Oxidized Positions of Fatty Acyl Moieties of Phosphatidyl-ethanolamine in Liposome Autoxidation

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Liposomes of soybean and egg yolk phosphatidylethanolamine (PE) were oxidized in the presence of copper. Fatty acyl moieties with hydroxy and hydroperoxy groups attached were converted to methyl esters. The methyl esters were separated in TLC and then separated on silicic acid thin layer plates containing AgNO3. After GLC purification, hydrogenated and trimethylsilylated samples were analyzed for positional isomers in MS. Fatty acids, 18:2 and 18:3 in soybean PE and 18:2, 20:4, and 22:6 in egg yolk PE were examined. A singlet oxygen mechanism was presumably involved in part, but to explain the whole isomer pattern, some yet unexplained mechanism appears to be involved.

Many researchers have observed that phosphatidylethanolamine (PE) undergoes much more rapid oxidation than phosphatidylcholine and it accumulates only a small amount of the peroxides in autoxidation.1,2) We have reported formerly that the rapid oxidation of PE emulsions can be explained in terms of a chelate-like affinity of the emulsion interface to copper ions.3) It has been suggested that singlet oxygen participates in the autoxidation of phospholipid liposome.4,5) The purpose of the present work is to find out the clue to the mechanism of autoxidation catalysis of PE emulsions by analyzing the isomer composition of the oxidized products of fatty acyl moieties of PE. The formation mode of positional isomers of hydroperoxides has been established in singlet oxygen oxidation6−8) and that in autoxidation is well known. In this investigation the isomer pattern obtained of the oxidized products was different both from that in the case of autoxidation and from that of singlet oxygen oxidation.

MATERIALS AND METHODS

Preparation of phosphatidylethanolamine liposome.

Soybean PE was prepared from crude soya lecithin (Kanto Chem. Co.) and egg yolk PE was prepared from hen eggs after Folch's extraction, by column chromatography on silicic acid as previously described,3) except for exchange of the chloroform for trichloroethylene. The purities were checked in the thin layer chromatography. After freeze-drying of the benzene solutions, the PE (2% w/v) was dispersed in 0.03M aqueous sodium cholate solution by homogenizing.9) The resulting liposome solution was passed through Sephadex G-50 columns in order to remove cholate and then the concentration was adjusted to 2% by evaporating. All the procedures were performed under oxygen-free conditions made by purging with nitrogen.

Oxidation of liposomes. A liposome solution (about 30ml) was divided and taken into ten vessels of the Warburg's manometer, and aqueous cupric sulfate or a combination of aqueous cupric sulfate and L-ascorbic acid adjusted to pH 5.0 was added. The vessels were shaken at 40°C. The oxidized liposome was lyophilized. When contained, the ascorbic acid in the oxidized solution was removed by passing through Sephadex G-50 columns prior to freeze-drying. The freeze-dried sample was dissolved in 2−3ml trichloroethylene, 1M sodium methoxide in methanol (50ml) and methyl acetate (5ml) were added; the mixture was allowed to stand at room temperature under a nitrogen atmosphere for 10min, and acidified by an addition of acetic acid. After an excess addition of water, the mixture was extracted with hexane. The hexane layer was then extracted with methanol containing 10% water. The methanol layer was diluted with 1 volume of water and extracted with benzene. The benzene layer
contained the methyl esters of the oxidized products and after several washings with water was evaporated under reduced pressure.

Separation to hydroperoxy and hydroxy fatty esters. The above concentrate of the oxidized products (from about 600 mg PE) was applied to a Kieselgel GF layer (0.25 mm, 20 x 20 cm). The layer developed with hexane-ethyl ether (2:1) and the area of hydroperoxy and hydroxy fatty acyl esters were detected under a UV lamp, scraped off, and extracted with ethyl ether (Fig. 1(a)). To both the hydroperoxy and hydroxy fatty acyl ester preparations was added triphenylphosphine (5 mg in 1 ml benzene) and they were purified in the same thin layer chromatography as above (Fig. 1(b)).

Separation of hydroxy fatty acyl esters into double-bond-number classes. Layers of Kieselgel H containing 10% silver nitrate were used. For the samples which came from soybean PE, duplicate development with hexane-ethyl ether (1:1) was able to resolve the linoleate and linolenate derivatives (Fig. 2(a)). For the samples which came from egg yolk PE, triplicate development with ethyl ether was necessary to resolve the linoleate, arachidonate, and docosahexaenoate derivatives (Fig. 2(b)). The zones were revealed under a UV lamp after spraying with 2',7'-dichlorofluorescein, and were extracted with ethyl ether after being moistened with methanol-water containing sodium chloride.

Gas liquid partition chromatography (GLC). The above samples were dissolved in ethanol and hydrogenated by 2 ~ 3 hr shaking in the presence of platinum black under an atmospheric pressure of hydrogen at room temperature. After the solvent was removed a trimethylsilylating reagent (TMS-HT Tokyo Kasei Co., trimethylchlorosilan and hexamethyldisilazan in pyridine) was added, and after 10 min standing without further separation procedures the mixture was injected into a gas chromatograph (Shimadzu GC 6A) under the following conditions: Column, 3 mm x 2 m glass column packed with 3% OV-1 on Chromosorb W (HP); carrier gas, He 50 ml/min; detector, thermal conductivity detector; temperature, 100°C to 300°C at a rate of 5°C/min for the soybean PE samples and 150°C to 300°C at a rate of 5°C/min for the egg yolk PE samples. The retention times were: 15 min for linoleate derivative and 15 min for linolenate derivative (split peaks) in soybean PE; 17 min for linoleate derivative, 20 min for arachidonate derivative, and 23 ~ 25 min for docosahexaenoate derivative (split peaks) in egg yolk PE. The separated peaks were collected and applied to Hitachi Mass Spectrometer RMU-6 using the direct inlet system (inlet temperature 150°C, and ionization potential 70 eV).

RESULTS AND DISCUSSION

Oxidation of the liposome of soybean PE was catalyzed by an addition of copper ions, 10 ppm, or a combination of copper ions, 10 ppm, and ascorbate 10⁻² M, pH 5.0 and for oxidation of the egg yolk PE liposome 100 ppm copper ions were added. In the case of egg yolk PE the accumulation of hydroperoxides was very low. As a preliminary experiment showed that a high oxidation rate favored the accumulation of peroxides, a high concentration of copper (100 ppm) was used in the case of egg yolk PE but the hydroperoxides failed to be obtained probably owing to their loss in the chromatographic procedure.

It was established in a preliminary experiment that oxidized fatty acyl moieties of lipids of a triglyceride type could be converted to methyl ester without considerable damage by treating the samples with 1 m sodium methoxide in methanol at room temperature for 10 min. The result of the preliminary experiment was as follows:
(1) The peroxide content of a methyl linoleate hydroperoxide sample was not diminished after this treatment.
(2) There was no change of a methyl linoleate hydroperoxide sample on a thin layer chromatogram after the treatment.
(3) Methyl esters of hydroperoxides were successfully obtained from oxidized cotton seed and linseed oils by this treatment.
(4) Methyl ester of linoleic acid hydroperoxide was converted to ethyl and propyl esters almost quantitatively by analogous procedures using ethyl and propyl alcohols, respectively.

No hydroperoxide was detected in the products from the liposome of soybean PE oxidized in the presence of copper and ascorbic acid after the transesterification. Removal of the ascorbic acid by passing the liposome through a Sephadex G-50 column prior to the transesterification also gave no hydroperoxide, indicating that there was no hydroperoxide accumulation in the presence of ascorbic acid. Although ascorbic acid is known to assist copper-catalyzed oxidation of PE its reducing activity presumably decomposes the
Phosphatidylethanolamine Fatty Moieties in Autoxidation

Fig. 1. Separation of Hydroperoxo and Hydroxy Fatty Methyl Esters by TLC.
(a): First separation. (b): Second separation after addition of triphenylphosphine.
1, polar fraction of transesterified PE; 1', hydroperoxy fatty acyl esters obtained by the first separation; 1", hydroxy fatty acyl esters obtained by the first separation; 2, authentic alcohol derived from methyl linoleate hydroperoxide; 3, authentic methyl linoleate hydroperoxide.

Fig. 2. Separation of Hydroxy Fatty Acyl Methyl Esters by Argentation TLC.
(a): Samples from soybean PE. (b): Samples from egg yolk PE.
1, hydroxy fatty acyl ester mixture (soybean); 2, alcohol derived from methyl linoleate; 3, alcohol derived from methyl linolenate; 4, hydroxy fatty acyl ester mixture (egg yolk); 5, alcohol derived from methyl arachidonate; 6, alcohol derived from methyl docosahexaenoate.

Fig. 3. Isomer Patterns of Attacked Fatty Acyl Moieties Observed in Cu-Catalyzed Oxidation of Soybean PE As Expressed by Summarized Mass Fragmentations.
(1), the position with trimethylsiloxy group attached; (2), the position of the active methylene from which the hydrogen abstraction should result in the formation of the isomers of (1) if normal radical mechanism is assumed. Oxidation time: 1.5 hr. Oxygen uptake: 6.8 mol% on the basis of total fatty acids. Change in fatty acid composition (%) by oxidation: 16:0, 23.9–24.7; 18:0, 3.0–2.6; 18:1, 8.7–8.3; 18:2, 58.6–48.5; 18:3, 5.7–4.3.

Hydroperoxides further.

After the transesterification most part of the methyl ester of the unchanged fatty acids was removed from the polar oxidized products by an extraction procedure in order to diminish the sample size in the following chromatographic procedures. In them a small size of the adsorbent was necessary to avoid degradation of the products.

Hydroperoxy (ROOH), and hydroxy (ROH) derivatives of fatty acid methyl esters were separated by thin layer chromatography as shown in Fig. 1. ROOH was converted to ROH with triphenylphosphine. Each of the ROHs from the above different origins was separated into classes of double-bond number by thin layer chromatography using silicic acid containing 10% silver nitrate. The method has
been applied to hydroxy derivatives of oleate, linoleate, and linolenate\textsuperscript{10} but not to those of higher unsaturated members, so that appropriate conditions of development were preliminarily determined. The samples from soybean PE were separated into ROHs of linoleate and linolenate and the samples from egg yolk PE were separated into ROHs of linoleate, tetraenoates, and hexaenoates as shown in Fig. 2. In egg yolk PE, tetraenoates consisting mainly of arachidonate and \(\omega_3\)-eicosatetraenoate were negligible in comparison with arachidonate, and only the \(\omega_3\)-isomer has been known as being docosahexaenoates.

The ROHs separated into different double-
bond-number classes were catalytically hydrogenated, converted to TMS-derivatives, purified by gas chromatography in order to remove components different in the carbon number, and then subjected to mass spectrometry. The conversion of the ROHs to the corresponding saturated derivatives made gas-chromatographic separation possible without overlapping of different carbon-number members. The hydrogenation also made interpretation of the mass spectra easy.

Attempts were made to compare the isomer patterns of hydroperoxy and hydroxy fatty acids in liposome oxidation of the PEs with those in normal autoxidation of fatty esters and their singlet oxygen oxidation.

Figures 3, 4 and 5 show the results of the liposome oxidations of soybean PE catalyzed with copper, of soybean PE catalyzed with copper and ascorbic acid, and of egg yolk PE catalyzed with copper, respectively. The oxidation times and the changes in fatty acid composition after oxidation are described in the legends. In these Figures the intensity of each peak was expressed as a percentage on the basis of the sum of the intensities of all the peaks which are expected to be found in normal autoxidation of the fatty acid, e.g., in the case of linoleate the sum of the intensities of peaks m/z: 173, 229, 259, and 315 was taken as 100%. Data obtained from the corresponding fatty acyl methyl esters by autoxidation were depicted in the Figures as dotted-lined peaks and in the following discussion, intensities of peaks obtained from liposome oxidation will be compared with those of the corresponding peaks of normal autoxidation.

Figures 3(a, c), and 4(a) show that the isomer composition of linoleate in the liposome oxidation did not differ from that in autoxidation. Figures 3(b, d), and 4(b) show that in the liposome oxidation, linolenate gave a considerable amount of the 10-isomer but no 15-isomer, suggesting that it can not simply be explained in terms of singlet oxygen mechanism, and the 12-isomer and the 16-isomer tended to increase and decrease, respectively, although there was some deviation among different runs and samples.

Of egg yolk PE, Fig. 5(a) shows that linoleate in the liposome oxidation gave a small amount of the 10-isomer. Figure 5(b) shows that arachidonate in the liposome oxidation gave small amounts of the 6- and 14-isomers, and the 9-isomer highly increased and the 15-isomer highly decreased, and slight increases in the 8- and 12-isomers were also observed. Figure 5(c) shows that docosahexaenoate in the liposome oxidation gave small amounts of the 5- and 19-isomers, and marked increases in the 8- and 11-isomers and the marked decrease in the 20-isomer occurred, and the 4-isomer decreased slightly. Although only hydroxy derivatives were obtained in many experiments, for the present it is considered valid that the hydroxy derivatives were derived from the corresponding hydroperoxy derivatives by secondary changes. The presence of the 10-isomer of linoleate, the 6- and 14-isomers of arachidonate, and the 5- and 19-isomers of docosahexaenoate suggests that singlet oxygen oxidation played a roll to some extent.

However, in both the cases of soybean and egg yolk PEs, differences in the isomer compositions from those of normally autoxidizing fatty esters were not explainable totally in terms of a simple combination of autoxidation and singlet oxygen oxidation mechanisms because there was a marked and unexpected change in isomer distribution. So, there must be an unknown mechanism which can explain such different reactivities with different positions on a fatty acid molecule in liposome PE. The mechanism will involve some novel active species other than ordinary free radicals and singlet oxygen, or some movement restriction of active species and fatty acyl chains by the interface.

Little has been known about mechanism dominating such interfacial oxidation but well-known loose specificity of lipoxigenase in positional isomer formation, in which the isomer composition varies widely by changing pH and other medium conditions, may give a suggestive analogy in further investigation.
Also, Chan and his coworker’s interesting study\(^\text{11}\) on metal-catalyzed oxidation of fatty soap micelles in which isomer compositions differed from those of autoxidation may be suggestive.

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REFERENCES