Changes in Molecular Size and Chemical Properties of Gelatin Caused by the Reaction with Oxidizing Methyl Linoleate

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Gelatin, soy protein, lysozyme, succinyl-casein and succinyl-egg albumin were allowed to react with methyl linoleate (ML) at a relative humidity (RH) of 0\% at 50°C for 7 days (protein : ML = 1 : 1). Gel filtration indicated that only gelatin was extensively fragmented. The gelatin was then incubated with ML under various conditions, and changes in the molecular sizes, the gel forming abilities and the chemical characteristics were investigated. The fragmentation of gelatin was increased by decreasing the RH and with the increase in the ratio of ML to protein. The melting point of gel in heating and cooling gelatin was decreased by increasing the fragmentation. The contents of amide and carbonyl groups increased and that of amino group decreased as the reaction progressed at RH 0\%, but no change in C-terminal amino acids was observed. Following the reaction at RH 0\%, many kinds of amino acid residues of gelatin were damaged, although in our previous paper [Matoba et al., Agric. Biol. Chem., 46, 979 (1982)] such was not detected in casein and egg albumin. From the above results, we conclude that gelatin is susceptible to fragmentation by reaction with oxidizing lipids and one possible mechanism of the degradation may be the \(-N-C-\) scission of peptide bonds as proposed by Zirlin and Karel [J. Food Sci., 34, 160 (1969)]. Complex reactions other than this scission may also occur.

The oxidation of lipids constituting unsaturated fatty acids causes chemical changes in proteins. This chemical reaction between proteins and oxidizing lipids is responsible for the deterioration of food proteins during processing, storage and cooking. Such chemical changes in proteins affect not only the functional properties of proteins owing to aggregation and fragmentation but also their nutritional quality based on the damage of the amino acid residues.1~7) There has been some information concerning the cross-linking (polymerization) and insolubilization of proteins caused by reaction with oxidizing lipids. In general, it is known that the polymerization increases with increasing water activity.3,8,9) On the other hand, investigations regarding the fragmentation have scarcely been reported in detail. Homma et al.10) observed fragmentation of kori-tofu proteins during storage at relative humidity (RH) 90\%. Zirlin and Karel11) reported fragmentation of gelatin caused by the scission of peptide bonds occurring in the dry state. However, we found an insignificant decrease in the molecular size of casein and egg albumin after the reaction at RH 0\%, although significant fragmentation of gelatin was detected.5)

In the present study, we investigated changes in the molecular sizes of several proteins after the reaction with oxidizing methyl linoleate (ML) in the dry state. Accordingly, we examined changes in the molecular sizes and the gel forming ability of gelatin after reaction with ML under various conditions. We also carried out some chemical analyses in order to explore possible mechanisms of the fragmentation.

MATERIALS AND METHODS

Materials. Gelatin (fine powder) and florisil were pur-

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chased from Nakarai Chemicals Ltd. Methyl linoleate was obtained from Tokyo Chemical Industry Co., Ltd. Casein (Hammarsten type), egg albumin (fine powder) and lysozyme were purchased from Merck. Sephadex G-50 was obtained from Pharmacia Fine Chemicals. A TSK G 3000 SW column was purchased from Toyo Soda Manufacturing Co., Ltd. Soybean protein was prepared according to the procedure of Ohtsuru et al. Succinylation of casein and egg albumin was carried out by the method of Franzen and Kinsella.

Reaction system of proteins with methyl linoleate. Protein (0.3 g) was added to 0.3 g of methyl linoleate (ML) in 15 ml of water (pH 7.0). The ML was passed through a column of florisil to reduce its initial level of oxidation. The mixture was emulsified in a homogenizer for 3 min at 10,000 rpm. The resulting viscous mixture was quickly frozen in liquid nitrogen, and then freeze-dried. The freeze-dried sample was further dehydrated overnight over P2O5 in vacuo. The resulting sample was then stored in a desiccator containing P2O5 (relative humidity (RH) 0%), saturated MgCl2 solution (RH 30%) or saturated (NH4)2SO4 solution (RH 80%) at 50°C for several days. After storage, the mixture was repeatedly extracted with acetone-methanol mixture (1:1, v/v) by mixing thoroughly with a pestle and mortar to remove lipid components from it. The organic phase was removed by centrifugation. The resulting precipitate, which was dried over P2O5 in vacuo, was used for the following experiments.

Gel filtration. Gel filtration on Sephadex G-50 was carried out as follows. The eluting buffer was 0.01 m phosphate, pH 7.0 containing 0.1 m NaCl. The sample (10 mg) was dissolved in 2 ml of the buffer containing 0.025% NaN3 by stirring at room temperature overnight. After centrifugation, the supernatant (1 ml) was applied to the column. The eluted fraction was spectrophotometrically detected at 215 nm.

Gel filtration on TSK G 3000 SW was carried out by high performance liquid chromatography (Hitachi 638-30) and detection was with a UV detector (Hitachi 638-0410) at 215 nm. The eluting buffer was 0.025 m phosphate, pH 6.8 containing 0.1% SDS and 0.2 m NaCl. The flow rate was 0.4 ml/min. The sample concentration was 0.04% and the injection volume was 0.025 ml. The solution was prepared by incubating the protein in 0.025 m phosphate, pH 6.8 containing 2% SDS and 0.2 m NaCl at 100°C for 5 min.

Chemical analysis. Nitrogen content was determined by the micro-Kjeldahl method using a Kjeldahl nitrogen analyzer (Mitsubishi KN-01). Amino acid analysis carried out after acid hydrolysis (6 N HCl, 110°C, 24 hr) with an amino acid analyzer (Hitachi 835). Amide group content was determined as ammonia when the amino acid analysis was performed. Amino group content was measured by the method of Goldfarb. C-Terminal analysis was carried out according to the procedure of Braum and Schroeder. Carbonyl group content was measured by the method of Garrison et al.

13C NMR analysis. The 13C NMR spectrum was measured on a Hitachi R-90 in D2O.

RESULTS AND DISCUSSION

Changes in molecular sizes of several proteins

In the previous paper, we reported insignificant changes in molecular sizes and amino acid composition of casein and egg albumin after reaction with oxidizing methyl linoleate (ML) at relative humidity (RH) 0%. However, both the proteins showed significant changes at RH 80%. Zirlin and Karell observed the scission of the peptide bonds of gelatin after reaction with ML at RH 0%, resulting in the decrease of its molecular weight, as found in our reaction system.

In the present experiment, we investigated whether proteins other than gelatin are fragmented after exposure to ML at RH 0% and 50°C for 7 days, using succinyl-casein and succinyl-egg albumin, lysozyme and soy protein. The gel filtration profiles of the reacted proteins on Sephadex G-50 are shown in Fig. 1. The reaction of these proteins with ML at RH 0% did not significantly cause the formation of low molecular weight peptides, al-

![Fig. 1. Gel Filtration on Sephadex G-50 of Several Proteins Following Reaction with ML.](image-url)
though in the case of gelatin fragmentation occurred, suggesting that the degree of fragmentation may change with the kind of proteins. After the reaction, the solubility of lysozyme and soy protein in buffer (0.01 M phosphate, pH 7.0 containing 0.1 M NaCl) decreased significantly, on the other hand, succinyl-casein, succinyl-egg albumin and gelatin were completely soluble. This decrease of solubility was observed even when these proteins were allowed to stand at 50°C and RH 0% in the absence of ML. Succinylation of casein and egg albumin was carried out to suppress the polymerization of the proteins that may be caused by the ε-amino groups, because the amino-carbonyl reaction (cross-linking of protein via bifunctional aldehyde and aldol condensation) is known to be one of the possible mechanisms of polymerization of the proteins after the reaction with oxidizing lipids.\(^2\)\(^,\)\(^3\)

**Fragmentation of gelatin**

Changes in molecular sizes of gelatin after the reaction with ML under various conditions were examined by high speed gel filtration on a porous silica gel column (TSK G 3000 SW) in SDS aqueous solution. This chromatography has been reported to show very high separation efficiency of proteins at high speed.\(^{17}\)

Determination of protein and peptide contents was performed by absorbance at 215 nm, which bases on the peptide bonds.

a) Effect of relative humidity. Gelatin was allowed to react with ML for 10 days at RH 0, 30, 50 and 80% and at 50°C. The chromatogram of the reacted gelatin is shown in Fig. 2. The fragmentation of gelatin was extensive at RH 0%, followed by the reaction at RH 30 and 50%. On the other hand, the reaction at RH 80% resulted in the insolubilization of gelatin owing to the formation of high polymers. When this samples (RH 80%) was analyzed by SDS polyacrylamide gel electrophoresis,\(^{18}\) highly polymerized bands, which could not pass through the stacking gel, were observed.

Zirlin and Kare\(^{11}\) measured the specific viscosity of gelatin after reaction at RH 0, 31.5 and 59.5%, showing that the molecular weights of samples at RH 0 and 31.5% significantly decreased and the decrease was more extensive in the sample at RH 0% than in that at RH 31.5%.

b) Effect of ratio of methyl linoleate to gelatin. Gelatin was allowed to react with different amounts of ML (ratio of ML to protein, 0.1 \~ 2.5) for 10 days at 50°C and RH 0%. The chromatogram of the reacted gelatin is shown in Fig. 3. This shows that the degradation of gelatin was extensive with an increase in the ratio of ML to gelatin.

c) Time course. Gelatin–ML mixture (1 : 1, w/w) was incubated at 50°C and RH 0% for periods of 0 to 10 days. The chromatogram of gelatin after the incubation is shown in Fig. 4. This indicates that the fragmentation of gelatin commenced at an early period (1 \~ 3 days) and reached the maximum level after 6 days.

**Melting points of gelatin gel**

Gel forming ability is one of the important functional properties of gelatin. In this experiment, melting points of gel in heating and cooling gelatin after the reaction with ML under various conditions were examined, as shown in Table I. When gelatin was allowed to react with different amounts of ML at RH 0% and 50°C for 10 days, the gelation after the reaction with a small amount of ML (gelatin : ML = 1 : 0.25, w/w) formed a gel having a lower melting point than control gelatin (no reaction), but no formation of gel occurred when the amount of ML was increased (ML to gelatin = 0.5). The gelatin after the reaction at RH 0% and 50°C for 1 to 10 days gave a gel having a similar melting point to that of the control gelatin up to 3 days of the reaction, but the sample after 4 days did not form gel. The gelatin after the reaction (protein : ML = 1 : 1, 50°C, 10 days) at RH 30 and 50% also did not form a gel. The sample at RH 80% did not give a clear solution. When a mixture of this sample in water was boiled, an elastic and gelationous coating was formed on the sur-
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Table I. Melting Points of Gelatin Gel Following Reaction with ML

Gelatin was allowed to react with ML at 50°C. Melting point was determined after gelatin solution (3%) was heated in boiling water and cooled in ice for 1 hr.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Melting point (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>26.6</td>
</tr>
<tr>
<td>ML ratio (RH=0%, 10 days) w/w</td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>13.2</td>
</tr>
<tr>
<td>0.50</td>
<td>No</td>
</tr>
<tr>
<td>0.75</td>
<td>No</td>
</tr>
<tr>
<td>1.0</td>
<td>No</td>
</tr>
<tr>
<td>Time course (RH=0%, ML ratio=1.0)</td>
<td></td>
</tr>
<tr>
<td>1 day</td>
<td>26.4</td>
</tr>
<tr>
<td>2 day</td>
<td>26.0</td>
</tr>
<tr>
<td>3 day</td>
<td>25.0</td>
</tr>
<tr>
<td>4 day</td>
<td>No</td>
</tr>
<tr>
<td>8 day</td>
<td>No</td>
</tr>
<tr>
<td>10 day</td>
<td>No</td>
</tr>
<tr>
<td>RH (ML ratio=1.0, 10 days) %</td>
<td></td>
</tr>
<tr>
<td>0%</td>
<td>No</td>
</tr>
<tr>
<td>30%</td>
<td>No</td>
</tr>
<tr>
<td>50%</td>
<td>No</td>
</tr>
<tr>
<td>80%</td>
<td>High</td>
</tr>
</tbody>
</table>

Fig. 2. High Performance Liquid Chromatography on TSK G 3000 SW of Gelatin Following Reaction with ML: Effect of RH.

Gelatin was allowed to react with ML (protein: ML = 1:1, w/w) at RH 0, 30, 50 or 80% and 50°C for 10 days. Numbers indicate RH values.

The melting point of the test tube. The melting point of this gelationous material was higher than that of the control gelatin, although this did not give a definitive melting point. As described above, the decrease of gel forming ability of the gelatin after the reaction with ML corresponded with the results of Figs. 2~4, showing that fragmentation of gelatin lowered the gel forming ability.

Zirlin and Karel also reported changes in melting point of gelatin gel, although their conditions of reaction with gelatin and ML were different from ours. They showed that melting points of gels (5%) of control gelatin and the reacted gelatin after the reaction (ML: protein = 0.2:1) at 50°C and RH 0% for 10 days were 30.0°C and 23.5°C, respectively.
They did not examine the gelation ability of gelatin other than the samples described above.

**Chemical analysis**

In order to explore the possible mechanism of the gelatin fragmentation caused by the reaction with ML, the following analyses of the reacted and control gelatin were carried out: changes in the contents of amide, carbonyl and amino groups, C-terminal amino acids and amino acid composition. The results are shown in Table II.

a) **Changes in amide, carbonyl and amino groups.** According to the discussion of Zirlin and Karel,\(^{11}\) the mechanism of the fragmentation of proteins caused by oxidizing lipids in the dry state is assumed to be as follows:

\[
\begin{align*}
\text{O} & \\
\text{R' & R'} \\
\text{R-C-N-C-R"} & \rightarrow \\
\text{H & H} & \\
\text{O} & \\
\text{R' & R'} \\
\text{R-C-N-C-R"} & \rightarrow \\
\text{H & O-OH} & \\
\end{align*}
\]

Peroxide is formed in \(\alpha\)-carbon of peptide bonds, then scission of the \(-N-C-\) bonds occurs by a mechanism similar to scission of hydroperoxides of unsaturated fatty acid\(^{19}\) or to that of proteins caused by radiation.\(^{20}\) If this mechanism occurred in the present reaction system, increase of the contents of amide and carbonyl groups in the reacted samples would be expected and these changes would be equimolar. Table II indicates that these contents increase as the reaction progressed, but these changes were not equimolar, suggesting that complex reactions occurred during the period of incubation with ML. However, we presume that the cleavage of \(-N-C-\) bonds (see the above scheme) is one of the possible mechanisms for the fragmentations of gelatin caused by the reaction with ML, because the qualitative changes in these groups showed the expected tendency. We also carried out \(^{13}\)C-NMR analysis of the control and reacted sample (gelatin : ML = 1 : 1, 50°C, 10 days and RH 0%) and demonstrated insignificant changes between the two samples.

Zirlin and Karel\(^{11}\) reported an increase of amide groups of gelatin after exposure to ML. However, they did not examine the change in carbonyl groups.

b) **Changes in C-terminal amino acids.** Changes in C-terminal amino acids of gelatin after the reaction with ML (gelatin : ML = 1 : 1
and 1:2.5) are shown in Table II. Most of the C-terminal amino acid detected was glycine. Change in the amount of C-terminal amino acid was not observed, indicating that the fragmentation of gelatin was not due to the cleavage of peptide bonds caused by hydrolysis.

c) Amino acid composition. The amino acid composition of gelatin after the reaction with ML is shown in Table III, indicating that many kinds of amino acid residues were damaged. In the previous paper, we reported insignificant changes in the amino acid compositions of casein and egg albumin after the reaction with ML (RH 0%, 50°C, 10 days, protein:ML = 1:1). The amino acid composition of gelatin significantly changes as a result of the reaction with ML, suggesting that gelatin is susceptible to the reaction with oxidizing lipids as shown in Fig. 1. However, at this moment it is not clear why gelatin is labile to oxidizing lipids, compared to the other proteins.

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