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Interaction between Lipid and Protein during Frozen Storage

II. Effect of non-polar and polar lipid on rainbow trout myofibrils during frozen storage

Sei-ichi ANDOU*, Kōzō TAKAMA** and Kōichi ZAMA**

Abstract

A model system was prepared to investigate the effect of lipid on myofibrils during frozen storage at −20°C. Changes on protein solubility, in vitro digestibility by trypsin and pepsin, and rheological properties were investigated.

The results were as follows: Salt soluble protein rapidly decreased during frozen storage and the patterns of protein insolubility were almost the same among each model system [untreated myofibrils (MF system), myofibrils treated with non-polar lipid (NPL system) and treated myofibrils with polar lipid (PL system)]. In the NPL system, the result of the tryptic digestibility of the precipitate formed during frozen storage and that of the arginine content and free amino acid composition of the tryptic hydrolysate of the precipitate suggested that the degree of protein structural change is small. The precipitate formed during frozen storage was treated with cold acetone to obtain the dehydrated precipitate. The tryptic digestibility of this dehydrated precipitate gave the highest value in the PL system and the lowest in the MF system.

From the differences of their digestibilities, it was considered that the water surrounding the sites attacked by the digestive enzyme is stabilized by lipid which leads to the control of freezing dehydration. These differences in digestibility also suggested that there is an important interaction between protein, water and lipid.

Introduction

Myofibrillar proteins which are the main components of fish muscle proteins are remarkably unstable and are easily denatured during frozen storage.

The interaction of myofibrillar protein with lipid is well known to lead to protein insolubility. Free fatty acids produced by the hydrolysis of lipid, and low aliphatic aldehydes and fatty acids produced from oxidized lipid are known to cause this protein insolubility.[1,2]

Information concerning the control of the denaturation of fatty fish muscle protein during frozen storage is reported[3)-5)]. Thus, it is considered that the effect of lipid on protein during frozen storage differs among investigators.

This paper shows the effect of lipid on myofibrils during frozen storage from the points of myofibrillar protein insolubility, in vitro digestibility by trypsin and

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pepsin, and rheological properties of the precipitate formed during frozen storage at −20°C.

Materials and Methods

1. Myofibrils were prepared from the dorsal muscle of rainbow trout (*Salmo gairdneri irideus* GIBBONS) as described by FUKAZAWA *et al.*[6]. In this experiment, myofibrils were suspended in 0.10 M KCl-0.039 M borate buffer solution (pH 7.1).

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

3. Generally, in the case of fatty fish having 3–5% lipid content, the lipid-protein content ratio is considered to be ca. 20% (w/w). Therefore, a model system containing 20% (w/w) lipid to protein ratio was used in this experiment.

Lecithin was used as an emulsifier in the NPL system and the weight ratio of lecithin to fatty acid methylester used was 1:9.

The preparation of myofibrils and the lipid mixed system was carried out as shown in Fig. 1. A peroxide free ether solution containing aliquot amount of lipid was added to a test tube (29 mm×130 mm). Ether was evaporated using a flow of N_2 gas. Then 5 ml or 10 ml aliquot of myofibril suspensions (protein concentration ca. 10 mg/ml) were added to the test tube, and mixed by automixer three times for 15 seconds with a 5 seconds interval between each mixing. These mixtures were stored at −20°C, taken out at definite time intervals and thawed at 4°C for 3 hours. Then these mixtures were extracted with 1.5 M KCl at 4°C overnight, and then centrifuged at 1,500 × g for 20 minutes.

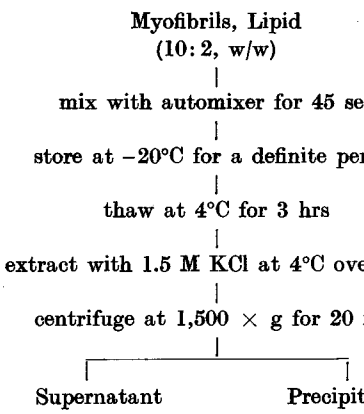

Fig. 1. The preparation of myofibrils and the lipid mixed system.

4. The extraction of lipid from each model system after frozen storage at −20°C was carried out according to Bligh-Dyer's method.[7]

5. The determination of protein content was carried out using the biuret method[8] and/or the micro-biuret method.[9]

6. The determination of in vitro digestibility by trypsin was performed as described by HORIGOME and KANDATSU.[10] The precipitate formed after centrifugation was digested by trypsin (2,000 units/g; Wako Pure Chem. Ind. Ltd.) at 37°C for 20 hours and the free amino acid content in the supernatant solution was determined by the ninhydrin method.[11]

Arginine content in the tryptic hydrolysate of the precipitate formed during frozen storage at –20°C was determined by the SAKAGUCHI's method.[12]

Free amino acid composition of the tryptic hydrolysate of the precipitate formed during frozen storage at –20°C was analyzed by gas-liquid chromatography[13].

The determination of in vitro digestibility by pepsin was performed as described by HORIGOME and KANDATSU[10]. The precipitate formed after centrifugation was digested by pepsin (1:10,000; Difco Lab.) at 37°C for 24 hours. The tyrosine and tryptophan contents in the supernatant solution were determined.[14]

7. Rheological properties such as the hardness and elasticity were measured with the use of a Rheolometer RMT–1300.

Results

1. Changes in the salt soluble protein during frozen storage at –20°C.

Salt soluble protein extracted with 1.5 M KCl remarkably decreased after 3 days storage at –20°C, and then gradually decreased in proportion to storage period. A similar tendency was observed in all the model systems used: untreated myofibrils (MF system), treated myofibrils with non-polar lipid (NPL system), and treated myofibrils with polar lipid (PL system).

Fig. 2 shows that ca. 20% of protein exists in the supernatant and ca. 80% of protein exists in the precipitate after centrifugation.

From these results, it could be considered that the protein in the supernatant was solubilized. On the other hand, the precipitate which included most of the insolubilized protein can also be assumed to be important in relation to the stability of food components. Therefore, the authors have attempted to investigate the properties of this precipitate.

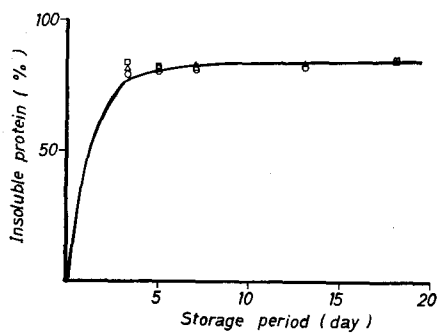

Fig. 2. Changes in insoluble protein of rainbow trout myofibrils during frozen storage at –20°C.
Insolubility values are relative to the initial concentration before freezing.
Symbols: ○ Untreated myofibrils, △ Treated myofibrils with non-polar lipid, □ Treated myofibrils with polar lipid

2. Lipid oxidation during frozen storage at –20°C.

Table 1 shows the carbonyl value of extracted lipid from the precipitate. Table 2 shows the recovery of lipid from each model system after frozen storage at –20°C.

These results showed that the carbonyl value did not increase during frozen storage and that lipid oxidation had little or no effect on protein insolubility during frozen storage under these experimental conditions.

Table 1. *Carbonyl value (meq/Kg) of lipid extracted from the precipitate.*

Myofibrils	Storage period (day)			
	3	7	15	22
untreated	—	—	—	—
treated with non-polar lipid	60.5	48.2	51.9	59.7
treated with polar lipid	129.8	115.7	142.1	138.5

Table 2. *Recovery of lipid from the model system after frozen storage at –20°C.*

Myofibrils	Storage period (day)		
	6	13	22
untreated	—	—	—
treated with non-polar lipid	88.4% (75.6%)	86.7% (70.1%)	90.2% (68.6%)
treated with polar lipid	87.8% (22.9%)	— (—)	87.1% (20.4%)

The values in parenthesis show lipid content in the precipitate.

3. *Digestibility of the precipitate formed during frozen storage at –20°C.*

As shown in Fig. 2, the degree of protein insolubility during frozen storage was almost the same among the model systems with and without lipid. On the other hand, however, as shown in Table 2, recovery of lipid from the precipitate remarkably differed depending on the kind of lipid added in the model systems.

Based on the large difference in the amount of lipid contained in the precipitate formed in this experiment, the authors concluded that the effect of lipid on protein in the precipitate formed during frozen storage may differ depending on the kind of lipid added. Therefore, the difference in the properties of the precipitate formed during frozen storage was investigated using in vitro digestibility by trypsin and pepsin.

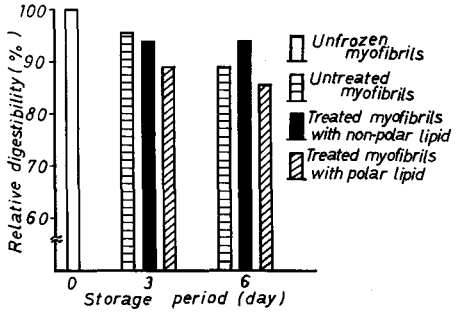

Fig. 3. Changes in relative digestibility by trypsin of the precipitate formed during frozen storage at –20°C.

(1) Fig. 3 shows the tryptic digestibility of the precipitate formed during frozen storage. A decrease in digestibility in proportion to storage period was observed in the MF and PL system, respectively. However, by comparison, the PL system gave a lower digestibility than the MF system. On the other hand, digestibility of the precipitate formed during frozen storage for 3 and 6 days showed no change in the NPL system and it was found to give the highest tryptic digestibility among the three model systems.

It is known that the sites attacked by trypsin are bond sites consisting of arginine, lysine and other amino acids. Therefore, the authors attempted to measure the amount of non-proteinic arginine content in the hydrolysate in order to determine the degree of digestibility by trypsin. Table 3 shows arginine content in the tryptic hydrolysate of the precipitate formed during frozen storage for 6 days. As shown in Table 3, the arginine content in the hydrolysate of the NPL system was found to be the highest among the model systems used. This supports the results shown in Fig. 3.

Table 4 shows the free amino acid composition in the tryptic hydrolysate of the precipitate formed during frozen storage for 6 days. In the NPL system, arginine and lysine contents were the highest among the model systems used. This results agrees with those shown in Fig. 3 and Table 3.

(2) Table 5 shows tyrosine and tryptophan contents in the peptic hydrolysate

Table 3. *Arginine content of the tryptic hydrolysate in the precipitate formed during frozen storage at -20°C for 6 days.*

Myofibrils	Arginine μg/protein mg
unfrozen	84.94
untreated	78.04
treated with non-polar lipid	90.42
treated with polar lipid	68.57

Table 4. *Amino acid composition of the tryptic hydrolysate in the precipitate formed during frozen storage at -20°C for 6 days.*

Amino acid	Amino acid μg/protein mg			
	Unfrozen myofibrils	Untreated myofibrils	Treated myofibrils with non-polar lipid	Treated myofibrils with polar lipid
Ala	0.925	0.882	1.019	0.982
Val	0.178	0.183	0.179	0.289
Gly	0.875	0.899	1.051	0.993
Ile	0.266	0.273	0.267	0.324
Leu	0.963	1.061	1.135	1.157
Ser	1.012	1.616	1.416	0.783
Met	0.277	0.233	0.188	0.082
Phe	0.922	0.820	0.840	0.867
Asp	0.878	0.902	0.980	1.027
Glu	1.009	1.036	1.162	1.174
Orn	1.040	1.069	1.480	1.384
Lys	8.416	7.884	9.224	8.864
Thr	0.302	0.248	0.242	0.197
Cys/2	0.889	0.821	0.802	0.935
Hyp	0.665	0.751	0.502	0.179
Pro	0.175	0.300	0.176	0.096
Tyr	1.286	1.178	1.198	1.046
Arg	5.300	6.491	7.134	5.422
Trp	0.311	0.532	0.257	1.179
His	0.510	0.446	0.237	0.301

of the precipitate formed during frozen storage for 6 days. Tyrosine and tryptophan contents were found to be the highest in the hydrolysate of the NPL system among the model system used.

Table 5. *Tyrosine and tryptophan content of the peptic hydrolysate in the precipitate formed during frozen storage at -20°C for 6 days.*

Myofibrils	Tyrosine (μg/protein mg)	Tryptophan (μg/protein mg)
untreated	40.16	34.53
treated with non-polar lipid	59.25	59.86
treated with polar lipid	51.85	37.51

(3) Table 6 shows the tryptic digestibility of the precipitate (which was dehydrated using cold acetone) formed during frozen storage for 6 days. The tryptic digestibility of the cold acetone dehydrated precipitate gave the lowest value in the MF system and the highest value in the PL system. This result differed from that of the tryptic digestibility of the precipitate (which was not dehydrated using cold acetone) formed during frozen storage for 6 days as shown in Fig. 3 and it was considered that water surrounding the protein molecule affected the digestibility.

Table 6. *The tryptic digestibility of the cold acetone dehydrated precipitate (-20°C, 6 days). The values are relative to the digestibility of unfrozen myofibrils.*

Myofibrils	Relative digestibility (%)
untreated	76.6
treated with non-polar lipid	83.6
treated with polar lipid	97.7

4. Rheological properties of the precipitate formed during frozen storage at -20°C.

Table 7 shows the hardness and elasticity of the precipitate formed during frozen storage. The elasticity values were almost the same for all the model systems used during frozen storage. On the other hand, the hardness values differed among the model systems used. The hardness in the NPL and PL system was half as much as that in the MF system when frozen stored for 15 days. However, measurements of the hardness values after 22 days at frozen storage showed a gradual increase in the NPL and PL system. It gave a value of ca. 60% (NPL system) and 80% (PL system) against the MF system (100%). From these results, the authors presumed that the hardness of the precipitate formed during frozen storage was controlled by the existence of lipid.

Table 7. *Changes in the hardness and elasticity of the precipitate. The hardness and elasticity values are relative to untreated myofibrils.*

Storage period (day)	Hardness			Elasticity		
	Untreated myofibrils	Treated myofibrils with non-polar lipid	Treated myofibrils with polar lipid	Untreated myofibrils	Treated myofibrils with non-polar lipid	Treated myofibrils with polar lipid
7	100	51.8	—	100	100	—
15	100	48.1	51.8	100	96.4	100
22	100	59.2	81.5	100	102.3	92.0

Each 1.3 g of wet precipitate formed during frozen storage was placed into a plastic cup (1.6 cm i.d. ×1.3 cm ht.), and measured with a Rheolometer.
Rheolometer RMT-1300, 12 cycles/min., sens 15 V, Clearance 1.5 mm

Discussion

In the previous paper,[15] it was found that fish dipped in olive oil have a small degree of protein denaturation during frozen storage as compared with those not dipped in olive oil as shown by the determinations of digestibility by pepsin and trypsin, and of available lysine content.

Also, the protective effect of oil treatment on fish muscle protein was investigated from the result of microscopic observation of frozen muscle sections, which were prepared from rainbow trout muscle dipped in olive oil and frozen stored for 37 days at –20°C.[16]

It is considered that these protective effects of lipid on protein denaturation during frozen storage are based on the interaction between protein and lipid.

This study was done in order to distinguish the relationship between myofibrils and non-polar lipid, and that of myofibrils and polar lipid from the points of myofibrillar protein solubility, digestibility by trypsin and pepsin, and rheological properties of the precipitate formed during frozen storage at –20°C.

Salt soluble protein rapidly decreased during frozen storage and 80% of the salt soluble protein became insoluble after 3 days storage. Since most of the protein became insoluble during frozen storage, it was considered valuable to investigate the properties of this insoluble protein (precipitate).

Also, the occurrence of lipid oxidation during frozen storage at –20°C was found to proceed slightly up to 3 weeks in storage as shown by the measurement of the carbonyl value (Table 1) and the recovery of ca. 90% of the added lipid (Table 2).

As shown in Fig. 2, the patterns of protein insolubility during frozen storage were similar among the model systems used, and it was assumed that the presence of lipid had no influence on salt soluble protein.

However, from the results of the following: a) tryptic digestibility of the precipitate formed during frozen storage, b) determination of the arginine content of the tryptic hydrolysate of the precipitate, and c) determination of free amino acid composition by gas-liquid chromatography of the tryptic hydrolysate of the precipitate, the authors assumed that the degree of protein structural change occurring during frozen storage was small in the NPL system. That is, the precipitate in the NPL system was easily digested by trypsin as compared with other model

systems. These results suggested that in the presence of non-polar lipid, the structures surrounding arginine and lysine, which are the sites attacked by trypsin, were stabilized in a state of easy digestion.

As for the peptic digestibility of the precipitate formed during frozen storage, the precipitate in the NPL system had been easily digested as well, and it was suggested that the structures surrounding tyrosine and tryptophan were also stabilized in a state of easy digestion.

On the other hand, the values of the tryptic digestibility of the precipitate (which was dehydrated using cold acetone) formed during frozen storage was found to be the lowest in the MF system and the highest in the PL system. These results differed apparently from those of the wet precipitate prior to dehydration. It was presumed that the structures surrounding the arginine and lysine were stabilized by the presence of polar lipid.

Also, the bound water content of the precipitate in each model system was estimated using cobaltous chloride method,[17] as shown in Table 8. The bound water content was found to be the highest in the PL system. By taking into consideration the fact that digestive enzyme action was related to the presence of water, it was obvious that the lecithin (polar lipid) plays an important role in the maintenance of bound water.

Generally, it is well known that heat denaturation of protein causes the unfolding of its high dimensional structure exposing the attacked sites, thus making itself easily digestible.[18] However, the digestibility of the precipitate formed during frozen storage was lowered. It is therefore obvious that the change in the high dimensional structure of protein during frozen storage differs from that of heat denaturation.

Also, in general, the wet precipitate formed during frozen storage had a high digestibility by trypsin as compared with the cold acetone dehydrated precipitate (Fig. 3, Table 6). It is considered that this was caused by the amount and state of the lipid in the wet and dehydrated precipitate. However, it is also considered that the digestibility by trypsin is connected with water content and the present state of water in the model systems. Since the digestibility of the cold acetone dehydrated precipitate was higher in the NPL and PL system than that in the MF system, it was considered that water surrounding the sites attacked by the digestive enzyme is held by lipid existence and therefore freezing dehydration is controlled. These results suggested that the interaction between protein, water and lipid is important.

This was apparently recognized in the hardness of the precipitate formed during frozen storage as a rheological properties. The fact that the hardness in the

Table 8. *Bound and total water contents of the precipitate formed during frozen storage at -20°C for 21 days.*

Myofibrils	Bound water (%)	Total water (%)
untreated	12.33	87.67
treated with non-polar lipid	21.95	95.12
treated with polar lipid	22.97	94.59

NPL and PL system was lower than in the MF system was connected with water content (Table 8). The reduction of the hardness in the precipitate formed during frozen storage was caused by the change in water content of the precipitate.

Further study is needed to explain the type of binding between lipid and protein, and the present state of water in the lipid-protein complex of the precipitate.

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