Effect of Accumulated Free Fatty Acid on Reduction of Salt Soluble Protein of Cod Flesh during Frozen Storage

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The effect of the free fatty acid (FFA) released from phospholipids by the action of lipolytic enzymes on protein denaturation of fish flesh during frozen storage was studied. The FFA content and the salt soluble protein extractability of commercially available frozen “surimi” were determined. The results obtained suggest that there may be a definite negative interrelation between the FFA content and the salt soluble protein extractability.

Minced cod flesh was washed thoroughly with cold water, mixed with phospholipase A2 specimen, and stored at -16°C for 24 weeks. The FFA content increased rapidly accompanied with marked decreases of polar lipids due to added phospholipase A2 during storage for first one week. The content of salt soluble protein increased during storage for up to 1–2 weeks and then decreased gradually to the minimum amount after storage for 6 weeks. The length of storage period up to the time when the content of salt soluble protein reached the minimum amount was shorter in the test-sample than in the control-sample without added phospholipase A2.

From these results, it is presumed that the FFA released from phospholipids by the action of added phospholipase A2 induces the reduction of salt soluble protein extractability, though some oxidation products of lipids may contribute to the reduction of salt soluble protein extractability to some extent.

It is well known that protein denaturation in fish flesh progresses gradually during storage at low temperature. DYER et al.1–4) pointed out that a large amount of free fatty acid (FFA) released from phospholipids by the action of lipolytic enzymes were concerned with this protein denaturation. KING et al.5) showed that addition of small amounts of linoleic and linolenic acids resulted in rapid insolubilization of cod actomyosin. On the other hand, OLLEY et al.6–8) reported that there was no distinct relationship between the rates of increase in the contents of FFA and of denaturation of fish protein and that protein denaturation progressed at higher rate in the fish species with higher percentages of C22:6 in the FFA liberated during frozen storage.

Many evidences that various oxidized lipids induce protein denaturation have been found in the literature. However, this fact is not contradictory with the above-mentioned presumption that FFA accumulated in fish flesh by the action of phospholipases might participate in denaturation of fish protein during frozen storage.

The present study was carried out in order to make clear the effect of the FFA released from phospholipids by the action of lipolytic enzymes on protein denaturation of fish flesh during frozen storage. First, commercially available frozen “surimi”, frozen minced fish meat, was analyzed on salt soluble protein and FFA contents. Second, commercial phospholipase A2 specimen was added to minced cod flesh which was previously washed thoroughly with cold water. Changes in contents of FFA and of the salt soluble protein in the cod flesh with added phospholipase A2 were determined during storage at -16°C for 24 weeks.

Materials and Methods

Materials

Several commercially graded frozen “surimi” prepared from Alaska pollack Theragra chalcogramma were used. Grades and lipid contents of the frozen “surimi” are shown in Table 1.

Cod Gadus morhua, weighing 6.0 kg, was purchased from Tokyo Metropolitan Central Wholesale Market.

Determination of Fracture Strength of “Kamaboko”

Frozen “surimi” was thawed at room temperature. After grinding for 20 min, the “surimi”
Table 1. Grades and contents of lipids of commercially available frozen “surimi”

<table>
<thead>
<tr>
<th>Frozen “surimi”</th>
<th>Grade*</th>
<th>Lipid content (mg/100 g)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TL</td>
</tr>
<tr>
<td>A</td>
<td>SA</td>
<td>390</td>
</tr>
<tr>
<td>B</td>
<td>FA</td>
<td>370</td>
</tr>
<tr>
<td>C</td>
<td>RA</td>
<td>676</td>
</tr>
<tr>
<td>D</td>
<td></td>
<td>784</td>
</tr>
<tr>
<td>E</td>
<td></td>
<td>1,070</td>
</tr>
<tr>
<td>F</td>
<td>2nd class</td>
<td>537</td>
</tr>
<tr>
<td>G</td>
<td></td>
<td>543</td>
</tr>
<tr>
<td>H</td>
<td></td>
<td>666</td>
</tr>
</tbody>
</table>

* Commercially available frozen “surimi” produced at the plant on ship are graded as follows: SA, special A; FA, fancy A; RA, regular A. These are higher in quality than those produced at the plant on land and graded in 2nd class.

Preparation and Storage of Minced Cod Flesh
Washed with Cold Water
The ordinary muscle of cod was minced by a meat chopper and washed thoroughly in cold water at 4°C according to the procedure outlined in Fig. 1. Liophylized powder of phospholipase A₂ from Crotalus adamanteus venom was purchased from Sigma Chemical Company. Phospholipase A₂ specimen, 1,000 units in specific activity, was suspended in 50 ml of water and mixed thoroughly with 1,200 g of minced and washed flesh (test-sample). An appropriate amount of water was added to control-sample. One hundred grams portions of the test- and of the control-samples were packed in polyethylene bags and stored at −16°C.

Determination of Salt Soluble Protein
Five grams of the sample after thawing was homogenized with 95 ml of 0.6 M KCl–50 mm phosphate buffer (pH 6.8) for 2 min. A 10 ml aliquot of the homogenate was pipetted into a centrifuge tube and kept at 4°C for 1 h. After centrifugation at 6,000 × g for 1 h, the supernatant was collected by decantation. The resulting precipitate in the tube was mixed with 10 ml of the buffer and centrifuged again in the same manner. The supernatants thus obtained were combined and diluted to 50 ml with the buffer. The protein content of the extract was determined by the micro-biuret procedure. A calibration curve was obtained using bovine serum albumin as a standard protein.

Extraction and Fractionation of Lipids
Total lipid (TL) was extracted from 70 g of the flesh according to the method of Bligh and Dyer. Separation of polar lipid (PL) from TL was carried out by a column chromatography on Bio-Beads S–X2.

HPLC of PL, TLC-Densitometry of PL and NL, and GLC of Fatty Acid Methyl Ester
High-performance liquid chromatography (HPLC) of PL, thin-layer chromatography (TLC)-densitometry of PL and non-polar lipid (NL), and gas liquid chromatography (GLC) of fatty acid methyl ester prepared from TL, PL, and NL were conducted under the same conditions as described in the previous paper.

Determination of TBA Number
TBA number was determined after the method of Sidwell et al. For this purpose, 5 g of the sample was used and the results obtained were expressed as optical density at 532 nm.
Results and Discussion

Relationship between FFA Content and Extractability of Salt Soluble Protein in Frozen “Surimi”

In this study, salt soluble protein extractability and elasticity of “kamaboko” prepared from frozen “surimi” were used as criteria of denaturation of protein: when the protein in frozen “surimi” undergoes denaturation, the salt soluble protein content and the elasticity of “kamaboko” prepared are reduced.

The contents of FFA and salt soluble protein in frozen “surimi” were determined and plotted in Fig. 2.

Fig. 2. Relationship between contents of FFA and of salt soluble protein of commercially available frozen “surimi”.

From the plots, an equation \( Y = -0.970X + 128 \) was obtained and a correlation coefficient \( r = -0.749 \).

Relationship between FFA Content in Frozen “Surimi” and Elasticity of “Kamaboko”

Relationship between FFA content in frozen “surimi” and elasticity of “kamaboko” prepared is shown in Fig. 3. From the plots in Fig. 3, an equation \( Y = -0.063X + 98 \) was obtained and a correlation coefficient \( r = -0.645 \), suggesting that “kamaboko” with stronger elasticity can be prepared from the frozen “surimi” containing FFA at lower concentration.

Fig. 3. Relationship between contents of FFA and fracture strengths of “kamaboko”.

These results indicate that the frozen “surimi” with higher extractability of salt soluble protein contains FFA at low concentration.
Changes in the Contents of Lipid Classes of Minced and Washed Cod Flesh during Frozen Storage

Changes in the contents of lipid classes of PL and NL in the test- and control-samples during storage at -16°C are shown in Figs. 4 and 5, respectively. PC and PE were predominant lipid classes in PL, accounting for 250 mg/100 g and 100 mg/100 g, respectively. NL of the flesh consisted predominantly of FFA, free sterol (ST), triglyceride (TG), hydrocarbon (HC) and sterol ester (SE).

In the test-sample, the contents of PC and PE decreased to 75 mg/100 g and 20 mg/100 g, respectively, during storage for first 1 week. Thereafter, the decrease of PC and PE almost ceased. Contrary to this, the contents of FFA increased rapidly to 125 mg/100 g during storage for first 1 week. After that, the rate of increase in FFA became markedly low.

In the control-sample, increase in FFA and decreases in PC and PE during frozen storage for 24 weeks were considerably slower than those in the test-sample. This seems to be due to removal of the most of endogenous lipolytic enzymes from cod flesh by washing with water.

Changes in the Elution Patterns of PL in HPLC

Changes in the elution patterns of the PL of the test- and control-samples are shown in Fig. 6. The PL in the sample before storage (0 week in Fig. 6) revealed two large peaks due to PC and PE.
and three small peaks of LPE, LPC and SPM on the chromatogram. In the test-sample, the peaks of LPC and LPE became striking after storage for 1 week, and the ratios of peak areas of LPC and of LPE to total peak area increased considerably. Thereafter, no marked changes in the elution patterns occurred. These results suggest that accumulated lyso-form phospholipids such as LPC and LPE are formed by the action of added phospholipase A2 specimen and that endogenous lysophospholipase is washed out to a large extent from the sample with cold water.

In the control-sample, on the other hand, no striking change in the elution patterns occurred during storage. However, the peak area ratio of PE to PC on the chromatogram increased gradually during storage: PE was hydrolyzed enzymatically faster than PC.

### Table 2. Changes in TBA numbers of the test- and control-samples during storage at −16°C

<table>
<thead>
<tr>
<th>Storage, week</th>
<th>TBA number (O.D. at 532 nm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>0</td>
<td>0.067</td>
</tr>
<tr>
<td>1</td>
<td>0.031</td>
</tr>
<tr>
<td>2</td>
<td>0.014</td>
</tr>
<tr>
<td>3</td>
<td>0.011</td>
</tr>
<tr>
<td>4</td>
<td>0.038</td>
</tr>
<tr>
<td>6</td>
<td>0.032</td>
</tr>
<tr>
<td>8</td>
<td>0.041</td>
</tr>
<tr>
<td>12</td>
<td>0.034</td>
</tr>
<tr>
<td>16</td>
<td>0.030</td>
</tr>
<tr>
<td>24</td>
<td>0.061</td>
</tr>
</tbody>
</table>

*See the text.*

### Effect of FFA on Protein Extractability

Changes in the salt soluble protein extractability in the test- and control-samples during storage were shown in Fig. 7. In the control-sample, salt soluble protein content increased rapidly to the maximum amount, accounting for 40.5 mg/g, after 2 weeks of storage and then decreased gradually during storage for up to 8 weeks. Similarly, in the test-sample to which phospholipase A2 specimen was added, salt soluble protein content came to the maximum, accounting for 37.5 mg/g, after 1–2 weeks of storage and then decreased.
rapidly to the minimum amount, 24.0 mg/g, during storage for up to 6 weeks. Both maximum and minimum amounts of salt soluble protein of the test-sample were smaller than those of the control-sample, respectively, and the length of storage period up to the time when the salt soluble protein content reached the minimum amount was shorter in the test-sample than in the control-one.

TBA number, shown as optical density at 532 nm in Table 2, seemed to increase slightly in both samples during frozen storage at \(-16^\circ\text{C}\), while no clear difference in the increasing rate of TBA number was found between them. Moreover, the ratios of percentages of C\(_{20:5}\) and of C\(_{22:6}\) to that of C\(_{16:0}\) in fatty acid compositions of TL\(_{22}\) decreased at a similar rate in both samples during frozen storage, as shown in Figs. 8 and 9. These results suggest that lipid oxidation in both samples progresses to some extent and that the rate of lipid oxidation in the test-sample is almost equal to that in the control-one. Thus, the distinct difference in the salt soluble protein extractability between the test- and control-samples observed during frozen storage for up to 8 weeks may be caused by the accumulated FFA released from PC and PE by the action of added phospholipase \(A_2\), though the various oxidation products also may contribute to the reduction of salt soluble protein extractability in the both samples during frozen storage.

The results obtained in this study strongly support the presumption that the FFA accumulated enzymatically in the flesh plays an important role in denaturation of protein during frozen storage of fish such as cod.

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References