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Interaction between Lipid and Protein during Frozen Storage
III. Interaction between water and lipid
surrounding myofibrillar protein

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Abstract

The binding type between lipid and myosin in the precipitate formed during frozen storage was measured by the binding ability method. It was considered that in the precipitate formed during frozen storage myosin interacted with lecithin (polar lipid) and fatty acid methylester (non-polar lipid) by electrostatic forces, and Van der Waals and hydrophobic forces, respectively.

The water content of the precipitate formed during frozen storage was found to be the highest in the treated myofibrils with non-polar lipid system (NPL) and the lowest in the untreated myofibrils system (MF). The state of water in the lipid-myofibrils complex of the precipitate formed during frozen storage was measured by DTA heat denaturation thermopprofile and IR spectrum. It was considered that tightly bound water, and loosely bound water was maintained in the precipitate of the treated myofibrils with polar lipid system (PL), and the NPL system, respectively.

It was presumed that interaction between myofibrillar protein and lipid during frozen storage exerted not only a direct effect but also an indirect effect, which showed that the water held in the lipid-protein complex stabilized the high dimensional structure of the protein.

As described in the previous paper¹⁾, a precipitation was caused when myofibrillar protein had been stored at -20°C , and the precipitated material contained about 80% of the protein. The material formed from the mixture of myofibrillar protein and lipid (NPL, PL) had less toughness compared with that of the mixture without lipid. It is possible that the formations of the precipitated materials in those frozen mixtures reflect the toughness of the fish muscle during frozen storage. The present study was undertaken to investigate potential interactions among myofibrillar protein, lipid and water at -20°C in order to evaluate the role of the lipid on the toughing process of the fish muscle during frozen storage.

Materials and Methods

I. The measurement of binding ability to dye.

1. The myosin was prepared from the dorsal muscle of rainbow trout (*Salmo gairdneri irideus* GIBBONS) by the method of Takashi *et al.*²⁾ In the experiment,

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myosin was dissolved in the buffer solution consisting of 0.5 M KCl and 0.05 M phosphate (pH 7.2).

ATP-sensitivity of myosin used in this experiment was less than 10.

2. Purified lecithin prepared from northern blenny roe and fatty acid methyl-ester prepared from squid oil were used as polar lipid and non-polar lipid, respectively.

3. Myosin and lipid mixed system were prepared as described in the previous paper¹⁾. This mixture was stored at -20°C , taken out at definite intervals and thawed at 4°C for 3 hours. The precipitated material formed during frozen storage (PPT-F) was separated by centrifuging at $20,000 \times g$ for 20 minutes.

The binding-amount of safranine O of a basic dye, orange G of an acidic dye to the PPT-F was measured by the method of Ohashi *et al.*³⁾

4. The determination of protein content was carried out by using the biuret⁴⁾ and/or the micro-biuret method.⁵⁾

II. Instrumental analysis of lipid-protein complex.

1. Myofibrils were prepared from rainbow trout muscle by the method described in the previous paper.¹⁾

2. Preparation of myofibrils and lipid mixed system, and extraction of lipid from each model system (MF, NPL and PL systems) after the frozen storage were carried out as described in the previous paper.¹⁾

3. Measurements of weight and water content of the PPT-F were carried out by the gravimetric procedures. Water holding capacity was expressed as the weight of water associated with unit protein in the PPT-F.

4. The PPT-F was dehydrated by treatment 4 times with each 40 ml of cold acetone and the remaining water was measured by Differential Thermal Analysis (DTA) and IR spectrum.

DTA heat denaturation thermopprofile and endothermic peak temperature were measured by Model DTA 8001S (Rigaku denki Co. Ltd.,) at $10^{\circ}\text{C}/\text{minute}$ heating rate from 30°C to 220°C .

IR spectrum was measured by KBr pellet method with Koken DS-301 IR spectrophotometer. The ratio of $E_{3100\text{cm}^{-1}}$ which is derived from bound water to $E_{3300\text{cm}^{-1}}$ which is derived from NH stretching vibration of protein was used as indicator of bound water in the dehydrated PPT-F.⁶⁾

Results

Tables 1 and 2 show the characteristics in the binding-amounts of safranine O and orange G to the protein in the PPT-F, respectively. As compared with other model systems, the binding-amount of safranine O and orange G were always small in the PPT-F obtained from the lipid mixed system.

The wet weight of the PPT-F, shown in Fig. 1, gave the highest value in the NPL system, and the lowest one in the MF system. The wet weight of the PPT-F was shown to decrease with increasing to the storage periods in each model system.

Fig. 2 shows the change of water content of the PPT-F. It was clearly shown that the pattern was similar to that of the weight of the PPT-F shown in

Table 1 Changes in the binding-amount of safranin O to myosin in the precipitate formed during frozen storage at -20°C .

Storage period (days)	Binding-amount of safranin O (M/protein $\text{g} \times 10^3$)		
	Untreated myosin	Treated myosin with non-polar lipid	Treated myosin with polar lipid
0	6.12	5.70	5.05
5	7.72	8.15	7.97
11	8.59	8.71	7.31
17	8.20	7.81	6.07
29	8.79	8.60	7.91

Table 2 Changes in the binding-amount of orange G to myosin in the precipitate formed during frozen storage at -20°C .

Storage period (days)	Binding-amount of orange G (M/protein $\text{g} \times 10^4$)		
	Untreated myosin	Treated myosin with non-polar lipid	Treated myosin with polar lipid
0	9.50	9.22	8.78
5	18.90	12.92	12.17
11	14.76	14.10	11.47
17	12.70	12.39	9.79

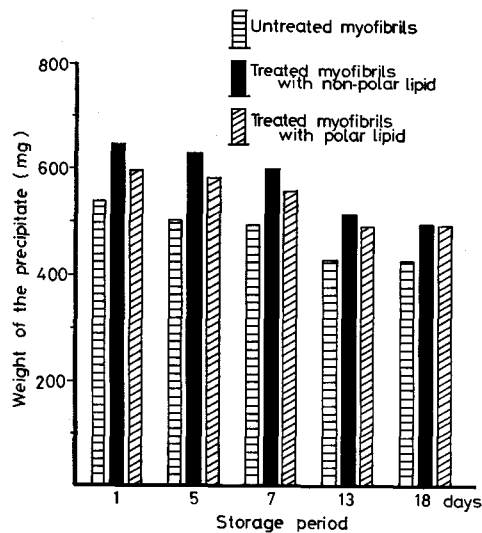


Fig. 1 Changes in the weight of the precipitate formed during frozen storage at -20°C .

Fig. 1. Therefore, it was presumed that the decrease of the weight of the PPT-F was due to the water content of the PPT-F.

Water holding capacity of the PPT-F, as shown in Fig. 3, was the largest in the

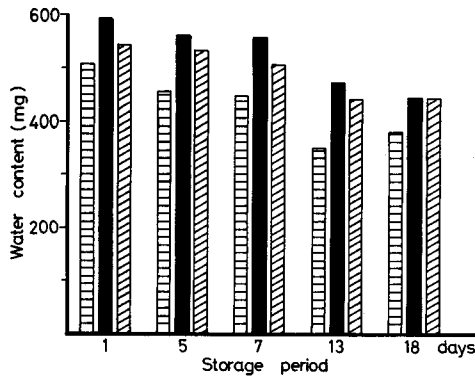


Fig. 2

Changes in the water content of the precipitate formed during frozen storage at -20°C. Symbols are the same as shown in Fig. 1.

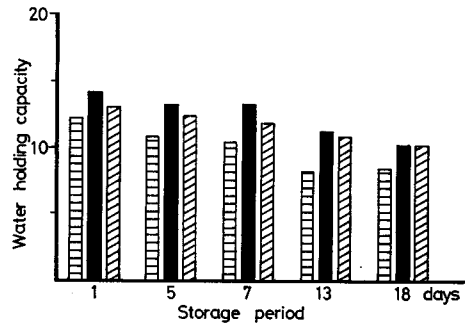


Fig. 3

Changes in the water holding capacity of the precipitate formed during frozen storage at -20°C.

The water holding capacity is represented as the water content (mg) to 1 mg protein. Symbols are the same as shown in Fig. 1.

NPL system, while the MF system was the smallest. The capacity decreased with prolonging to the storage periods in each model system.

Table 3 shows the changes in the lipid content of the PPT-F. The lipid content in the PPT-F gave the higher value in the NPL system compared with the PL system, and decreased with prolonging to the storage periods in both systems.

Table 3 Changes in the lipid content of the precipitate formed during frozen storage at -20°C. The lipid content is relative to the initial amount of the lipid before freezing.

Storage period (days)	Lipid content (%)	
	Treated myofibrils with non-polar lipid	Treated myofibrils with polar lipid
0	100	100
3	73.9	30.1
7	71.8	25.0
15	71.3	—
22	68.6	20.4

The typical DTA thermograms of the dehydrated PPT-F, which was formed during frozen storage for 3 days, are shown in Fig. 4. Table 4 shows endothermic peak temperature of the dehydrated PPT-F. There were two apparent endothermic peaks; i.e. 86-108°C and 170-193°C, in each model system. The temperature of endothermic peak found in the MF system was lower than that of the NPL system, and the highest in the PL system among the model systems.

The IR spectra of the dehydrated PPT-F are shown in Fig. 5. Table 5 shows the ratio of $E_{3100cm^{-1}}$ to $E_{3300cm^{-1}}$. The ratio gave the lowest value in the MF

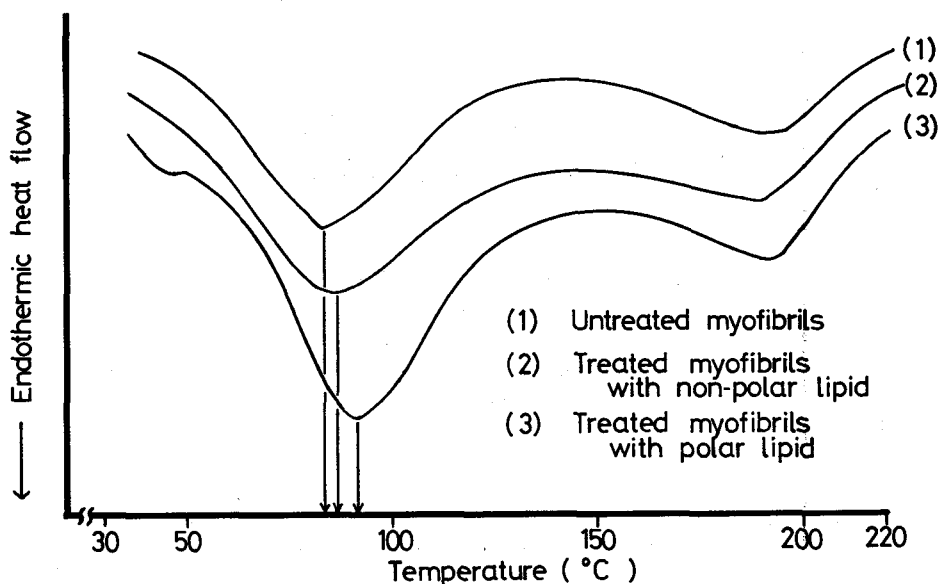


Fig. 4 Typical DTA heat denaturation thermoprofiles of the cold acetone dehydrated precipitate formed during frozen storage for 3 days. Sample weights: 20 mg, Standard sample: α -alumina, Sensitivity of DTA: 50 μ V, Heating rate: 10°C/min.

Table 4 Changes in the endothermic peak temperature of the dehydrated precipitate by cold acetone.

Storage period (days)	Untreated myofibrils			Treated myofibrils with non-polar lipid			Treated myofibrils with polar lipid		
	First peak	Second peak	Third peak	First peak	Second peak	Third peak	First peak	Second peak	Third peak
3-5	—	86-87	190	—	88-90	190	50-55	91-94	192-193
7-13	—	86-89	170 091	—	90	190	50-52	100-108	190

Before freezing (control)

First peak: —, Second peak: 90.6, Third peak: 190

system, against the highest in the PL system. And it meant that the tightly bound water was the smallest in the former system and the largest in the latter one. The value of the ratio decreased with prolonging of the storage periods in each model system.

Discussion

Shenouda *et al.*⁷⁾ reported that the interactions between lipid and protein were dependent on different types of forces, namely electrostatic forces between

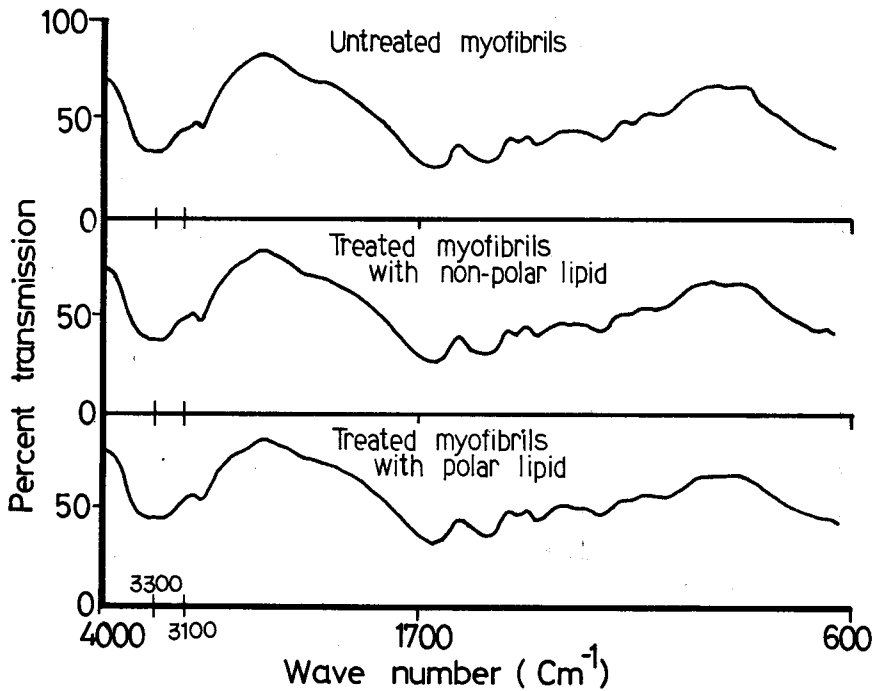


Fig. 5 IR spectra of the cold acetone dehydrated precipitate (-20°C, 7 days).

Table 5 Changes in the ratio of $E_{3100\text{cm}^{-1}}$ to $E_{3300\text{cm}^{-1}}$ of the cold acetone dehydrated precipitate.

Myofibrils	$E_{3100\text{cm}^{-1}}/E_{3300\text{cm}^{-1}}$	
	Storage period (days)	
	1	7
untreated	0.7187	0.6936
treated with non-polar lipid	0.7563	0.6967
treated with polar lipid	0.7858	0.7126

Before freezing (control)

$E_{3100\text{cm}^{-1}}/E_{3300\text{cm}^{-1}}$: 0.7588

the charged groups, Van der Waals forces between the hydrocarbon chains, hydrogen bonding and hydrophobic forces.

The measurement of dye binding ability could make clear the state of polar amino acid residues which existed on the surface of protein. Aoki⁸⁾ investigated the reactive residues of protein in the gel formation of soy bean protein using the dye binding ability method. Niwa *et al.*⁹⁾ used the same method to investigate the attitudes of protein side chains in the setting of "Kamaboko".

As shown in Tables 1 and 2, the binding-amounts of safranin O and orange G to the PPT-F were apparently less in the PL system compared with that of the MF one. Lecithin molecule has a phosphate which dissociates negatively and has

a choline-N which dissociates positively in a pH 7.2 medium. If lecithin did not bind to myosin at all, the binding-amounts of safranin O and orange G should be higher in the PL system compared with other model systems. As described above, however, the binding-amounts of both dyes were less in the PL system. This probably suggests that the major force responsible for the interaction between lecithin and myosin in the PL system is electrostatic. On the other hand, the binding-amounts of both dyes in the NPL system was somewhat smaller compared with the MF system and larger compared with the PL one. Therefore, it was considered that myosin interacted with lipid mainly by Van der Waals forces and hydrophobic ones in the PPT-F obtained from the NPL system.

In the previous paper¹⁾, it was pointed out that there were important interactions among protein, water and lipid from the results of *in vitro* digestibilities by trypsin and pepsin, and rheological properties of the PPT-F. Therefore, the state of water in the lipid-myofibrils complex of the precipitate formed during frozen storage (PPT-F) was measured by DTA heat denaturation thermoprofile and IR spectrum.

As shown in Figs. 1, 2, 3 and 4, water holding capacities were higher in the NPL and PL systems than that in the MF system. Therefore, these show that the interaction between water and myofibrillar protein is enhanced by lipid. This was apparent from the results of the photograph of the exterior of the PPT-F, as shown in Fig. 6, and of the phase-micrograph of the PPT-F, as shown in Fig. 7. The precipitate in the MF system was shown as the network of thick fibers, and it was presumed that the damage of myofibrillar protein in the MF system during frozen storage was larger than other model systems.

Shenouda *et al.*¹⁰⁾ reported that in lipid-protein interaction agitation caused the exposure of hydrophobic regions on actin molecules, which led to an increase in the bound neutral lipids and a decrease in the bound polar lipids. In the present study, lipid contents in the PPT-F were higher in the NPL system than the PL system (Table 3), and this was the similar result with that of Shenouda *et al.*¹⁰⁾

Water holding capacity of the PPT-F fell as the lipid intake of the PPT-F

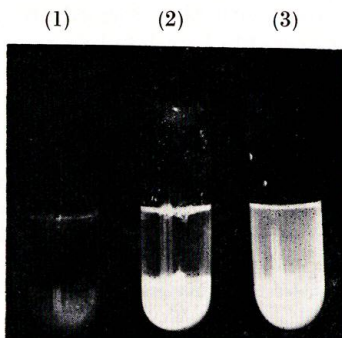


Fig. 6 Photograph of the precipitate formed during frozen storage (-20°C , 10 days).

- (1): Untreated myofibrils (2): Treated myofibrils with non-polar lipid
(3): Treated myofibrils with polar lipid

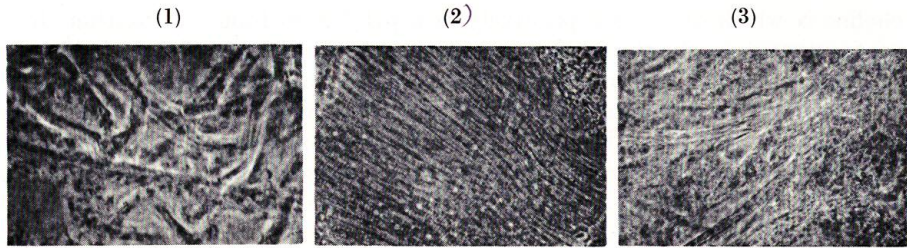


Fig. 7 Phase-micrograph of the precipitate formed during frozen storage (-20°C , 21 days). ($\times 520$)

- (1): Untreated myofibrils (2): Treated myofibrils with non-polar lipid
(3): Treated myofibrils with polar lipid

decreased, and it was considered that the existence of water related closely to the interaction between myofibrillar protein and lipid.

Takagi¹¹⁾ reported that there were three types of water in "Kamaboko" by measuring with DTA analysis. Karmas *et al.*¹²⁾ reported that there were two endothermic peaks ($65-73^{\circ}\text{C}$, $82-83^{\circ}\text{C}$) in the protein by measuring with DSC analysis and that the endothermic peak especially ranging $82-83^{\circ}\text{C}$ was derived from the changes of water structures.

As shown in Table 4, the temperature of the second endothermic peak found in the NPL and PL systems was higher than that in the MF system, and the highest was particularly in the PL system. Therefore, it was presumed that the most tightly bound water existed in the PL system as compared with other model systems. This was also suggested by IR spectrum to some extent and by the measurement of bound water content in the previous paper.¹⁾

The results in the previous¹⁾ and present papers suggested that water holding capacity of the PPT-F was enhanced by the existence of lipid and it was presumed that myofibrillar protein interacted with non-polar and polar lipids by Van der Waals and hydrophobic, and electrostatic forces, respectively. On the other hand, tightly bound water was maintained in the PPT-F of the PL system, against loosely bound water in that of the NPL system. And these were considered to be reasons why the PPT-F in the systems with lipids was more digestible and elastic compared with that formed from the system without lipid, and also these reasons could probably be related to protein stabilities of the frozen stored fatty fish muscle.

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