Lipid Degradation Products Capable of Reacting with Amino Acid— Identification of 4-Hydroxy-2-Hexenal, 9-Formyl Methyl- 8-Nonenoate, and 10-Formyl Methyl-9-Decenoate from Autoxidized Methyl Linolenate

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Gel chromatography of autoxidized methyl linolenate on Bio-Beads S-X3 gave a degradation product fraction which was discolored by shaking with 0.5 M Gly at 45°C for 90 min.

IR spectra obtained before and after the discoloration reaction, showed that the absorptions at 5.96 μ due to C=O of conjugated carbonyl, 6.13 μ due to conjugated C=C and 10.3 μ due to *trans* C=C were remarkably reduced by the discoloration reaction.

After conversion of carbonyl group into dimethylhydrazone and of hydroxyl group into TMS ether, the degradation products were analyzed by GLC and GC-MS. Some of conjugated carbonyls that decreased after the discoloration reaction were identified as 4-hydroxy-2-hexenal, 9-formyl methyl-8-nonenoate and 10-formyl methyl-9-decenoate.

Autoxidation of lipids containing significant quantities of polyunsaturated fatty acids results in severe impairment of quality in foods. Among the undesirable characteristics caused by the autoxidation, such as toxicity, off-flavor, and lowering of nutritive value, the discoloration is one of the serious problems in food technology. Much evidence has indicated that carbonyl compounds, especially unsaturated ones, are responsible for the discoloration¹⁻³⁾, but few substances relating to the discoloration have been identified from autoxidized lipids.

In the previous paper, we separated the components of autoxidized methyl linolenate into polymer, dimer and monomer by gel chromatography, and found that some degradation products, which were capable of reacting with amino acid to produce hydrophilic discolored substances at 45°C, were eluted in a later part of monomer fraction⁴⁾. In this paper, some of these degradation products believed to react with amino acid are identified.

Materials and Methods

Autoxidation of methyl linolenate Methyl linolenate, prepared from linseed oil by methanolysis followed by urea fractionation⁵⁾ and vacuum distillation, was autoxidized in a loosely stoppered flask at 40°C for 4 days with stirring. GLC-purity of the methyl linolenate was 84%, and the rest was methyl linoleate. POV of the autoxidized one was 2500 meq/Kg.

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Preparation of degradation product fraction The autoxidized methyl linolenate was fractionated into polymer, dimer and monomer by gel chromatography on Bio-Beads S-X3 with benzene as solvent. The degradation product fraction (DP), which eluted in a later part of monomer fraction and was capable of reacting with amino acid⁴⁾, was collected.

Discoloration of DP with Gly To 22.5 mg of DP, 6 ml of methanol and 3 ml of 0.5 M Gly solution (pH 7.0) were added. The mixture was shaken for 90 min at 45°C. To the discolored mixture, 1.5 ml of water and 12 ml of chloroform were added. After having been shaken for a min and allowed to stand for 10 min, the mixture was separated into two parts, hydrophilic (upper phase) and lipophilic (lower phase) parts. Both parts were diluted to 25 ml with methanol, and optical densities were measured at 430 nm. After removal of the solvent under aspirator pressure from the lipophilic part, the 2nd and 3rd discoloration reactions were carried out following the same procedure as described above.

The lipophilic part thus obtained after the 2nd discoloration reaction was referred to as "after discoloration". "Control" was the lipophilic part obtained by shaking with water instead of 0.5 M Gly.

IR analysis IR spectra of "before discoloration (DP)", "after discoloration" and "control" were measured in chloroform with a EPI-G21 Hitachi IR spectrometer.

Preparation of derivatives (1) Dimethylhydrazone (DMH) Ten mg of the lipophilic part was dissolved in 0.5 ml of N,N-dimethylhydrazine, and the solution was allowed to stand at room temperature for 2 hr⁶⁾. (2) Trimethylsilyl (TMS) ether Hydroxyl compounds in the lipophilic part were silylated with hexamethyldisilazane and trimethylchlorosilane in pyridine⁷⁾. (3) Reduction with NaBH₄ Ten mg of the lipophilic part was dissolved in 3 ml of methanol, cooled to 0°C and reduced with 30 mg of NaBH₄⁸⁾. After 20 min at 0°C and 40 min at room temperature, the solution was acidified with 3% HCl/methanol. Methyl borate produced and HCl were removed under aspirator pressure, and NaCl produced was excluded by centrifugation from small amount of the methanol solution.

GLC analysis DMH, TMS and DMH-TMS derivatives of the lipophilic parts were analyzed with a Shimadzu GC-4BPF gas chromatograph equipped with two 2 m × 4 mm I.D. glass columns packed with 5% SE-30 on 60-80 mesh Shimalite W. The flow rate of N₂ carrier gas was 40 ml per min, and the temperature was linearly programmed from 100 to 220°C at 2°C per min.

GC-MS analysis GC-MS analysis was carried out with a JMS-07 Nihon Densi mass spectrometer equipped with a 2 m × 3 mm stainless steel column packed with 5% SE-30 on 60-80 mesh Shimalite W and programmed from 100 to 220°C at 2°C per min. Ionizing current was 300 μ A and ionizing voltage was 21 eV.

Results

Discoloration of DP with Gly DP prepared by the gel chromatography was discolored with Gly and the result is shown in Table 1. Optical density of the hydrophilic part was much higher than that of the lipophilic one. Visual color of the hydrophilic one was reddish brown in the 1st and light purple in the 3rd discoloration reaction.

Table 1. Discoloration of DP, a part of monomer fraction from autoxidized methyl linolenate

Discoloration times	O.D. at 430 nm	
	Hydrophilic part	Lipophilic part
1st	0.111	Nil (<0.02)
2nd	0.068	Nil
3rd	0.026	Nil

IR analysis IR spectra of "before discoloration" and "after discoloration" were measured. No significant change was observed in absorptions at $2.8\text{--}3.0\ \mu$ due to OH, $3.3\text{--}3.6\ \mu$ due to CH, $5.81\ \mu$ due to C=O, and $8.1\text{--}9.1\ \mu$ due to C-O, on the other hand, absorptions at $5.96\ \mu$ due to conjugated C=O, $6.13\ \mu$ due to conjugated C=C, and $10.3\ \mu$ due to *trans* C=C were reduced remarkably after the discoloration reaction (Fig. 1).

Fig. 1. IR spectra of "before discoloration", "after discoloration" and "control".

Before discoloration: DP, after discoloration: lipophilic part obtained after the discoloration reaction, control: lipophilic part obtained after shaking with water instead of 0.5 M Gly.

GLC analysis After conversion of carbonyl group into DMH followed by conversion of hydroxyl group into TMS ether, "control" and "after discoloration" were analyzed

by GLC on 5% SE-30. Since it is assumed that little change occurs in peak-G of methyl linolenate during the discoloration reaction, then shadowed peaks in Fig. 2 are significantly decreased by the discoloration reaction; especially almost all of peak-A, E and F disappear. Peak-A was considered to be DMH-TMS ether, because this appeared only after treatment with dimethylhydrazine and subsequent trimethylsilylation. Peak-E and F were considered to be DMHs because the both peaks appeared after the treatment with dimethylhydrazine but no change in retention time was observed by subsequent trimethylsilylation.

Fig. 2. Separation of the degradation products by GLC.

GLC were carried out after treatment with *N,N*-dimethylhydrazine and trimethylsilylation. Shadowed peaks in "control" were significantly decreased after the discoloration reaction.

Fig. 3. Separation of the degradation products by GLC.

GLC was carried out after reduction with NaBH_4 and trimethylsilylation. Shadowed peaks were significantly decreased after the discoloration reaction. Mother compounds of peak-a, c, d and f are identical with those of peak-A, C, D and F respectively.

As for the peak-B, C and D in Fig. 2, no significant change was observed after the discoloration reaction. They were characterized by the behaviors on the GLC column. Peak-B was TMS ether, and peak-C and D were DMHs (Figs. 2 and 3).

Identification of GLC-peaks by MS analysis (1) Degradation products that decreased after the discoloration reaction **peak-A**: Molecular ion is m/e 228. The fragment ions at m/e 73 ($\text{Si}(\text{CH}_3)_3^+$), 75 ($(\text{CH}_3)_2\text{SiOH}^+$) and 139 ($\text{M}-(\text{CH}_3)_3\text{SiO}$) showed the presence of TMS ether group in the molecule⁹. Major peak at $M-44$ ($-\text{N}(\text{CH}_3)_2$) showed the presence of DMH group. The peak at m/e 199 ($M-29$) was derived from α -cleavage of the TMS ether group (Fig. 4, A). These results suggested that peak-A was DMH-TMS ether of 4-hydroxy-2-hexenal. Further evidence for the presence of this hydroxy aldehyde is shown in Figs. 3 and 4, a. Mass spectrum of **peak-a**, which was obtained after reduction with NaBH_4 and subsequent conversion of the alcohol into the TMS ether, showed the absence of the peak at $M-44$, and the presence of the fragment ions at m/e 73, 75, 147 ($(\text{CH}_3)_2\text{SiOSi}(\text{CH}_3)_3^+$) and 170 ($M-90$) which were typical ions of TMS ethers^{9,10}. The position of original hydroxyl group was recognized again to be at C-4 and the double bond was between C-2 and C-3, because the large peak due to α -cleavage at saturated side of the TMS ether group was found at $M-29$ (m/e 231). **peak-E**: Molecular ion is m/e 240 and base peak is m/e 111 (Fig. 4, E). The peaks at $M-15$ ($-\text{CH}_3$), $M-31$ ($-\text{OCH}_3$) and m/e 59 (H_3COCO^+) showed the presence of methyl ester¹¹, and $M-44$ and m/e 44 showed the presence of DMH group in the molecule. Base peak at m/e 111 indicated that the double bond was located between C-8 and C-9. This ion of base peak is also observed in the authentic compound of DMH of 2-hexenal. Thus, this compound was identified as DMH of 9-formyl methyl-8-nonenoate. **peak-F**: Fragmentation pattern was similar to that of peak-E, except that the molecular ion is m/e 254 (Fig. 4, F). This compound was identified as DMH of 10-formyl methyl-9-decenoate.

(2) Degradation products that did not decrease after the discoloration reaction **peak-B**: Molecular ion was not seen, but molecular weight could be determined from the ion at m/e 231 ($M-15$) (Fig. 5). Fragment ions at m/e 73 and $M-47$ ($-\text{CH}_3$, $-\text{CH}_3\text{OH}$) showed the presence of TMS ether group, and $M-31$ and m/e 59 showed the presence of methyl ester in the molecule. Thus, this compound was TMS ether of 8-hydroxy methyl octanoate. **peak-C**: Molecular ion is m/e 214. Fragment ions at $M-31$ and m/e 59 showed the presence of methyl ester, and $M-44$ and m/e 44 showed the presence of DMH of carbonyl group in the molecule. A series of peaks corresponding to homologous ions $(\text{CH}_2)_n\text{CH}=\text{NN}(\text{CH}_3)_2^+$ was also observed. This compound was identified as DMH of 7-formyl methyl heptanoate. **peak-D**: Molecular ion is m/e 228, and fragmentation pattern was similar to that of peak-C. Thus, this compound was DMH of 8-formyl methyl octanoate.

The other shadowed peaks which decreased after the discoloration reaction were

Fig. 4. Mass spectra of DMH, TMS and DMH-TMS derivatives of the degradation products that decreased after the discoloration reaction.

also observed in Fig. 2, but they could not be identified because of their small contents and poor separation on the GLC column.

Fig. 5. Mass spectra of DMH and TMS derivatives of the degradation products that did not decrease after the discoloration reaction.

Discussion

Conjugated carbonyl compounds in DP disappeared after the discoloration reaction with Gly, and some of them were identified as 4-hydroxy-2-hexenal, 9-formyl methyl-8-nonenol and 10-formyl methyl-9-decenoate. 4-Hydroxy-2-hexenal may be produced *via* scission of 13,16-dihydroperoxy-9,11,14-octadecatrienoate and reduction of 16-hydroperoxy group to hydroxyl group during autoxidation. Similar compounds of hydroxy or hydroperoxy conjugated aldehydes have been detected in autoxidized methyl linoleate. Yoshioka and Kaneda isolated 4-hydroperoxy-2-hexenal as toxic substance¹²⁾. Shauenstein *et al.* isolated 4-hydroxy-2-octenal and other hydroperoxy conjugated aldehydes as biochemically active substances^{13,14)}.

A mechanism for the formation of 10-formyl methyl-9-decenoate may be *via* scission

of 11-hydroperoxy-9,12,15-octadecatrienoate or 11-hydroperoxy-9,12-octadecadienoate (Methyl linolenate used in this experiment contained 16% of methyl linoleate.). As for 9-formyl methyl-8-nonenoate, which has a double bond between C-8 and C-9, however, we could not explain the formation mechanism upon the generally accepted theory of autoxidation.

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