Monohydroperoxides Formed by Autoxidation and Photosensitized Oxidation of Methyl Eicosapentaenoate

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Methyl eicosapentaenoate (methyl 5,8,11,14,17-eicosapentaenoate) was subjected to autoxidation and methylene blue sensitized photooxidation. Methyl eicosapentaenoate monohydroperoxides, the primary products of the autoxidation and photosensitized oxidation, were isolated by silica gel column chromatography, and characterized by ultraviolet, infrared and nuclear magnetic resonance spectra. The isomeric composition of the monohydroperoxides were determined by gas chromatography-mass spectrometry as follows: the 5-, 8-, 9-, 11-, 12-, 14-, 15- and 18-isomers (autoxidation), and the 5-, 6-, 8-, 9-, 11-, 12-, 14-, 15-, 17- and 18-isomers (photosensitized oxidation). Methyl eicosapentaenoate was readily oxidized both by autoxidation and by photosensitized oxidation.

Unsaturated lipids are subjected to lipid peroxidation which leads to nutritional damage in foods and physiological damage in living organisms. The oxidation mechanisms of unsaturated lipids by the free radical reaction and by the singlet molecular oxygen ($^1\text{O}_2$) have been investigated.^{1~3)} Polyunsaturated fatty acids consisting of triacylglycerols and phospholipids produce positional isomers of monohydroperoxide as the primary oxidation products.

Eicosapentaenoic acid (5,8,11,14,17-eicosapentaenoic acid) has been reported to contribute to a low incidence of acute myocardial infarction and atherosclerosis as well as to the bleeding tendency.^{4,5)} Eicosapentaenoic acid is considered to be susceptible to lipid peroxidation because of the presence of five double bonds in the molecule. Fukuzumi *et al.*⁶⁾ reported the peroxide concentrates from autoxidized methyl eicosapentaenoate, but they could not determine the structures of the peroxides. Noble and Nawar^{7,8)} reported the volatile decomposition products of methyl docosahexaenoate after autoxidation, and proposed mechanisms for the formation of the volatile compounds. However, little is

known about the peroxidation mechanism of eicosapentaenoic acid.

This paper reports the autoxidation and the $^1\text{O}_2$ oxidation of methyl eicosapentaenoate. Methylene blue sensitized photooxidation was employed as the $^1\text{O}_2$ generating system.⁹⁾ The monohydroperoxides were isolated, and their structures are characterized. The rate of peroxidation of methyl eicosapentaenoate is compared with those of the other unsaturated fatty acid methyl esters.

MATERIALS AND METHODS

Materials. Methyl eicosapentaenoate concentrate was kindly provided by Taiyo Fishery Co., Ltd. Methyl eicosapentaenoate was purified by reverse-phase high performance liquid chromatography (HPLC) on a column (4.6 × 250 mm) of LiChrosorb RP-18 (Merck, 10 μm in particle size) using methanol-water (95:5, v/v) as the solvent. The purity of the methyl eicosapentaenoate was >99% by gas liquid chromatography. Methyl linoleate and methyl linolenate were purchased from Tokyo Kasei Co., and methyl arachidonate was prepared from pig liver phospholipids.¹¹⁾ These methyl esters were also purified by HPLC as described above. Other reagents used were of analytical grade.

Peroxidation procedure. Methyl eicosapentaenoate

(300 mg) in a glass vial was autoxidized at 37°C with bubbling air for 20 hr. The rate of autoxidation of methyl eicosapentaenoate, methyl arachidonate, methyl linoleate and methyl linoleate was determined in a thin lipid film. 50 μ l of 10^{-2} M methyl ester in ethanol solution was added to a test tube (1.5 cm in diameter), and then the solvent was removed by evaporating *in vacuo* to form a thin lipid film. The sample was placed in an incubator maintained at 37°C in the dark. At intervals, each sample was dissolved in 5 ml of ethanol, and the amount of conjugated diene was measured at 235 nm.

Methyl eicosapentaenoate was photo-oxidized in an ethanol solution containing methylene blue as the photosensitizer. Methyl eicosapentaenoate (150 mg) was dissolved in 50 ml of ethanol containing methylene blue (2 mg). The solution in a reaction vessel was placed in a water bath at 25°C, and shaken mechanically. The light source was a 60 W tungsten projection lamp at a distance of 20 cm from the vessel. The reaction was carried out for 6 hr. The rate of photosensitized oxidation of methyl eicosapentaenoate, methyl arachidonate, methyl linoleate and methyl linoleate was determined in an ethanol solution. 10^{-2} M methyl ester in an ethanol solution containing 10^{-4} M methylene blue was added to a reaction vessel, and irradiated with a 60 W lamp at 25°C. At intervals, 50 μ l of the sample was dissolved in 5 ml of ethanol, and measured at 235 nm.

Preparation and characterization of monohydroperoxides. The peroxidized methyl eicosapentaenoate was analyzed by thin layer chromatography (TLC), using a Merck pre-coated silica gel 60 plate, with hexane-ethyl ether-acetic acid (60:40:1, v/v). Lipids were detected with 50% H_2SO_4 in saturated K_2CrO_7 solution, and peroxides were detected with 5% KI in methylcellosolve solution. Monohydroperoxides were separated by silica gel column chromatography with stepwise elutions of 5 and 30% ethyl ether in hexane. The unoxidized lipid was eluted with the 5% ethyl ether fraction, and monohydroperoxide eluted with the 30% ethyl ether fraction.

The isolated monohydroperoxides were characterized by ultraviolet (UV) spectra, infrared (IR) spectra and nuclear magnetic resonance (NMR) spectra. The NMR spectra were recorded at 90 MHz with a Hitachi R-22 spectrometer for solutions in CCl_4 , tetramethylsilane being used as the internal standard. The monohydroperoxides were reduced by NaBH_4 or hydrogenated by a stream of hydrogen gas according to the method described by Terao and Matsushita.¹¹⁾ The reduced or hydrogenated product was converted to a trimethylsilyl (TMS) derivative and analyzed by gas chromatography-mass spectrometry (GC-MS). GC-MS was carried out on a Hitachi M-52 apparatus. Operational conditions were the same as described previously.¹²⁾

RESULTS

Autoxidation of methyl eicosapentaenoate

Methyl eicosapentaenoate was autoxidized for 20 hr and the oxidation products were analyzed by TLC. Figure 1 shows the densitometric pattern of autoxidized methyl eicosapentaenoate on TLC. Methyl eicosapentaenoate and many products were observed on the plate. The oxidation products were shown to be peroxides by the KI test.¹³⁾ The most mobile oxidation product, A, was isolated by silica gel column chromatography. The UV spectrum of compound A gave an absorption maximum at 235.5 nm in ethanol ($\epsilon = 23,000$) due to conjugated diene. The IR spectrum of compound A gave a strong absorption at 3400 cm^{-1} , attributed to the hydroperoxy group.⁶⁾ Furthermore, the NMR spectrum of compound A showed a broad signal downfield, δ ca. 8.5 ppm, as a result of the hydroperoxy proton.¹⁴⁾ After the addition of D_2O to the NMR tube, the signal diminished to the base line. The proton number in the region of the hydroperoxy proton calculated from an integral based on the terminal methyl proton (δ 0.9~1.1) was 0.9. Thus, compound A was identified to be methyl eicosapentaenoate monohydroperoxide.

The TMS derivative of NaBH_4 -reduced

FIG. 1. Densitometric Pattern of Autoxidized Methyl Eicosapentaenoate on the TLC Plate.

An aliquot of the autoxidized methyl eicosapentaenoate (37°C for 20 hr) was spotted on a TLC plate. The plate was developed with a solvent of hexane-ethyl ether-acetic acid (60:40:1, v/v). After it had been sprayed with 50% H_2SO_4 in saturated K_2CrO_7 solution, the plate was heated for 10 min and monitored by a densitometer at 350 nm.

FIG. 2. Gas Chromatograms of the TMS Derivatives of Monohydroperoxide Formed by the Autoxidation of Methyl Eicosapentaenoate after NaBH_4 Reduction (A1) and Hydrogenation (A2).

compound A was analyzed by GC-MS (Fig. 2-A1). Two vicinal peaks, a and b, appeared on the chromatogram. The mass spectra of peaks a and b were almost the same. The presence of the characteristic ions at m/z 404 (M^+), 389 ($[\text{M}-\text{CH}_3]^+$) and 314 ($[\text{M}-90]^+$, loss of trimethylsilanol) indicated a hydroxy methyl eicosapentaenoate TMS derivative. When the double bonds were saturated by hydrogenation, the TMS derivative showed two peaks, c and d, on the chromatogram (Fig. 2-A2). From the mass spectra, peaks c and d were identified to be hydroxy methyl eicosanoate TMS derivatives;¹⁵⁾ m/z 399 ($[\text{M}-\text{CH}_3]^+$), 383 ($[\text{M}-\text{CH}_3\text{O}]^+$) and 367 ($[\text{M}-(\text{CH}_3+\text{CH}_3\text{OH})]^+$). Characteristic fragmentation patterns indicated that peak c was a mixture of C-5 (m/z 203 and 313), C-8 (m/z 245 and 271), C-9 (m/z 257 and 259), C-11 (m/z 229 and 287), C-12 (m/z 215 and 301), C-14 (m/z 187 and 329) and C-15 (m/z 173 and 343), and peak d was C-18 (m/z 131 and 385) positional isomers of the TMS group. From the above spectral data, compound A was identified to be isomeric methyl eicosapentaenoate mono-

FIG. 3. Time Course of Autoxidized Methyl Eicosapentaenoate, Methyl Arachidonate, Methyl Linolenate and Methyl Linoleate.

The neat fatty acid methyl esters were autoxidized at 37°C . ●—●, methyl eicosapentaenoate; ○—○, methyl arachidonate; △—△, methyl linolenate; □—□, methyl linoleate.

FIG. 4. Densitometric Pattern of the Photosensitized Oxidation of Methyl Eicosapentaenoate on the TLC Plate. An aliquot of the photosensitized oxidation products of methyl eicosapentaenoate (25°C for 6 hr) was spotted on a TLC plate. The conditions of TLC separation and detection were the same as described in Fig. 1.

hydroperoxides.

Figure 3 shows the rate of autoxidation of the methyl eicosapentaenoate, methyl arachidonate, methyl linolenate and methyl linoleate thin films at 37°C . Methyl eicosapentaenoate was rapidly autoxidized. On the other hand, the other unsaturated esters scarcely oxidized during this incubation time.

Photosensitized oxidation of methyl eicosapentaenoate

Methyl eicosapentaenoate was photo-

oxidized for 6 hr in an ethanol solution containing methylene blue as the photosensitizer. Figure 4 shows the densitometric pattern of photo-oxidized methyl eicosapentaenoate on TLC. Two spots corresponding to methyl eicosapentaenoate and an oxidation product, **B**, appeared on the plate. Compound **B** was positive to the KI test, and showed an absorption maximum at 235.5 nm in ethanol ($\epsilon = 20,000$). The IR spectrum gave a strong absorption at 3400 cm^{-1} . The NMR spectrum showed a broad hydroperoxy proton signal at δ ca. 8.5 ppm and the number of the hydroperoxy proton was 1.1. Thus, compound **B** was identified to be methyl eicosapentaenoate monohydroperoxide.

The TMS derivative of NaBH_4 -reduced compound **B** was analyzed by GC-MS (Fig. 5-B1). Two vicinal peaks, a and b, appeared on the chromatogram. The mass spectra of the two peaks, showing almost the same result as described in the TMS derivative of NaBH_4 -reduced compound **A**, indicated a hydroxy methyl eicosapentaenoate TMS derivative. When the TMS derivative of hydrogenated compound **B** was analyzed by GC-MS, three peaks, c, d and e, appeared on the chromatogram (Fig. 5-B2). From the mass spectra, peaks c, d and e were identified to be hydroxy methyl eicosanoate TMS derivatives; m/z 399 ($[\text{M}-\text{CH}_3]^+$), 383 ($[\text{M}-\text{CH}_3\text{O}]^+$) and 367 ($[\text{M}-(\text{CH}_3+\text{CH}_3\text{OH})]^+$). Characteristic fragmentation patterns indicated that peak c was a mixture of C-5 (m/z 203 and 313), C-6 (m/z 217 and 299), C-8 (m/z 245 and 271), C-9 (m/z 257 and 259), C-11 (m/z 229 and 287), C-12 (m/z 215 and 301), C-14 (m/z 187 and 329) and C-15 (m/z 173 and 343), peak d was C-17 (m/z 145 and 371), and peak e was C-18 (m/z 131 and 385) positional isomers of the TMS group. From the above data, compound **B** was identified to be isomeric methyl eicosapentaenoate monohydroperoxides.

Figure 6 shows the rate of methylene blue sensitized photooxidation of methyl eicosapentaenoate, methyl arachidonate, methyl linolenate and methyl linoleate in ethanol solutions. The formations of the conjugated

FIG. 5. Gas Chromatograms of the TMS Derivatives of Monohydroperoxide Formed by the Photosensitized Oxidation of Methyl Eicosapentaenoate after NaBH_4 Reduction (B1) and Hydrogenation (B2).

FIG. 6. Time Course of Photosensitized Oxidation of Methyl Eicosapentaenoate, Methyl Arachidonate, Methyl Linolenate and Methyl Linoleate.

The reaction mixture containing 10^{-2} M fatty acid methyl ester and 10^{-4} M methylene blue in ethanol solution was exposed to irradiation at 25°C . ●—●, methyl eicosapentaenoate; ○—○, methyl arachidonate; △—△, methyl linolenate; □—□, methyl linoleate.

diene were linear with the irradiation time. Methyl eicosapentaenoate showed the greatest formation of conjugated diene of the four

unsaturated esters.

DISCUSSION

The primary oxygenated product of unsaturated lipids is monohydroperoxide. Many workers have reported the autocatalytic oxidation of oleate, linoleate, linolenate and arachidonate, and have characterized monohydroperoxide isomers^{11,16~20)} and their further oxygenated compounds.^{11,21~23)} When methyl eicosapentaenoate was autoxidized, the eight regioisomers of monohydroperoxide, that is, the 5-, 8-, 9-, 11-, 12-, 14-, 15- and 18-isomers, were formed as the result of O₂ attack on the pentadiene radicals.^{1,2)} Further oxygenated compounds were also detected on the TLC plate (Fig. 1). However, their structures are still uncertain. Arachidonic acid is the precursor of dienoic prostaglandins,²⁴⁾ and eicosapentaenoic acid is the precursor of trienoic prostaglandins.²⁵⁾ The nonenzymatic peroxidation of arachidonic acid has been reported to form prostaglandin-like compounds.²⁶⁾ Therefore, the autoxidation of eicosapentaenoic acid may form prostaglandin-like compounds.

The lipid peroxidation is also caused by ¹O₂ which can be produced by photosensitized oxidation.²⁷⁾ The isomeric compositions of monohydroperoxides formed by the ¹O₂ oxidation of unsaturated lipids have been investigated.^{15,28,29)} Singlet molecular oxygen reacts directly with double bonds by concerted addition, the so-called "ene type" reaction.^{1,2)} The ten regioisomers of monohydroperoxide, that is, 5-, 8-, 9-, 11-, 12-, 14-, 15- and 18-isomers (conjugated isomer), and 6- and 17-isomers (nonconjugated isomer), were formed from methyl eicosapentaenoate as the result of the binding of ¹O₂ to the double bonds at the 4, 5, 8, 11, 14 and 17 positions. The difference in the isomeric compositions of the monohydroperoxides between the autoxidation and the ¹O₂ oxidation was that the nonconjugated diene isomers, 6- and 17-isomers, were specific to ¹O₂ oxidation in the peroxidation of methyl eicosapentaenoate. Furthermore, monohydro-

peroxide was the only product of the ¹O₂ oxidation (Fig. 4).

Methyl eicosapentaenoate contains five double bonds in the molecule. Consequently, methyl eicosapentaenoate was less stable on autoxidation and ¹O₂ oxidation (Figs. 3 and 6). In particular, the autoxidation proceeded much faster than with the other unsaturated fatty acid methyl esters. The results indicate that methyl eicosapentaenoate is rapidly peroxidized for deterioration. Research on antioxidants may unravel how to protect against the peroxidation of methyl eicosapentaenoate.

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