with an ion-exchange column of Shodex QA-824 (Showa Denko, 0.8 × 7.5 cm). The column was equilibrated with 5 mM Na phosphate, pH 6.8. The flow rate was maintained at 1 ml/min. Elution of the protein was done with a gradient formed from a starting buffer of 5 mM Na phosphate, pH 6.8, and a final buffer of 220 mM Na phosphate, pH 6.8, with continuously monitoring at A_{280}. Protein concentrations for pure lipoxygenase-2 and -3 were measured spectrophotometrically using A_{280} (1%) = 14.0.[21] Concentrations of lipoxygenases were calculated from the reported molecular weights (*i.e.*, 97,053 for lipoxygenase-2 and 96,541 for lipoxygenase-3).[1] Lipoxygenase activity was assayed by monitoring either the absorbance of linoleic acid 13-hydroperoxide at 234 nm with a Shimadzu UV-160A spectrophotometer or the consumption of oxygen with an oxygen electrode (Yellow Springs Instruments) as described.[21]

Cooxidation reaction. The standard reaction mixture contained 56.5 μM all-*trans*-retinoic acid (stock solution; 11.3 mM in DMSO), 125 μM linoleic acid (stock solution; 10 mM in 2.8% Tween 20, prepared as described[21]) and an appropriate amount of the purified enzyme in 50 mM Na phosphate, pH 6.5, containing 1 mM DETAPAC (diethylenetriaminepentaacetic acid). Cooxidation reaction was started by the addition of an enzyme solution and proceeded with stirring at 25°C under air. Respective reaction conditions are described in the legends for Figures. Continuous measurement of the cooxidation reaction was done with the following changes of $A_{382.5}$ (λ_{max} of all-*trans*-retinoic acid in the reaction buffer) with the spectrophotometer. For spectrum analyses (200–450 nm) or HPLC analyses, a portion (140 μl) of the reaction mixture was withdrawn at a given time and mixed with 860 μl of MeOH. HPLC analysis was done on a Hitachi L-6000 with an UV detector set at 325 nm. A Wakosil 5C18 column (Wako Pure Chemicals, 4.0 × 250 mm) was used at a flow rate of 0.75 ml/min of 86/14 mixture of MeOH and 70 mM AmOAc, pH 6.65.[22] The MeOH solution (20 μl) was directly injected into the HPLC column. In some cases, an HPLC system with a photodiode array detector (Hewlett Packard 1040A) was used. Methyl-esterification of the reaction products was done with ethereal diazomethane after extracting the compounds with AcOEt.

Preparation of 5,6-epoxyretinoic acid. All-*trans*-methylretinoate was prepared from all-*trans*-retinoic acid with diazomethane. To a mixture of methylretinoate (220.7 mg, 0.703 mmol), 20 ml of CH_2Cl_2, and 10 ml of saturated $NaHCO_3$ aqueous solution was added in small portions of *m*-chloroperbenzoic acid (Fluka, 173 mg, 1.00 mmol) with ice cooling.[23,24] After this was stirred for 1 h on an ice bath, the organic layer was separated, washed twice with 0.2 M KOH followed by water, and dried over Na_2SO_4. Removal of the CH_2Cl_2 gave a yellow residue that formed fine crystals if concentrated from its MeOH solution. Flash chromatography of the residue on silica gel (Kiesel gel 60, Merck) using 6% ether in hexane as eluent resulted in the isolation of the pure 5,6-epoxy methylretinoate (186.3 mg, 0.565 mmol) with yield of 80.3%. UV λ_{max} ($CHCl_3$) 346.5 nm; NMR δ_H ($CDCl_3$): 0.92 (3H, s, 5-CH_3), 1.09, 1.13 (6H, 2s, 1-CH_3), 1.95 (3H, s, 9-CH_3), 2.33 (3H, s, 13-CH_3), 3.70 (3H, s, –OCH_3), 5.78 (1H, s, CHC=O), 5.94–6.33 (4H, 4d, vinyls), 6.95 (1H, dd, C-11 H); CIMS: *m/z* (NH_3) 331.5 (M+1), 348.5 (M+18); EIMS: *m/z* (%) 330 (M^+, 14.6), 315 (6.8), 299 (0.9), 271 (11.8), 191 (13.3), 177 (22.2), 149 (40.9), 107 (36.2), 91 (49.7). 5,6-Epoxy methylretinoate (30 mg, 90.8 μmol) was further deesterified with 10% (w/v) KOH for 1 h[25] to form 5,6-epoxyretinoic acid (18.3 mg, 57.8 μmol, with yield of 63.7%). UV λ_{max} ($CHCl_3$) 346.0 nm. The amounts of retinoic acid, 5,6-epoxyretinoic acid and 5,6-epoxy methylretinoate were measured with their reported absorption coefficient, *E* (1% in $CHCl_3$) = 1260 (363 nm), 1360 (346 nm), and 1562 (345 nm),[25] respectively.

Separation of the optical isomers of methyl 5,6-epoxyretinoate. 5,6-Epoxyretinoic acid formed by the cooxidation reaction was purified by reversed-phase HPLC described as above. The epoxide was treated with diazomethane to form 5,6-epoxy methylretinoate. Chiral phase HPLC analysis was done on a Shimadzu LC-5A with an UV detector set at 342.5 nm. A Ceramospher Chiral RU-1 column (Shiseido, 4.6 × 250 mm) was used at a flow rate of 0.7 ml/min of MeOH. To identify the absolute configuration of the poxy function, the two optical isomers of synthetic 5,6-epoxy methylretinoate were isolated with the chiral-phase HPLC, and their CD spectra were recorded.

Results

Purification of soybean lipoxygenase-2 and -3

To separate soybean lipoxygenase-2 and -3, the HPLC method of Ramadoss and Axelrod[5] was modified. The mixture of lipoxygenase-2 and -3 obtained by classical DEAE chromatography[20,21] was separated by HPLC using a QAE column. At least four peaks of linoleic acid-induced O_2 uptake activity at pH 6.5 were obtained (Fig. 1). By this procedure, peaks A and B were purified to 1.7 and 0.53 μKat/mg protein, respectively; they were homogeneous by SDS–polyacrylamide gel electrophoresis. Although both the fractions were more active with linoleic acid (LA) as a substrate at pH 6.5 than with arachidonic acid (ARA), the LA/ARA ratio of fraction A (8.8) was much higher than

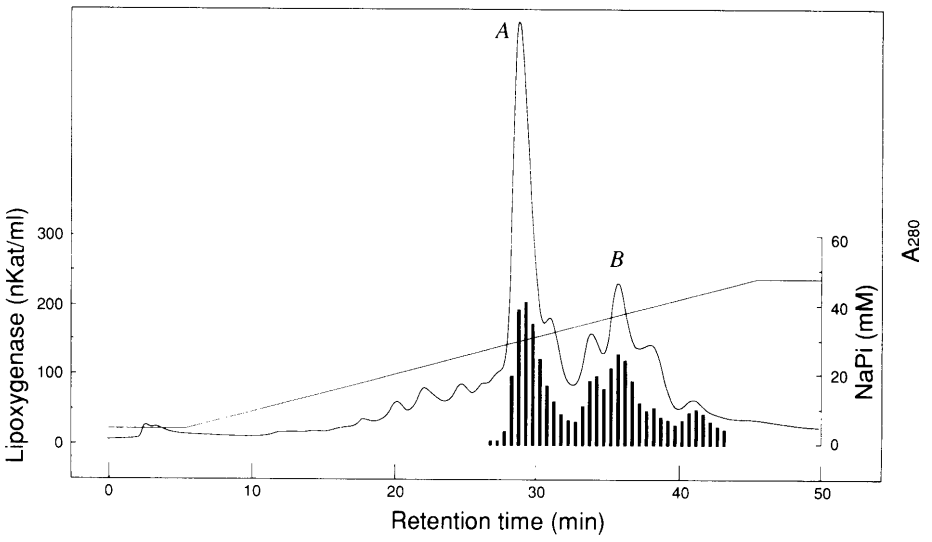


Fig. 1. HPLC Fractionation Profile of DEAE-Purified Lipoxygenase Fraction.
The bars represent linoleic acid-induced O_2 uptake activity.

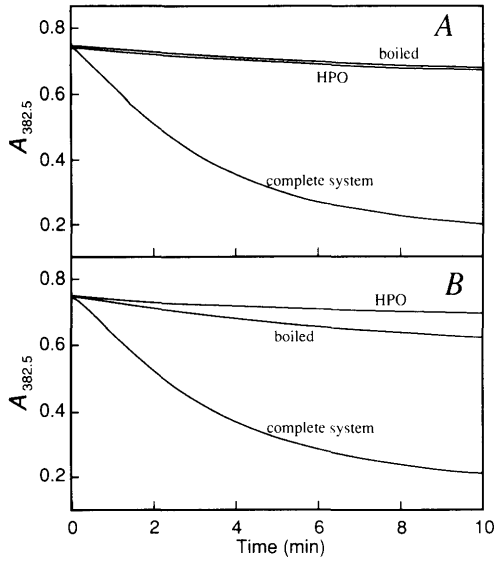


Fig. 2. Course of Degradation of All-*trans*-retinoic Acid.

All-*trans*-retinoic acid (22.3 μM) and linoleic acid (100 μM) were incubated with lipoxygenase-3 (panel A, 147 nM) or lipoxygenase-2 (panel B, 29.2 nM) in 50 mM Na phosphate, pH 6.5 containing 1 mM DETAPAC at 25°C under air. The reaction was started by the addition of enzyme, and the change in $A_{382.5}$ was continuously checked. HPO, linoleic acid was replaced by an equal concentration of linoleic acid 13-hydroperoxide; boiled, lipoxygenase was replaced by a sample boiled for 15 min.

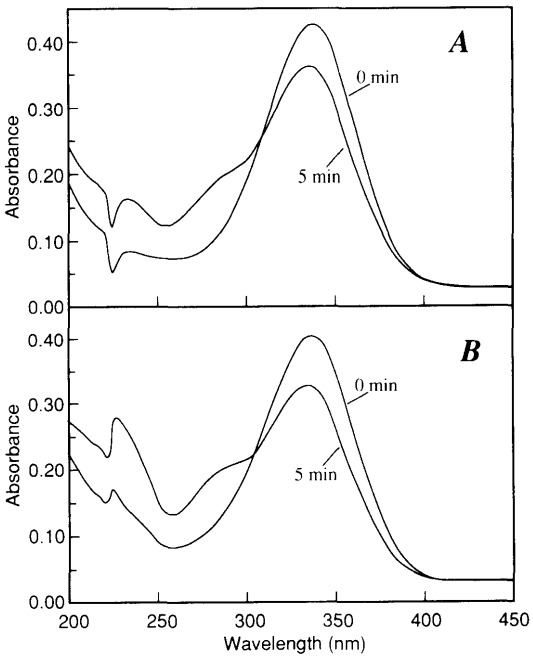


Fig. 3. Changes in the Chromophore of All-*trans*-retinoic Acid during Cooxidation Reaction.

All-*trans*-retinoic acid (56.5 μM) and linoleic acid (125 μM) was incubated either with lipoxygenase-3 (110.3 nM, panel A) or lipoxygenase-2 (110.3 nM, panel B) in 50 mM Na phosphate, pH 6.5 containing 1 mM DETAPAC at 25°C under air. The reaction was started with the addition of the enzyme. A portion (140 μl) of the mixture was withdrawn just after the addition of the enzyme or after 5 min and mixed with 860 μl of MeOH, then the spectrum was recorded.

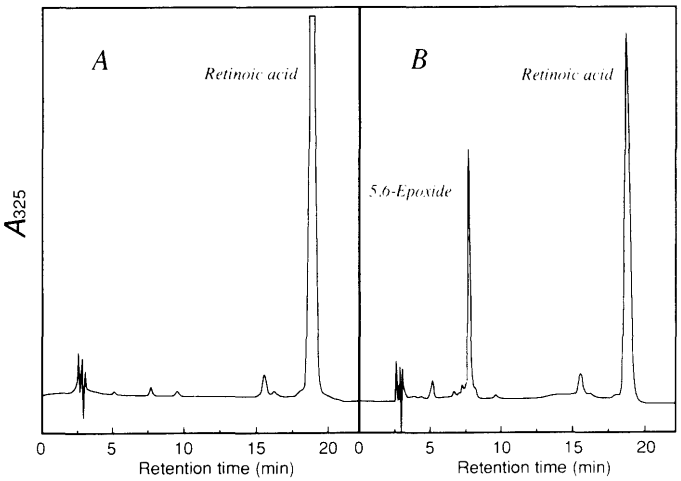


Fig. 4. HPLC Analysis of Cooxidation Reaction.

Lipoxygenase-2 (36.5 nM) was incubated with all-*trans*-retinoic acid (56.5 μM) and linoleic acid (125 μM) at 25°C under air. A portion (140 μl) of the mixture was withdrawn just after the addition of the enzyme (panel A) or after 5 min (panel B) and mixed with 860 μl of MeOH. The MeOH solution (20 μl) was directly resolved with reversed-phase HPLC. The cooxidation reaction done with lipoxygenase-3 under the same conditions (but the concentration of the enzyme increased to 184 nM) showed almost the same chromatogram.

that of fraction B (2.6). Ketodiene formation following changes in A_{280} was more pronounced with fraction A than with fraction B ($\Delta A_{280}/\Delta A_{234}$ ratio of A and B were 0.66, and 0.35, respectively). Although minor differences from the proposed criteria[5] for the identification of the respective soybean lipoxygenase isoenzymes existed, the above-mentioned observations, which agreed with the elution sequence from an ion-exchange HPLC column, made it reasonable to identify peaks A and B as lipoxygenase-3 and -2, respectively.

Spectrophotometric analysis of cooxidation

As shown in Fig. 2, both the isoenzyme effectively cooxidized retinoic acid under aerobic conditions. A slight lag was evident with lipoxygenase-2. Calculation from the initial highest velocity of the degradation gave 23.1 and 5.14 nKat/mg protein as specific activities of cooxidation catalyzed by lipoxygenase-2 and -3, respectively. With both the isoenzymes the disappearance of the absorbance originating from retinoic acid was dependent on the presence of linoleic acid. The effect was significantly decreased when the native enzyme was replaced by a heat-denatured preparation, or linoleic acid 13-hydroperoxide was used instead of linoleic acid.

Some compounds such as nordihydroguaiaretic acid have been reported to have pseudoperoxidase activity for soybean lipoxygenase-1.[26,27] In our study, neither retinoic acid nor linoleic acid 13-hydroperoxide was degraded when they were simultaneously incubated with lipoxygenase-2 or -3. Thus, pseudoperoxidase activity could be excluded.

Spectral analysis of the reaction mixture showed a significant change in the retinoid chromophore upon addition of either lipoxygenase-2 or -3 (Fig. 3). In both cases, no distictive isosbestic points could be observed, which suggested that a variety of products was formed from retinoic acid during reaction. These changes were eliminated when heat-denatured enzyme or linoleic acid 13-hydroperoxide was substituted for the native one or linoleic acid, respectively. The reason for the appearance of absorbance at 280 nm was not studied in detail. However, GC-MS analyses of the reaction mixture found several keto-diene compounds, which may have originated from linoleic acid (see later).[21,28]

Chromatographic analysis

To identify the primary product of cooxidation of retinoic

acid, the lipoxygenase-2 catalysis was stopped after 5 min by adding MeOH, and the mixture was separated with reversed phase HPLC. As shown in Fig. 4, the amount of retinoic acid decreased and a more polar compound appeared at the retention time of 8 min. Almost the same chromatogram was observed when lipoxygenase-3 was used. The formation of the major peak was not observed when linoleic acid was omitted, or when the enzyme or linoleic acid was replaced by a heat-denatured enzyme or linoleic acid 13-hydroperoxide. Photodiode array detection found an absorption maximum of 324.5 nm for this major cooxidation product. For structural elucidation, derivatization to its methyl ester and fractionation by reversed phase HPLC were done. Together with a higher retention time, a new absorption maximum at 340.5 nm was observed. CIMS analysis of the isolated and methylated product showed ions of high intensity at m/z (NH_3) 331.5 (M+1) and 348.5 (M+18). The ^{1}H-NMR spectrum of the methylated product was almost the same as that obtained with synthetic 5,6-epoxy methylretinoate. These data showed the formation of 5,6-epoxyretinoic acid as a product of retinoic acid cooxidation. This evaluation was verified by comparison of chromatographic and spectral data of the reaction product with those of synthesized 5,6-epoxyretinoic acid.

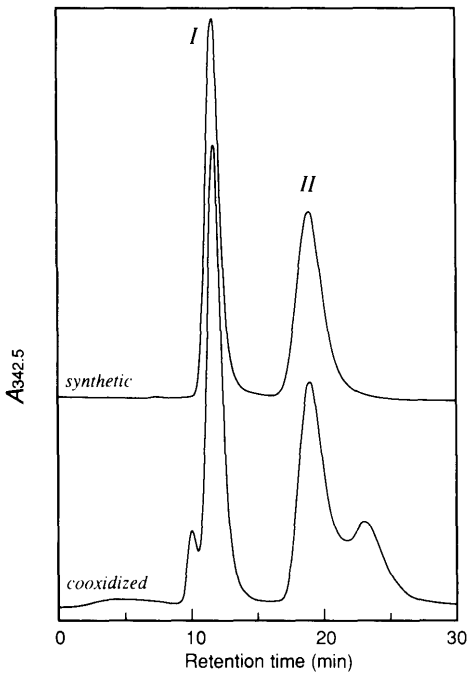


Fig. 5. Chiral-phase HPLC Analysis of 5,6-Epoxy Methylretinoate.

Synthetic 5,6-epoxy methylretinoate (upper trace, the peaks eluted fast and late were defined as peaks I and II, respectively) and methylated reaction products of lipoxygenase-3-catalyzed cooxidation of all-*trans*-retinoic acid (lower trace) were resolved by HPLC on chiral-phase column. The origin of the two minor peaks visible with lower trace has not been studied. Almost the same chromatogram was obtained with reaction products of lipoxygenase-2-catalyzed cooxidation (data not shown).

Optical purity of 5,6-epoxyretinoic acid

There was no information available on the resolution of optical isomers of 5,6-epoxyretinoic acid or its methyl ester. Thus, we tried to separate them by HPLC on a chiral phase. As shown in Fig. 5, synthetic 5,6-epoxy methylretinoate could be nicely separated into its optical antipodes (peaks I and II) using a Ceramospher Chiral RU-column. Comparison of the CD-spectra of the optical isomers (Fig. 6) with data previously reported for structurally related compounds such as 5*S*,6*R*-epoxy-4*S*-hydroxy retinol[29] indicated that peak I represented the (+)-5*R*,6*S*- and peak II the (−)-5*S*,6*R*-epoxy methylretinoate. Chiral phase HPLC analysis of the methylated 5,6-epoxide isolated from

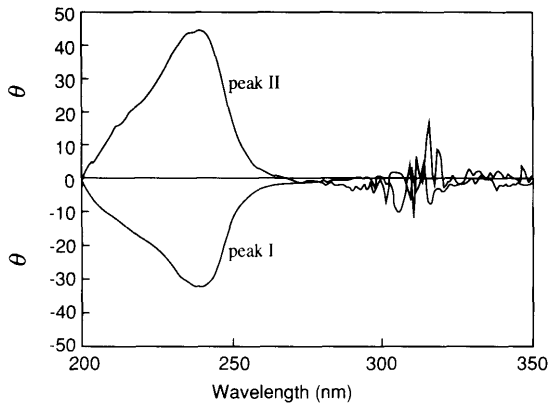


Fig. 6. CD Analysis of Resolved Optical Isomers of 5,6-Epoxy Methylretinoate.

Each optical isomer resolved by chiral-phase HPLC was used.

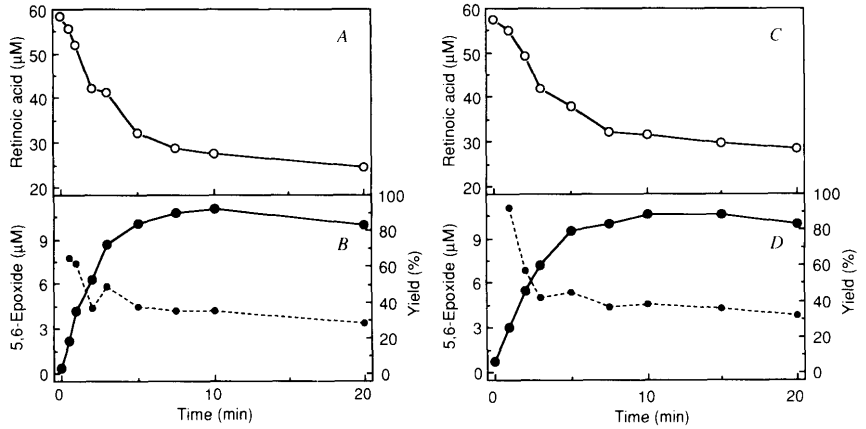


Fig. 7. Time Courses of Lipoxygenase-catalyzed Cooxidation.

All-*trans*-retinoic acid (56.5 μM) was incubated with either lipoxygenase-2 (36.5 nM, panels A and B) or lipoxygenase-3 (183.8 nM, panels C and D) in the presence of linoleic acid (125 μM) in 50 mM Na phosphate, pH 6.5, containing 1 mM DETAPAC at 25°C under air. A portion of the mixture (140 μl) was withdrawn and mixed with 860 μl of MeOH, then analyzed by quantitive HPLC. A and C, all-*trans*-retinoic acid; B and D, 5,6-epoxyretinoic acid. Dotted lines in panels B and D represent the yields (%) of 5,6-epoxyretinoic acid from retinoic acid.

the reaction mixture of cooxidation with either lipoxygenase-2 or -3 found equal amounts of both enantiomers (Fig. 5, lower trace). Obviously, the epoxidation during lipoxygenase-catalyzed cooxidation proceeded non-stereospecifically, *i.e.*, without specific interaction between the enzyme and the co-substrate.

Quantitative analyses of epoxidation

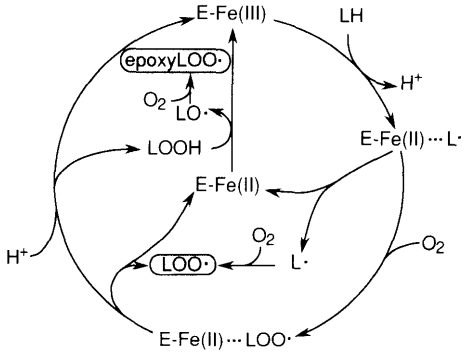
To analyze the stoichiometry of the epoxidation of retinoic acid during lipoxygenase-catalyzed cooxidation, the decrease of retinoic acid and the increase of 5,6-epoxyretinoic acid were simultaneously checked by HPLC. Figure 7 shows that the degradation of retinoic acid proceeded in almost the same course, as detected by spectrophotometric analysis (*cf.* Fig. 2). By HPLC analysis the specific activities of cooxidation by lipoxygenase-2 and -3 were calculated to be 25.7 and 9.26 nKat/mg protein, respectively. These values agreed with those estimated by spectrophotometric analysis. With both the isoenzymes, the progressive curves of 5,6-epoxide formation reached the saturation point slightly earlier than those of retinoic acid degradation. In both cases, the yields of 5,6-epoxide formation were about 30–40%, although the yields of 5,6-epoxide were higher in the earlier period of the cooxidation reaction and decreased gradually with time. These suggested that the primary major oxidation product was further degraded into other secondary products, which have little or no absorption at 325 nm, or retinoic acid was directly degraded into such other decomposition products. After methylation of the reaction mixture obtained from lipoxygenase-catalyzed cooxidation of retinoic acid, GC-MS analysis allowed the (tentative) identification of the following products (ions of high intensity are presented with relative intensity in parentheses); methyloctanoate (87 (33.9), 74 (100)), 2,4-dodecadienal (95 (8.5), 81 (100), 67 (18.6), 55 (16.9), 41 (37.3)), 9-oxo-methylnonanoate (143 (12.7), 115 (7.6), 111 (25.0), 87 (54.7), 83 (42.4), 74 (80.0), 59 (46.2), 55 (100)), 5,6-epoxy-β-ionone (135 (8.9), 123 (54.7), 43 (100)), 6-methyl-8-(1,1,5-trimethyl-5,6-epoxy)-3,5,7-octatrien-2-one, 13-oxo-9,11 (or 9-oxo-10,12)-methyl-octadecadienoate (308 (2.4), 237 (10.2), 166 (21.1), 151 (26.4), 109 (23.7), 95 (77.8), 81 (100)), 5,6-epoxy methyl-retinoate (330 (14.6), 315 (6.8), 299 (0.9), 271 (11.8), 1919 (13.3), 177 (22.2), 149 (40.9), 107 (36.2), 91 (49.7)), and several other unknown compounds. The compounds with straight chains were presumably derived from linoleic acid. They were predominant products observed after aeorobic reaction of lipoxygenase-2 or -3 with linoleic acid used as a sole substrate (results not shown). The C_{13} or C_{18} aldehydes were thought to be formed by oxidative cleavage at the side chain of 5,6-epoxyretinoic acid. Further characterization of these compounds are under way.

Discussion

In spite of the vast information available on the cooxidation of polyenes by lipoxygenases, data about the characterization of products and the reaction mechanism are rather scarce. Previously, the formation of xanthoxin and 3-hydroxy-5,6-epoxy-β-ionone, with yields as 2 and 8%, respectively, from violaxanthin by crude soybean lipoxygenase has been described.[16] Recently, Parry and Horgan reported that C_{25} and C_{27} apo-aldehydes, or C_{15} allenic apo-aldehydes were formed in less than 5% yield during cooxidation of C_{40} xanthophylls with soybean lipoxygenase-1.[17] These compounds are thought to be derived by an oxidative attack on the central part of the conjugated double bond of carotenoid, but El-Tinay and Chichester[30] indicated that the initial site of oxygen attack on the β-carotene molecule occurs at the terminal double bonds. This indication is reasonable because the terminal double bonds should have the highest electron density, while the density is progressively depleted as the central double bond is approached. All-*trans*-retinoic acid is structurally related to carotenoids, but has a simpler structure. Furthermore, its oxidative metabolism has been extensively examined, and several oxidation products such as 4-hydroxy-, 4-oxo,[13] 5,6-epoxy-, and 5,8-epoxyretinoic acid[12,22] have been identified. Therefore, we selected it as a model compound for the study of the lipoxygenase-catalyzed cooxidation with the aim to isolate and characterize its primary oxidation product.

Soybean lipoxygenase-1 has been shown to reduce fatty acid hydroperoxide into fatty acid alkoxyl radicals oxidizing reversible inhibitors such as catechols or hydroxylamines.[26,27,31] If this peroxidase-like cycle could be operated with retinoic acid, 4-hydroxy derivatives of retinoic acid should be expected to be formed as in the case with prostaglandin H synthase.[22] The results obtained in our study showed that all-*trans*-retinoic acid is not a substrate of the pseudoperoxidase activity of lipoxygenase-2 and -3. Thus, psueoperoxidase activities of these isoenzymes are not involved in the cooxidation of polyenes. Polyenes have been reported to inhibit lipoxygenase activity,[32] but their inhibitory mechanism has to be different from those of catechols or hydroxylamines.

Epoxidation of a polyene compound has been reported to proceed by a fatty acid peroxyl radical; *e.g.*, 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene has been described to be epoxidized by a peroxyl radical formed from fatty acid hydroperoxide by hematin.[33] α-Tocopherol has been found to be epoxidized by a *t*-butylperoxyl radical.[34] In addition, the formation of 5,6-epoxide from 13-*cis*-retinoic acid has also been derived by a peroxyl radical mechanism.[22] Thus, it is quite conceivable that a peroxyl radical formed from fatty acid is also operative in the lipoxygenase-catalyzed epoxidation of all-*trans*-retinoic acid. The proposed reaction mechanism is shown in Scheme. There are two sites where peroxyl radicals may be formed. One is generated after H-abstraction from



Scheme Reaction Mechanism of Lipoxygenase under Air.
L, fatty acid; E, lipoxygenase.

bis-allylic methylene in the fatty acid substrate followed by reaction with oxygen. This peroxyl radical is thought to be released from the lipoxygenase-intermediate complex attacking the $C_{5,6}$-double bond of the co-substrate. The other is considered to be formed from the product, *i.e.*, fatty acid hydroperoxide, which is reduced to an alkoxyl radical by oxidized state of lipoxygenase. The alkoxyl radical would immediately rearrange to an epoxyalkyl radical, which in turn react with oxygen to form epoxy-peroxyl radical. Both of these are thought to be primary oxidants forming 5,6-epoxide from retinoic acid. As the yield of 5,6-epoxide just after starting the reaction was high, and the lifetime of a peroxyl radical is much longer than that of alkyl or alkoxyl radicals, involvement of the latter radicals in the cooxidation reaction could be ruled out. The observation that the 5,6-epoxide formed during cooxidation was racemic confirms that there is no specific interaction between the co-substrate and the enzyme. However, to understand the reaction mechanism of the cooxidation more precisely, further investigations including detection of intermediate radical species and kinetic analyses on the reaction are inevitable.

Acknowledgments. This work was partly supported by the Monbusho International Scientific Research Program:Joint Research, No. 03044105. The financial support of Deutsche Forschungsgemeinschaft, Bonn, is gratefully acknowledged (SFB 347).

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