HISTOLOGICAL AND BIOCHEMICAL STUDIES ON POST-MORTEM TENDERIZATION OF FISH MUSCLE

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INTRODUCTION

Raw fish muscle is often consumed as "sashimi" or "sushi" in Japan, and now they have been accepted widely in the world. Generally, fish muscle decreases its freshness more rapidly than the muscle of domestic animals such as beef and pork. Great interests, therefore, have been taken in the freshness of fish muscle. A number of studies have been reported[1-6] so far to evaluate the freshness of raw fish. For example, Saito et al.[7] developed K value, which can be evaluated from contents of ATP and its related compounds, for estimating freshness of fish. The value is widely known as an index of fish freshness among several indices.

Rigor mortis, a phenomenon of decrease in elasticity of fish body, is also known as an important index for the freshness.[8-18] Bito et al.,[8] and Yamada et al.[9] studied the change in rigor intensity during ice storage for several fish species and showed that the onset of rigor varied from species to species. Reportedly, for such several fish species as red sea bream,[10,11] flatfish,[12] sardine,[13,14] carp[15,16,17] and yellowtail,[18] the onset of rigor depends on the exhaustion of ATP in muscle.

Muscle firmness is also an important factor to evaluate the quality of raw fish muscle as a food. It is generally accepted that cultured fish muscle is softer than wild fish one. In this point of view, Hatae et al.[19] have compared the texture of wild and cultured fishes, such as red sea bream, flounder, and yellowtail, by the sensory and instrumental methods, and suggested that the cultured fish muscle is inferior to the wild

one in firmness and elasticity. Therefore, soft muscle of cultured fish is regarded as a serious problem in fish culture. It has been reported that firmness of muscle decreases during chill storage.[20-33] The postulated cause of the post-mortem tenderization is as follows: (1) weakening of rigor which occurs between myosin and actin in chicken[20,21] and rabbit muscle[20,22-25] (2) weakening of Z-disc structure of myofibril which was indicated by the fragmentation test[26-30] (3) degradation of titin (connectin), analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).[31,32] However, the cause of post-mortem tenderization of muscle is not clearly resolved because these reports have not performed any textural study.

As to the relationship between rigor mortis and post-mortem tenderization of muscle, men have an impression that fish muscle is hard when the fish is in rigor, and after resolution of rigor, the fish muscle becomes tender. Toyohara and Shimizu[33] have evaluated the rigor mortis by rigor index,[8] measuring muscle firmness in parallel. According to the results, the onset of rigor mortis does not correspond to the change in muscle firmness during storage. Then, they have suggested that rigor mortis does not have an influence to post-mortem change in muscle texture.

There have been several reports on the post-mortem tenderization of fish muscle. In those studies, however, evaluation of muscle firmness is performed only in a few reports.[18,28,33] In addition, little histological reports on the storage of fish muscle are available. It is still unclear that

which portion of fish muscle causes post-mortem tenderization. In the biochemical aspects, breakdown of some muscle proteins during storage have been reported in relation to muscle firmness,[29,31,32,34,35] but no direct evidences is presented as to the participation of the muscle components in the change of muscle firmness. The purpose of this study is to clarify which portion or component of muscle causes post-mortem tenderization of fish muscle using histological and biochemical methods combined with textural procedures.

CHAPTER 1

Textural Studies on Post-mortem Tenderization

At the beginning of this study on the mechanism of post-mortem tenderization of fish muscle, it is important to describe the phenomenon correctly. As to fish muscle firmness, difference in fish-species,[28] influence of thaw rigor,[36] and effect of killing methods[18] have been reported. On the other hand, rigor mortis, another important phenomenon which occurs during storage, has been studied on relationship with exhaustion of ATP,[12,14,15] affect of storage temperature,[33] and difference in rigor onset among fish-species.[8]

In the present chapter, validity of instrumental method for evaluating muscle firmness and correspondence with rigor mortis are described.

Section 1. Change in Fish Muscle Firmness during Chill Storage

Firmness is a an important factor because of its close relation to freshness for evaluating the quality of raw fish muscle, such as sashimi,. It is indispensable to establish a convenient method for evaluating muscle firmness in the study of post-mortem tenderization of fish muscle during chill storage. Sensory tests have been performed to evaluate muscle firmness.[19] However, trained panelists are needed to carry out a sensory test. In addition, the results which are obtained by sensory tests have variations among individuals. To improve these

problems of sensory test, some attempts for measuring muscle firmness by instrumental methods have so far been reported.[18,28,33,37,38] Sawyer et al.[37] and Nakayama et al.[38] have examined sensory and textural properties; the purposes of their studies were to compare the difference in textural properties among fish species.

This section describes the relationship between breaking strength determined using a rheometer and estimated by a sensory test to evaluate the change in muscle firmness during chill storage.

Materials and Methods

Samples

Live rainbow trout (Oncorhynchus mykiss, 23-25 cm in body length and 250-300 g in body weight) were killed at selected time intervals and stored at 5 ^{o}C to serve muscle specimens immediately, 12, 18, 24, 48, and 72 h after death for the puncture and sensory tests at a time.

Breaking Strength

Slices of 10 mm in thickness, simulating sashimi, were dissected from the middle part of the dorsal muscle by cutting vertically to the orientation of muscle fibers. A cylindrical plunger (8 mm in diameter, simulating a molar tooth) was pierced into the slice parallel to the orientation of muscle fibers at a speed of 60 mm/min and the maximum force recorded by a rheometer (Yamaden model 3305, Tokyo) was regarded as the breaking

strength. Each value was expressed as the mean of 5-8 determinations ± S.D. Sensory test was performed by biting the slice by the molar teeth and the rank of firmness, from 1 to 6 in decreasing firmness, was determined. This sensory test was done by five trained panelists.

Results and Discussion

Table 1 shows the results for muscle firmness determined by rheometer and sensory tests. Rank correlation coefficient between the order number determined by the breaking strength value and the average order determined by the sensory test was calculated

Table 1. Firmness orders of rainbow trout muscle determined by the puncture and sensory tests

	Hours after death					
	0	12	18	24	48	72
Breaking strength (g±S.D.)	1098±207.7	845±173.9	680±56.3	622±63.4	596±125.5	510±77.6
Order of the breaking strength	1	2	3	4	5	6
Order determined by the sensory test						
Panelist A	1	4	3	2	6	5
Panelist B	3	1	2	4	5	6
Panelist C	1	3	2	4	6	5
Panelist D	1	3	4	5	2	6
Panelist E	1	2	4	5	6	3
Average order determined by the sensory test (±S.D.)	1.4±0.8	2.6±1.0	3±0.9	4±1.1	5±1.6	5±1.1

Order of muscle firmness was determined from "1" to "6", where "1" was the firmest and "6" the softest.

by the formula according to the method of Spearman.[39] The value of the correlation coefficient obtained was 0.961. Moreover, according to the examination table,[40] the result obtained was at 2 % level of significance. This result clearly suggests that the breaking strength is valid to evaluate the change in muscle firmness during chill storage.

In a study which concerns with post-mortem tenderization, it is important to evaluate muscle firmness by an instrumental method. The breaking strength values which have been measured in this study have little variations among researchers. It is concluded that measurement of the breaking strength is a convenient method in place of sensory measurement of the muscle firmness.

Section 2. Relationship between Firmness of Muscle and Rigor Mortis

It has generally been believed that the texture of fish muscle in rigor state is tough and then gradually tenderized according to the resolution of rigor. There are several reports on the post-mortem rigor of fish body[8-17] but rheological study was not carried out in these studies. On the other hand, Toyohara and Shimizu,[33] Oka et al.[18] examined the relationship between the post-mortem rigor and the tenderization of muscle, and demonstrated that muscle began to tenderize prior to the initiation of rigor. Therefore it was suggested that the tenderization of fish muscle occurred in the early stage of storage independently with the rigor of the fish body. To confirm whether this is true for other fish species, the relationship between the degree of rigor and the firmness of muscle on eight fish species was investigated in the present study.

Materials and Methods

Materials

Fishes were obtained alive from a city market, decapitated and stored at 5 ^{o}C. All fishes used in the present study were cultured. Table 2 summarizes the fish species examined in the present study. Two fishes having almost the same body length and same weight were used for each species to measure the rigor index and the breaking strength.

Table 2. Body length and weight of the fishes examined in the present study

	Species	Body length (cm)	Body weight (g)
Plaice	Paralichthys olivaceus	35 - 39	710 - 773
Parrot bass	Oplegnathus fasciatus	27 - 30	625 - 640
Yellowtail	Seriola quinqueradiata	48 - 50	1810 - 1900
Carp	Cyprinus carpio	39 - 41	1300 - 1350
Red sea bream	Pagrus major	36 - 38	1225 - 1300
Striped grunt	Parapristipoma	26 - 27	280 - 285
Tiger puffer	Fugu rubripes	30 - 32	800 - 840
Rainbow trout	Oncorhynchus mykiss	25 - 28	450 - 465

Rigor index and breaking strength

Rigor index was measured by the method of Bito et al.[8] Fish was put on a horizontal table protruding the half of the body length from the edge of the table. The distance from the horizontal line to the base of the tail (L) was measured at selected time intervals after death, and the rigor index was calculated by applying these values to the following equation.

$$\text{Rigor index} = \frac{L_0 - L}{L_0} \times 100$$

Where, L_0 is the value immediately after death.

Breaking strength was measured by the method mentioned in Section 1.

Results and Discussion

Figs. 1-8 show the relationship between the breaking strength and the rigor index in flatfish (Fig. 1), parrot bass (Fig. 2), yellowtail (Fig. 3), carp (Fig. 4), red sea bream (Fig. 5), striped grunt (Fig. 6), tiger puffer (Fig. 7) and rainbow trout (Fig. 8). The fishes except flatfish, tiger puffer and carp

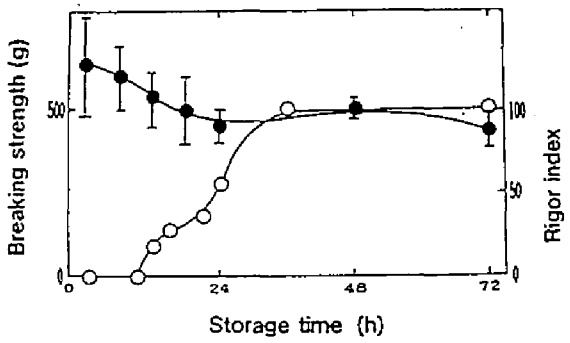

Fig. 1. Change in breaking strength (●) of muscle and rigor index (○) of flatfish during storage at 5 °C.

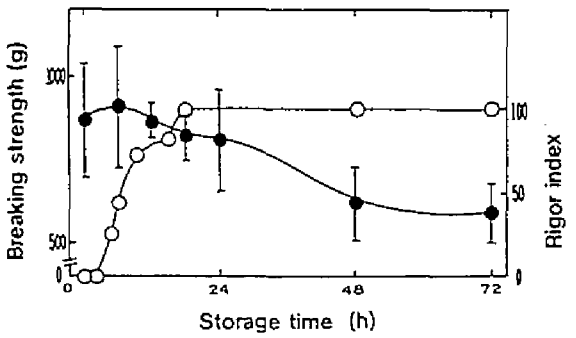

Fig. 2. Change in breaking strength (●) of muscle and rigor index (○) of parrot bass during storage at 5 °C.

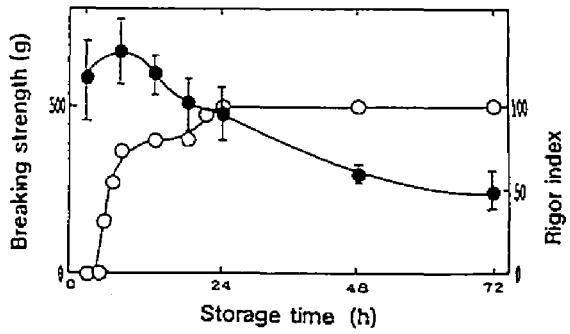

Fig. 3. Change in breaking strength (●) of muscle and rigor index (○) of yellowtail during storage at 5 °C.

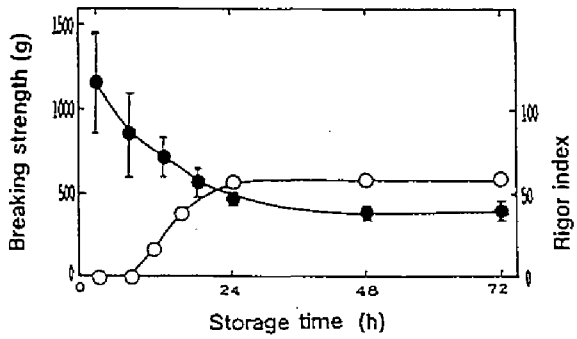

Fig. 4. Change in breaking strength (●) of muscle and rigor index (○) of carp during storage at 5 °C.

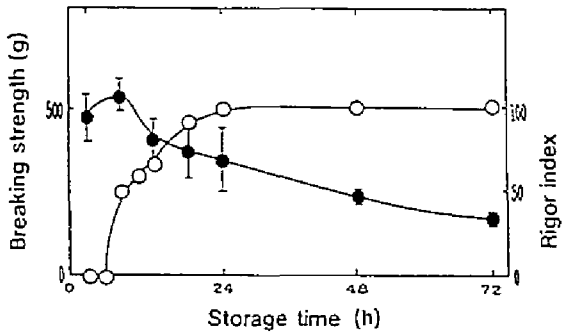

Fig. 5. Change in breaking strength (●) of muscle and rigor index (○) of red sea bream during storage at 5 °C.

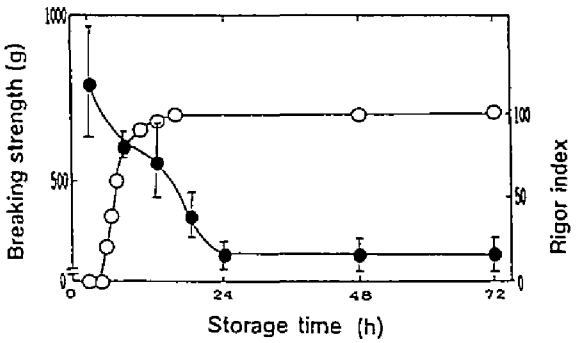

Fig. 6. Change in breaking strength (●) of muscle and rigor index (○) of striped grunt during storage at 5 °C.

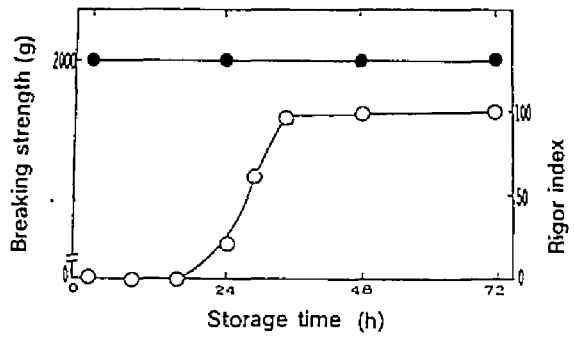

Fig. 7. Change in breaking strength (●) of muscle and rigor index (○) of tiger puffer during storage at 5 °C. The values of the breaking strength were expressed as 2,000 g for the sake of convenience because the muscle was not broken when compressed 2,000 g, the maximal force of the rheometer.

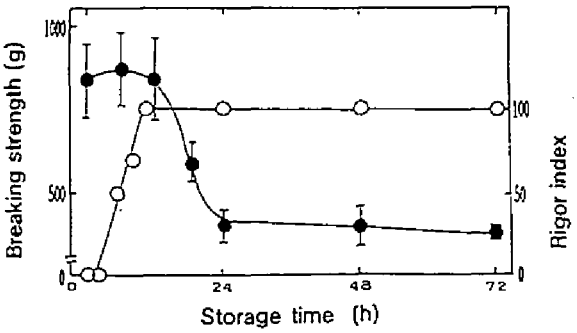

Fig. 8. Change in breaking strength (●) of muscle and rigor index (○) of rainbow trout during storage at 5 °C.

attained to the full rigor within 24 h after death. On the other hand, flatfish (Fig. 1) and tiger puffer (Fig. 7) attained to the full rigor later than other fishes. This could be ascribed to the irregular body shapes of these fish species. Carp attained to maximum rigor ca. 24 h after death (Fig. 4), but the rigor index in maximum rigor did not reach 100, presumably due to its relatively small degree of muscle contraction and/or its relatively thick body shape.

It must be also stressed that all fishes did not show the resolution of rigor during storage even for 72 h. Bito et al.,[8] however, reported that flatfish and tiger puffer showed the resolution of rigor after 30 h at atmospheric temperature. This was probably because the storage temperature of the experiment of Bito et al.[8] was higher than that of ours. In fact, Iwamoto et al.[12] reported that flatfish stored at 5 ^{o}C did not show the resolution of rigor within 72 h after death.

Temporal increase in the breaking strength ca. 6 h after the beginning of storage was observed for parrot bass (Fig. 2), yellowtail (Fig. 3), red sea bream (Fig. 5), and rainbow trout (Fig. 8). It seems likely that the initiation of muscle rigor mortis might be responsible for this temporal increase in the breaking strength because the increase in rigor index also started after 6 h.

As generally accepted, the texture of fish muscle varies according to fish species. This has already been ascertained quantitatively by Hatae et al.[28] on five fishes, i.e., skipjack, flyingfish, common horse mackerel, flatfish, and channel rock fish. The diversity in firmness of muscle among fish species was also clearly demonstrated as the difference in breaking strength in the present study. Parrot bass, carp, striped grunt, tiger puffer, and rainbow trout could be classified as fishes with tough texture, while red sea bream and yellowtail as those with relatively soft texture. Red sea bream, however, seems to be generally regarded as a fish species having relatively tough texture. Such a disagreement between the generally accepted image and the present result is in part due to

the materials used in this study are cultured as pointed out by Hatae et al.[19]

It is also noteworthy that the degree of the post-mortem decrease in breaking strength was varied among fishes. Remarkable decreases in breaking strength were demonstrated for carp (Fig. 4) and striped grunt (Fig. 6). Conversely, decreases in breaking strength of flatfish (Fig. 1), parrot bass (Fig. 2) and tiger puffer (Fig. 7) were less. In particular, no organoleptically noticeable textural change was observed for tiger puffer throughout the storage period (data not shown).

It is of particular interest that relatively large fluctuations of standard deviation in the values of breaking strength was demonstrated in all fishes within 24 h, especially in immediately after death. This is probably because the firmness of muscle at the early stage of storage could be ascribed not only as to the micro-structures, such myofibrils, but also to the macro-structures, such as myocommata and/or extracellular matrix connecting muscle fibers. "Micro" or "macro" used in this context indicates the difference in structural size compared to the diameter (8 mm) of the plunger. In accordance with the storage after 24 h, the fluctuation became small, suggesting that the contribution of the macro-structures to the firmness is lowered probably due to the loss of the integrity of these structures. Relatively small fluctuation in the later stage was probably because only micro and homogeneous structures, such as myofibrils, would contribute to the firmness of muscle at later stage.

In the present study, it was strongly suggested that the tenderization of fish muscle during chill storage proceeds independently with the rigor mortis. This result is inconsistent with the general view that the muscle in full rigor is tough and then becomes tender according to the resolution of rigor. Therefore, it is postulated that the degree of rigor is not always a good index for the texture quality of raw fish muscle particularly in the early stage of storage.

CHAPTER 2

Histological Studies on Post-mortem Tenderization

Several reports have been reported as to a structural change which corresponds to post-mortem tenderization.[41-43] Hay et al.[42] have reported that Z-disc structure of myofibril becomes decay, during storage in chicken muscle. Tachibana et al.[43] have shown the cutting of myofibril at Z-disc in red sea bream. Bremner and Hallet,[44,45] Hallet and Bremner[46] have studied on gaping by using scanning and transmission electron microscopes, and they have suggested the relation of gaping to post-mortem tenderization. In these studies, however, muscle firmness has not been measured. So, it is still unclear that whether the phenomena which they have shown have close relation with post-mortem change of muscle firmness.

In this chapter, light and electron microscopic observations were performed combined with measuring muscle firmness.

Section 1. Detection of Structural Change of Rainbow Trout Muscle by Using a Compression Test

Firmness of fish muscle is an important factor that determine the quality of raw muscle. Fish muscle, except for tiger puffer muscle, tenderizes within a day as shown in CHAPTER 1. Several studies have been reported as to the cause of post-mortem tenderization.[20-32] It should be stressed, however, that no direct evidence is available to connect various post-mortem

changes of muscle with the post-mortem tenderization because textural studies have not been performed in these studies. On the other hand, Hatae et al.[28] have demonstrated by the textural study and the analysis of fish muscle protein on SDS-PAGE that the tenderization of post-mortem fish muscle cannot be explained by the degradation of primary structure of protein molecule.

In the present study, a histological study combined with a compression test was performed parallel to the measurement of the breaking strength to clarify which cellular portion in muscle tissue was responsible for the firmness and what kind of structural changes could occur after death of fish.

Materials and Methods

Materials

Live rainbow trout (650-700 g in body weight and 35-38 cm in body length) was obtained from a local market, decapitated and filleted immediately. Each fillet was packed in a polyethylene bag and stored at 5 ^{o}C.

Measurement of the breaking strength

Breaking strength was measured as described in Section 1 of CHAPTER 1.

Compression test

To know which cellular portion in the muscle tissue is likely to be responsible for the firmness on biting, observations by using a light microscope after a compression test were carried

out. The compression test was done by compressing a 10 mm muscle cube at 200 g/cm^2 for 10 sec using a cylindrical plunger (40 mm in diameter) parallel to the orientation of muscle fibers. The cube was excised from the dorsal muscle in the middle part of the body immediately after death using a microtome blade (FEATHER S35 type).

Similar compression test was done at 100 g/cm^2 at selected intervals to clarify which cellular portion in muscle tissue would become weak during storage.

To establish these two compressing conditions, several conditions (10-300 g/cm^2, 1-10 sec) were examined in the preliminary experiments. In the case of excess weight, muscle structure was broken completely and it was difficult to decide which portion of muscle caused the firmness and its change. Small weights and shorter compressing time showed no structural difference from the non-compressed muscle. Then, the two compressing conditions were selected to demonstrate the differences in the histological observations most clearly. Histological observations were performed simultaneously with the compression test.

Preparation of the samples for the histological observations

Histological observations were carried out with light microscope (LM, Nikon, FX-PH-21) and scanning electron microscope (SEM, Hitachi, S-450). Thin sections for LM observation were prepared as follows. A small piece of muscle excised from a cube by a microtome blade was fixed in Bouin fixative and embedded in paraffin (mp 56-58 ^{o}C). Thin sections of 7 microns were prepared

with a microtome (Yamato Kohki, PR-50) and stained with hematoxylin and eosin.

Samples for SEM observation were prepared as follows. Each sample excised from the compressed muscle cube was fixed overnight in a phosphate buffered mixture of 2.5 % glutaraldehyde and 4 % paraformaldehyde, pH 7.4, according to the method of Reese and Karnovsky.[47] Then, each sample was washed in 0.2 M sodium phosphate buffer (pH 7.4), and processed by the conductive staining method,[48] dehydrated in ethanol series. After dehydration, samples were frozen in liquid nitrogen, and then fractured by striking with a cooled sharp blade which was positioned over the sample. The fractured sample was substituted by 2-methyl-2-propanol, freeze-dried, and then observed using a SEM with an accelerating voltage of 20 kV.

Results

Change in firmness of muscle after death

Change in firmness was represented as the change in breaking strength. As shown in Fig. 9, the breaking strength was almost constant until 12 h after death, but remarkably decreased between 12 and 24 h, followed by the gradual decrease during prolonged storage.

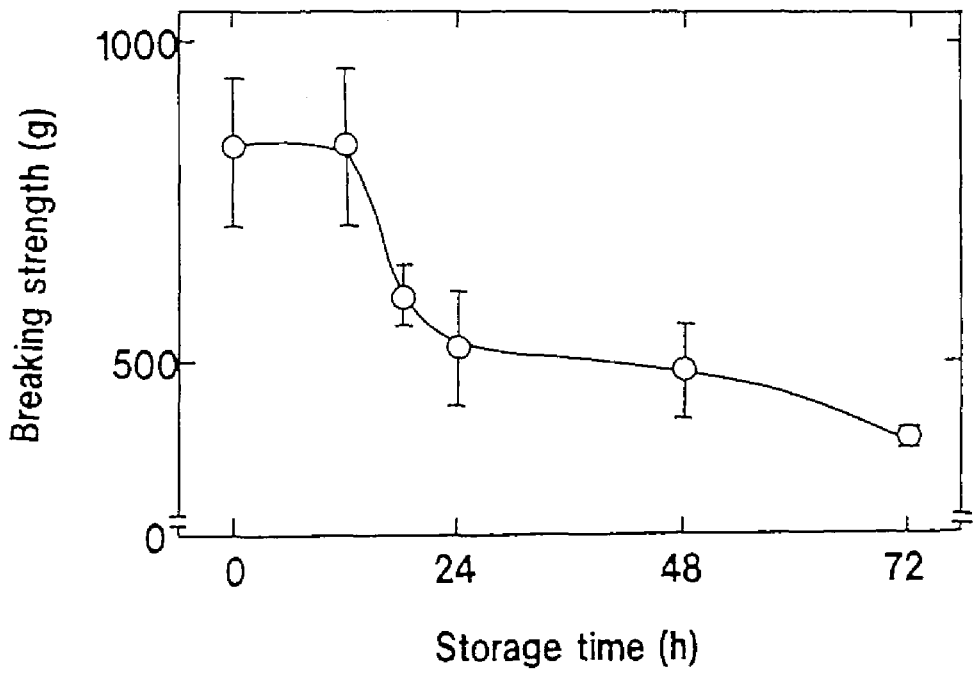

Fig. 9. Change of the firmness of rainbow trout muscle during storage at 5 °C. The firmness was measured as breaking strength as described in the text. Values are the average (± S.D.) of 5-8 determinations.

Histological examination of the cellular portion that might bear the firmness

Figs. 10A and 10B show the LM pictures of the transverse sections of the muscle immediately after death. These pictures demonstrated the detachment of muscle fibers by compressing clearly. It is, therefore, suggested that the connective tissue structure existing among muscle fibers would largely take part in maintaining the firmness of raw fish muscle.

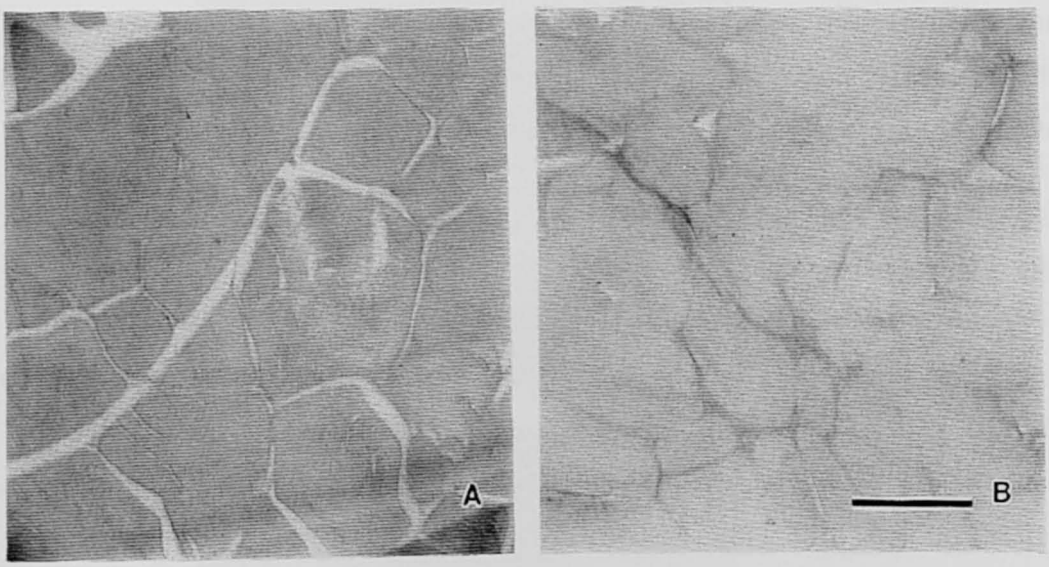

Fig. 10. Light micrographs of rainbow trout muscle immediately after death with compressing at 100 g/cm^2 for 10 sec (A) or without compressing (B). Bar=100 microns.

Post-mortem histological change during chill storage without the compression test

Fig. 11 shows the LM pictures of post-mortem muscle tissue without compressing. As illustrated in these pictures, conspicuous structural change which could explain the marked change in muscle firmness, as shown in Fig. 9, was not observed during storage for 72 h. This result disagreed with our postulation that considerable disintegration in pericellular connective tissue would have been observed corresponding to the marked decrease of the breaking strength. It was, therefore, speculated that the degree of disintegration was too low to be detected by the magnification applied in the present study. Therefore, the compression test was carried out to reveal any structural changes by microscopic observations.

Post-mortem histological change during chill storage with the compression test

As shown in Fig. 12A, no marked disintegration was observed for very fresh muscle even after the compression test at 100 g/cm^2 for 10 sec. However, the formation of clear space between muscle fibers was observed for the muscle stored for 24 h after death as given in Fig. 12B. Further disintegration was observed for the muscle stored for 72 h (Fig. 12C). Fibrous structure, presumably being constituted of connective tissue, was demonstrated in the inter-muscular clear space in the magnified picture observed by SEM (Fig. 13).

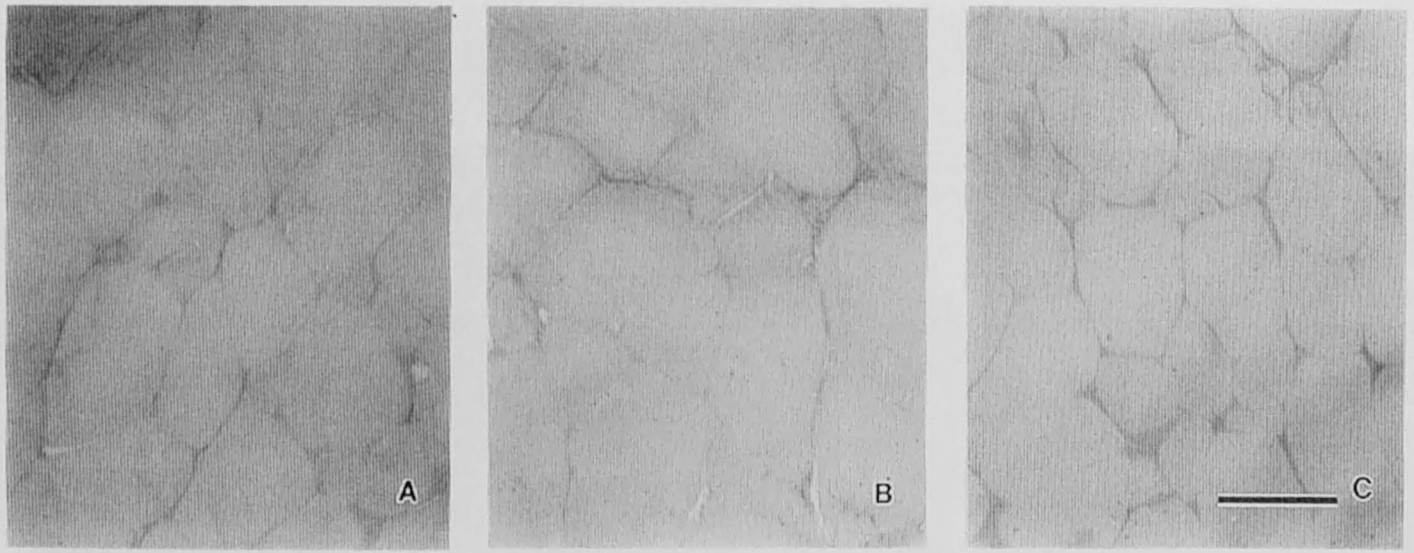

Fig. 11. Light micrographs of rainbow trout muscle during storage at 5 ^{o}C without compressing. (A) Immediately after death, (B) 24 h storage and (C) 72 h storage. Bar=100 microns.

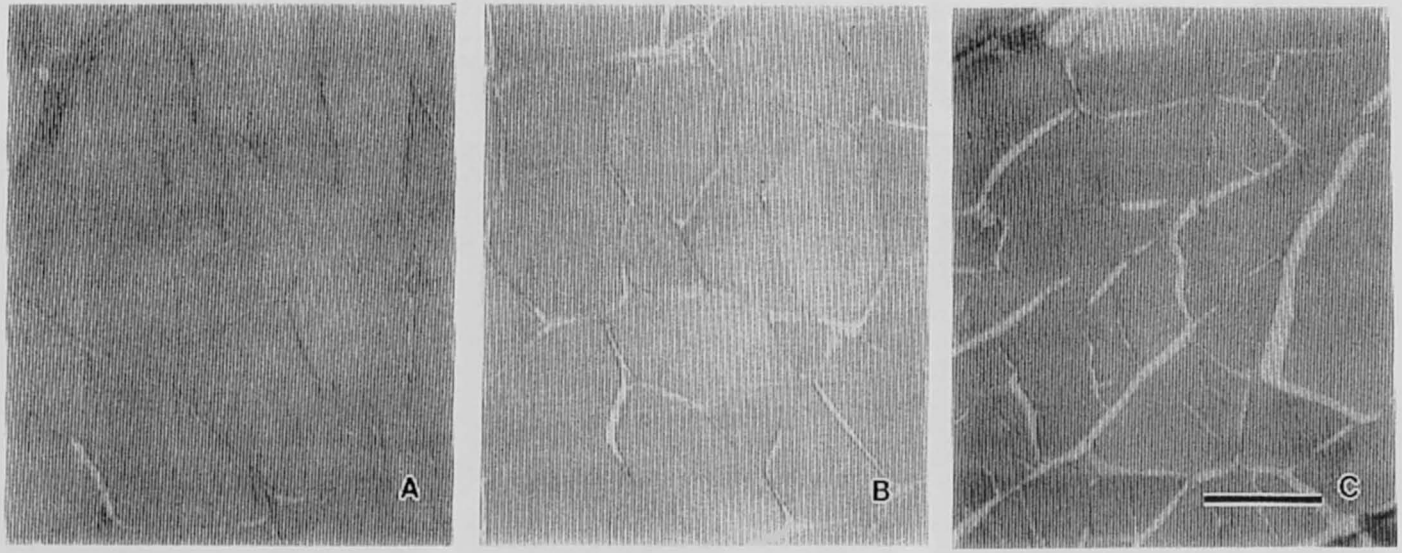

Fig. 12. Light micrographs of rainbow trout muscle during storage at 5 ^{o}C with compressing at 50 g/cm^{2} for 10 sec. (A) Immediately after death, (B) 24 h storage and (C) 72 h storage. Bar=100 microns.

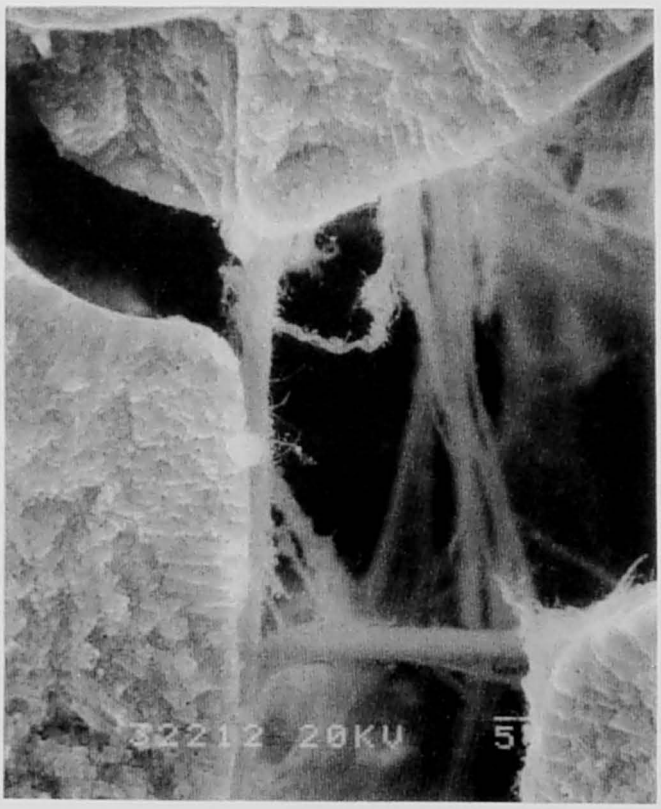

Fig. 13. Scanning electron micrograph of rainbow trout muscle stored at 5 ^{o}C for 72h with compressing at 50 g/cm^{2} for 10 sec. Bar=5 microns.

Discussion

The present study suggests that the gradual weakening of the pericellular connective tissue between muscle fibers would be mainly responsible for the post-mortem tenderization of rainbow trout muscle from the reasons as follows: (1) the firmness of muscle seems to depend on the integrity of the pericellular connective tissue (Fig. 10) and (2) during chill storage, the degree of the disintegration of the connective tissue by compressing (Fig. 12) coincides with the loss of firmness (Fig. 9). Therefore, it seems likely that the pericellular connective tissue plays an important role in maintaining the firmness of fish muscle.

The pericellular connective tissue has been supposed to be mainly constructed by collagen.[49] In addition, the content of collagen has been reported to be related with the firmness of fish muscle.[50,51] Together with these studies, it can be suggested that collagenous fibers connecting muscle fibers shown in Fig. 13 might largely take part in keeping the firmness of fish muscle. Nevertheless, any detectable morphological change of cellular structure corresponding to the tenderization of muscle was not observed without compressing (Fig. 11). This may be possibly because the changes, if any, would be too small to be detected by relatively lower magnification which was used in the present study. It seems also likely that the detachment of the collagen fibrils from the basement membrane of muscle fibers,[44-46] might be responsible for the tenderization.

The tenderization of fish muscle occurs more readily at

higher temperature.[33] This finding suggests the possibility that the disintegration might be due to some biochemical reaction such as proteolysis. Moreover, no change of banding pattern on SDS-PAGE analysis has not been detected for post-mortem tilapia muscle stored for 96 h.[33] However, the breakdown of a 40,000 dalton component to a 38,000 dalton component has been detected by more efficient analytical method, two-dimensional gel electrophoresis combined with silver staining.[52] Therefore, it is very interesting to speculate that the 40,000 dalton component might localize in the junction between collagen fibrils and the basement membrane, and that the breakdown of the component might lead the loss of the integrity of the pericellular connective tissue.

Section 2. Firmness Change of Several Fish Muscles Caused by Weakening of Pericellular Connective Tissue

It has been suggested that post-mortem tenderization of rainbow trout muscle is mainly due to the weakening of the pericellular connective tissue in Section 1. On the other hand, firmness of fish muscles and decreasing rate of the firmness during storage are diverse among fish species as shown in Section 2 of CHAPTER 1.

In the present study, an attempt was made to determine whether the weakening of the pericellular connective tissue causes the post-mortem muscle tenderization in common with other fish species by the histological observation after compression test which was examined in Section 1.

Materials and Methods

Materials

Three specimens of cultured yellowtail (Seriola quinqueradiata, 48-50 cm, 1800-1900 g), flatfish (Paralichthys olivaceus, 35-39 cm, 710-750 g), red sea bream (Pagrus major, 35-37 cm, 1050-1250 g), and tiger puffer (Takifugu rubripes, 27-30 cm, 720-780 g) were obtained alive from a city market, decapitated and stored at 5 ^{o}C.

Muscle Firmness

Muscle firmness was determined as the breaking strength as described in Section 1 of CHAPTER 1.

Compression Test

Compression test was performed as described in Section 1. As already shown for rainbow trout muscle (Section 1), the destroyed portion after compressing causes post-mortem tenderization of muscle.

Light Microscopy

Light microscopic observations were performed as described in Section 1.

Results and Discussion

Fig. 14 shows the change in breaking strength of the muscle of four fish species. Yellowtail and flatfish muscles tenderized

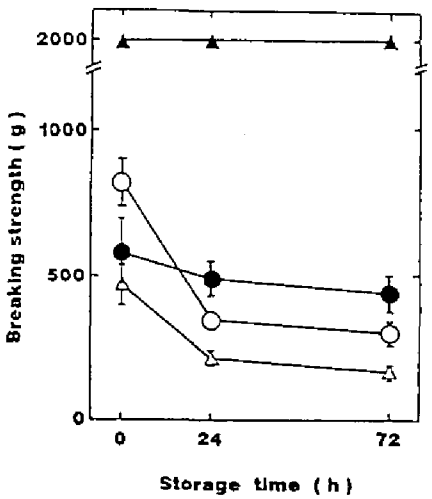

Fig. 14. Change in breaking strength of fish muscle during storage at 5 °C. Samples are identified by the following symbols: ○, yellowtail; ●, flatfish; △, red sea bream; ▲, tiger puffer. In case of tiger puffer muscle, the values of the breaking strength were expressed as 2,000 g for the sake of convenience because the muscle was not broken when compressed at 2,000 g, the maximal force of the rheometer.

from 0 to 24 h after death (CHAPTER 1 Section 2). Light microscopic observation on the muscle tissue demonstrated that muscle fibers were detached each other by compressing at 24 h after death compared to the muscles immediately after death (Figs. 15 and 16). This finding suggests that the strength of pericellular connective tissue which exists among muscle fibers was weakened during storage as shown in rainbow trout (Section 1). In red sea bream (Fig. 14), the breaking strength decreased from 0 to 24 h after death in common with yellowtail and flatfish, but the muscle fibers were detached by the compression

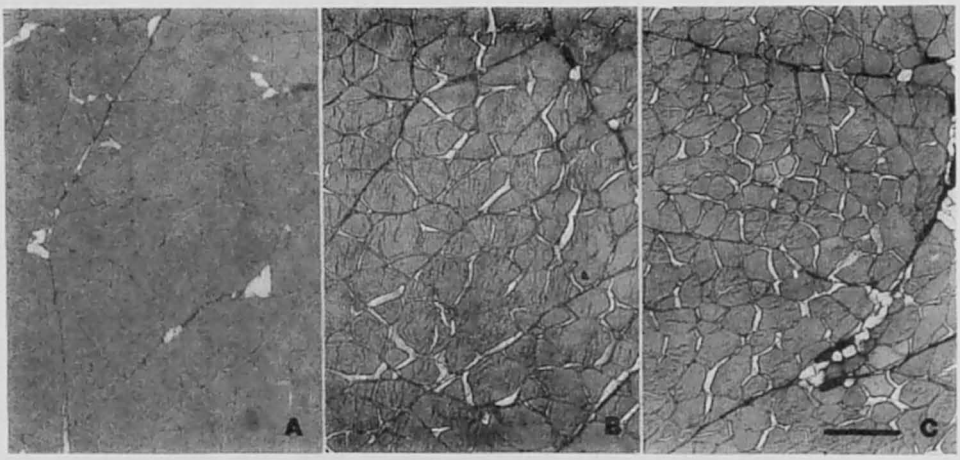

Fig. 15. Light micrographs of yellowtail muscle compressed at 100 g/cm^2 at each time of storage. (A) immediately, (B) 24 h, and (C) 72 h after death. Muscle fibers were detached each other in 24 h, and 72 h storage period corresponding to decrease in breaking strength. Bar represents 100 microns.

immediately after death (Fig. 17). This weakness of the binding strength between muscle fibers would result from relatively low firmness of red sea bream muscle (Fig. 13). Since structural difference of muscle could not be clearly observed over 72 h storage, a milder compression condition should be adopted to observe the structural change of red sea bream muscle during storage.

On the other hand, decrease in the breaking strength was hardly detected in tiger puffer (Fig. 14), indicating that the puffer muscle showed little tenderization during storage as also

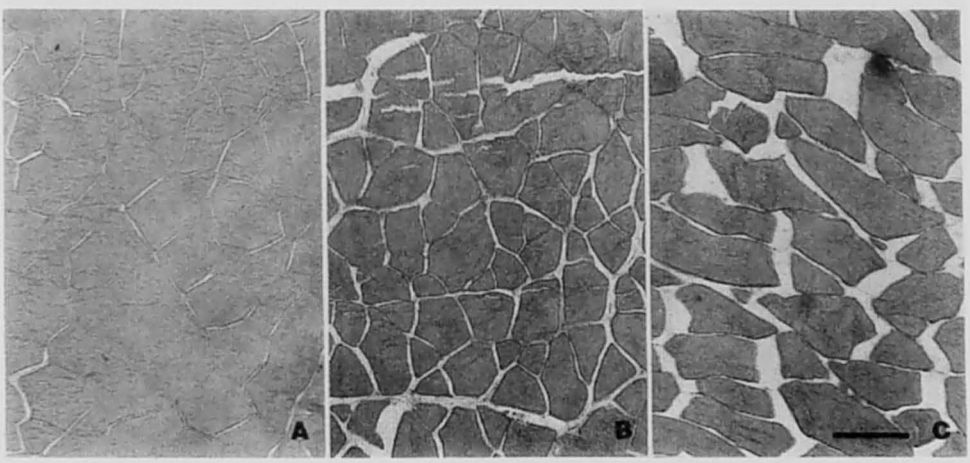

Fig. 16. Light micrographs of flatfish muscle compressed at 100 g/cm^2 at each time of storage. (A) immediately, (B) 24 h, and (C) 72 h after death. Bar represents 100 microns.

shown in Section 2 of CHAPTER 1. According to LM observations (Fig. 18), only a little detachment was recognized among muscle fibers by the compression even after 72 h-storage. These results suggest that the pericellular tissue which connects muscle fibers in tiger puffer muscle highly resists against the compression compared with that in yellowtail, flatfish, and red sea bream muscles.

From the results reported herein, it was shown that the weakening of pericellular connective tissue would greatly associate with the post-mortem tenderization of fish muscle. As

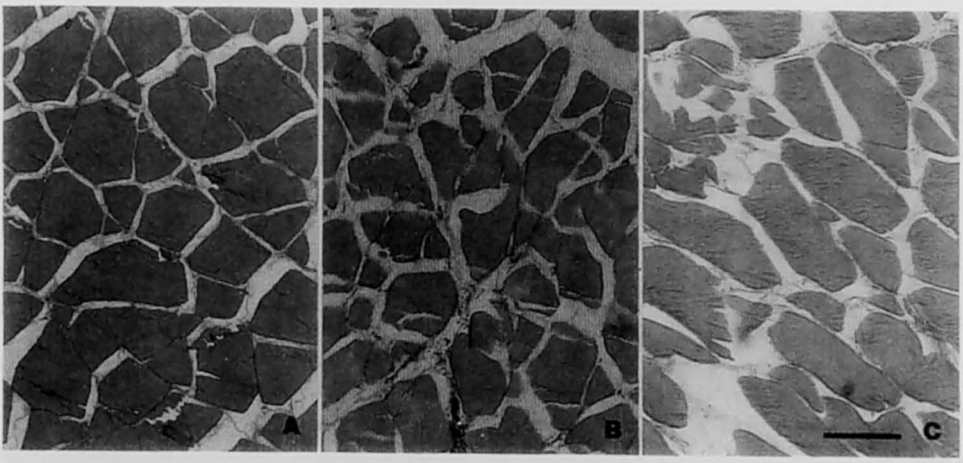

Fig. 17. Light micrographs of red sea bream muscle compressed at 100 g/cm^2 at each time of storage. (A) immediately, (B) 24 h, and (C) 72 h after death. Muscle fibers were detached each other even immediately after death; structural difference could not be clearly observed at 72 h. This weakness of the attachment among muscle fibers against the external force would result from relatively low firmness of red sea bream muscle. Bar represents 100 microns.

to the cause of the post-mortem tenderization, the weakening of Z-discs of myofibrils has been suggested so far.[26-32] If this is true, the weakening of Z-discs during storage of muscle would result in weakening muscle fibers, i.e. the bundle of myofibrils during storage of muscle. However, in the muscle deformed significantly by the compression, muscle fibers did not segment even after 3 days storage (data not shown). These findings suggest that the strength of myofibrils is hardly concerned with the post-mortem tenderization.

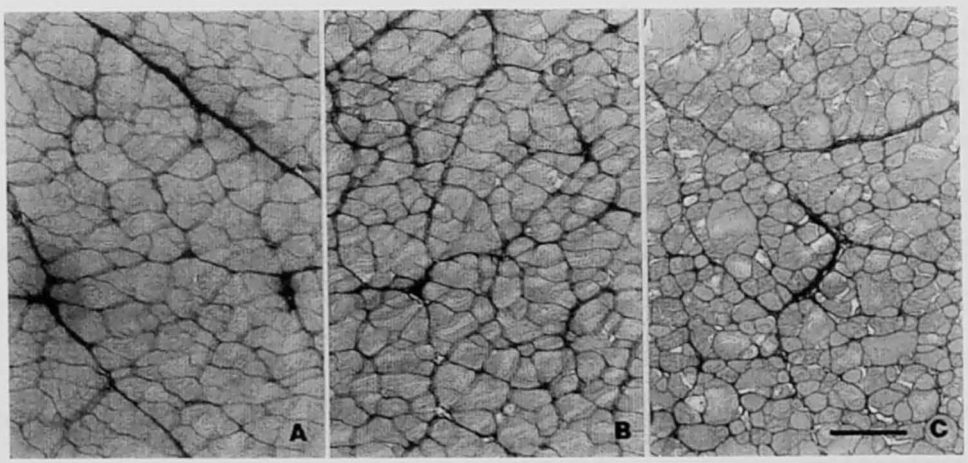

Fig. 18. Light micrographs of tiger puffer muscle compressed at 100 g/cm^2 at each time of storage. (A) immediately, (B) 24 h, and (C) 72 h after death. Only a little detachment was observed among muscle fibers even after 72 h storage. Bar represents 100 microns.

Bremner and Hallet,[44,45] Hallet and Bremner[46] have reported that disappearance of collagen fibrils, which connected muscle fibers to myocommata was observed in hoki (Macruronus novaezelandiae) and spotted trevalla (Seriolella punctata) muscles during chill storage. They suggest that the disappearance of collagen fibrils would concern to post-mortem tenderization. The disappearance of collagen fibrils possibly occurs in the fish species examined in the present study.

As to the cause of weakening of the pericellular connective tissue, physical strength produced by muscle contraction after death could be considered. Onset of rigor mortis agreed appreciably with that of decrease in breaking strength (CHAPTER 1 Section 2), muscle contraction would induce the physical strength between muscle fibers and consequently cause the disintegration and weakening of pericellular connective tissue. Toyohara and Shimizu,[33] however, have measured the breaking strength of tilapia (Oreochromis niloticus) muscle stored as fillet and round, and found no noticeable difference between fillet and round in change of the breaking strength during storage. These findings suggest that muscle contraction, namely rigor mortis, has little direct relationship to the post-mortem tenderization.

Section 3. Change in Three-dimensional Structure of Pericellular Collagen Fibrillar Network in Muscle

LM observations have clarified that post-mortem tenderization of fish muscle is due to weakening of pericellular connective tissue of muscle (Section 1 and 2). On the other hand, observation by using a scanning electron microscope (SEM) is much more useful than LM for a study on a three-dimensional structure of muscle. Recently, several studies have been reported on the observations of three-dimensional structure of connective tissue of some organs by using a cell maceration/SEM method.[53-61] Additionally, a close relationship between firmness of fish muscles and three-dimensional structure of pericellular connective tissue has been reported.[62]

The purpose of the present study is to observe the structural change in pericellular collagen fibrillar network of fish muscles during chill storage, and to discuss the relationship between the structure of pericellular connective tissue and post-mortem tenderization.

Materials and Methods

Samples

Yellowtail (2.4-2.6 kg, 50-52 cm), tiger puffer (690-720 g, 30-32 cm), and rainbow trout (140-160 g, 19-21 cm) were obtained alive from a local market. After decapitation, the fishes were filleted, and each fillet was packed in a polyethylene bag and stored at 5 ^{o}C.

Breaking Strength

Breaking strength of muscle was measured by the method as shown in Section 1 of CHAPTER 1 except for using a cylindrical plunger of 3 mm in diameter.

Cell-maceration/SEM method

Cell-maceration/SEM method[62] was applied to observe the three-dimensional structure of collagen fibrillar network of muscle with a slight modification. Dorsal muscle was cut into 3 mm cubes and those cubes were fixed in Karnovsky fixative. After fixation, samples were immersed in 10 % NaOH solution for 5-7 days at room temperature. After rinsing in 1 % NaCl for 1 h, conductive stain[48] was carried out. The samples were dehydrated in ethanol series, frozen in liquid nitrogen, and fractured with a cooled sharp blade. The fractured samples were substituted by 2-methyl-2-propanol, freeze dried, and observed by using a SEM (JEOL, JSM-T200 and 5400/LV) with an accerating voltage of 10 kV.

Results

Breaking strength values decreased during 24 h-storage in rainbow trout and yellowtail muscles, but tiger puffer muscle showed no decrease in breaking strength as described in Section 2 (data not shown).

Figures 19-21 show the results by using the cell-maceration/SEM method. In this study, 1 % NaCl is used for washing samples instead of distilled water. This is because the

samples expand and degrade in distilled water during preparation except for tiger puffer muscle (data not shown). This degradation would be due to a difference of osmotic pressure between 10 % NaOH and distilled water. The expansion or degradation did not occur in the preparation by 1 % NaCl.

In rainbow trout muscle (Fig. 19A), collagenous fibrils are observed and they are arranged parallel each other. There are many particles (5-10 microns in diameter) on the surface of the pericellular connective tissue (Fig. 19B, arrows). They are assumed to be phagocytes and/or fibroblasts which exist in connective tissue.[63,64] The pericellular connective tissue is thinner compared to fresh muscle after 24, and 72 h-storage (Fig. 19C and D). In a broken portion of the pericellular connective tissue, particles of 1 micron in diameter are observed (arrows). These particles would be degradation products of pericellular connective tissue because the particles cannot be seen in the muscle immediately after death (Fig. 19A). Among the particles, collagenous fibrils exist in an irregular form.

In the collagen fibrillar network of yellowtail muscle (Fig. 20A), the holes after elution of muscle fibers are about 50 microns in diameter. Thick (arrow) or thin (arrowhead) collagenous fibrils are observed in the pericellular connective tissue. After 24 and 72 h-storage (Fig. 20B and C), thick collagenous fibrils are less observed compared with the fresh muscle. The pericellular connective tissue becomes thinner and thin collagenous fibrils are clearly observed.

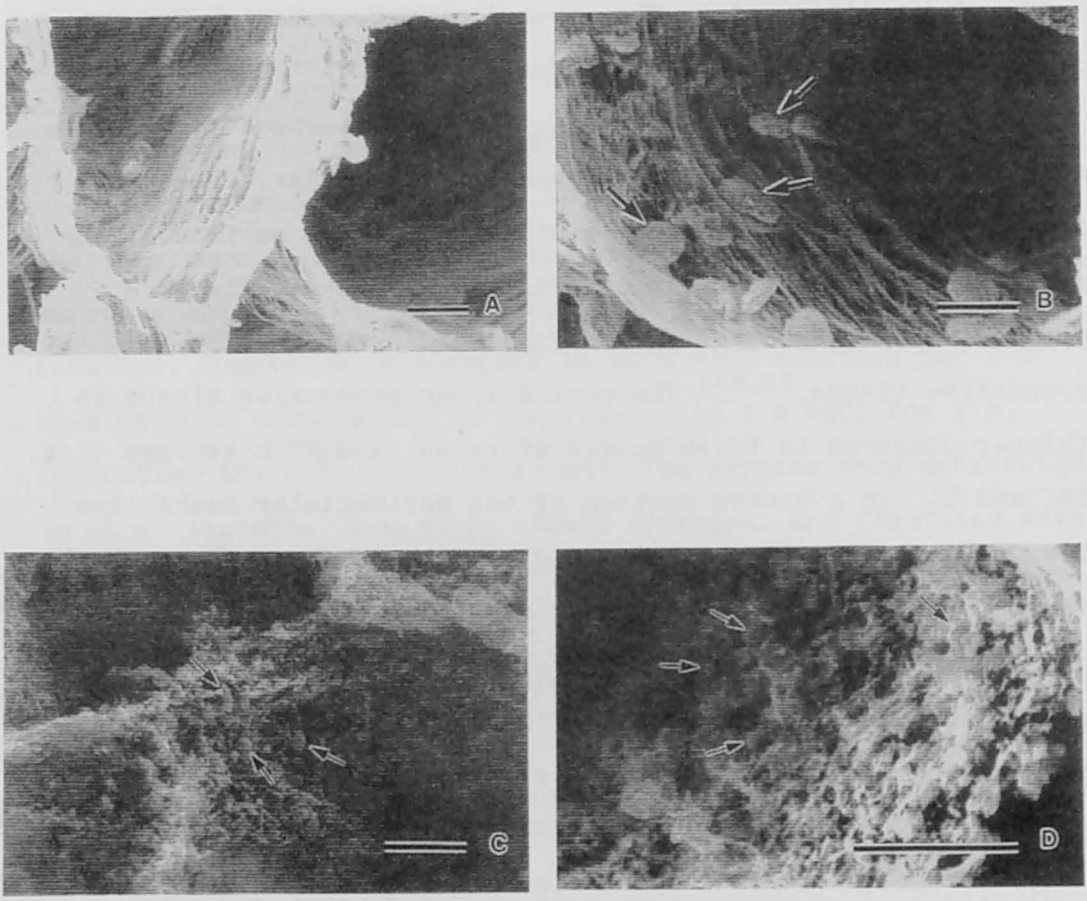

Fig. 19. Pericellular connective tissue of rainbow trout muscle. (A and B) fresh, (C) 24 h, and (D) 72 h-storage muscles. In the fresh muscle (A and B), collagenous fibrils are observed, and on the fibrils, phagocyte-like cells exist (arrows). After storage (C and D), pericellular connective tissue are broken at places, and there exists many particles which are about 1 micron in diameter (arrows). Bars=10 microns.

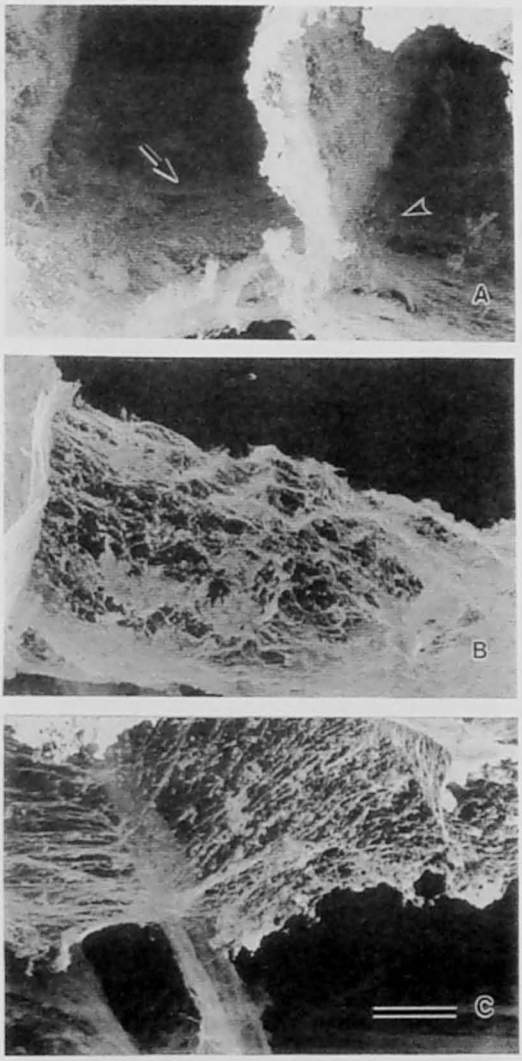

Fig. 20. Pericellular connective tissue of yellowtail muscle. (A) fresh, (B) 24 h, and (C) 72 h-storage muscles. Thick (arrow) and thin (arrowhead) collagenous fibrils are observed (A). In the stored muscle (B and C), the connective tissue becomes thinner and thin fibrils are clearly observed. Bar=10 microns.

In case of tiger puffer muscle (Fig. 20A), the holes after elution of muscle fibers are about 20-50 microns in diameter. The pericellular connective tissue looks like uniform membranous structure, and collagenous fibrils which construct the pericellular connective tissue are observed in higher density. In the 24 and 72 h-storage muscle, however, degradation of the collagen fibrillar network are not observed. This would correspond to the stability of the pericellular connective tissue during storage as shown in Section 2 of CHAPTER 2.

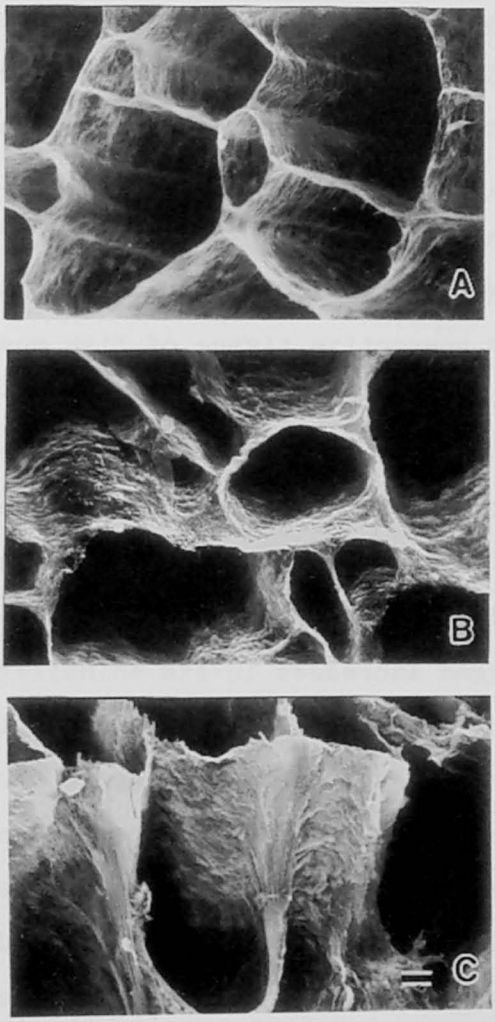

Fig. 21. Pericellular connective tissue of tiger puffer muscle. (A) fresh, (B) 24 h, and (C) 72 h-storage muscle. No structural change is observed in the pericellular connective tissue even after 72 h-storage. Bar=10 microns.

Discussion

The results of this study clarified the structural change in the collagenous fibrillar network, corresponding to the post-mortem tenderization.

As to the post-mortem change of pericellular connective tissue, LM observations have been performed as shown in Section 1 and 2 of CHAPTER 2, but it has had many difficulties to observe the structural change three-dimensionally. The present method enables a wide range observations in three-dimensional structure of the pericellular connective tissue of fish muscle.

In rainbow trout and yellowtail muscles, pericellular connective tissue seems to be thinner during storage. This would be because constituents of the pericellular connective tissue became soluble and was eluted by the sample preparation. Montero and Borderias,[65] Montero and Mackie[66] have reported that intramuscular collagen increases in its solubility during storage. In addition, Sato et al.[67] have shown that solubility of type V collagen increases corresponding to the post-mortem tenderization of rainbow trout muscle. These changes in collagen solubility would be related to the structural changes in the pericellular connective tissues in yellowtail and rainbow trout muscles in the present study. In case of tiger puffer muscle, both of post-mortem tenderization and disintegration of collagenous fibrillar network could not be observed (Fig. 21). This stability of collagenous fibrillar network would be the cause of non-weakening of pericellular connective tissue during storage (CHAPTER 2 Section 2). The reason why tiger puffer

muscle's connective tissue is stable is not evident. However, there is a possibility that pericellular collagen of tiger puffer might have some differences in its content and/or characteristics such as sensitivity to proteases compared to other fish species. Then, it is necessary to compare the characteristics of pericellular collagen between tiger puffer and other fishes.

Section 4. Structural Change of Collagen Fibrils in Pericellular Connective Tissue of Rainbow Trout Muscle

As shown in Section1-3, it has been resolved that weakening of pericellular connective tissue causes post-mortem tenderization of several fish muscles except for tiger puffer. The weakening of the pericellular connective tissue has been detected by using the compression test or NaOH-maceration/SEM method. However, the reason why the weakening was caused is not still evident. This would be because the weakening of the pericellular connective tissue is due to a fine structural change which can not be observed by LM or SEM.

In this study, it was the purpose to observe the fine structural change of the pericellular connective tissue by using a transmission electron microscope (TEM). Additionally, structure of Z-disc of myofibrils which has been reported as the major cause of post-mortem tenderization[43] was observed.

Materials and Methods

Materials

Live rainbow trout Oncorhynchus mykiss (550 g in body weight and 38 cm in body length) was obtained from a city market, decapitated and filleted immediately. Each fillet was packed in a polyethylene bag and stored at 5 ^{o}C.

Measurement of breaking strength

Breaking strength was measured by the method as shown in

Section 3.

Transmission electron microscopy

The dorsal muscle was fixed in the Karnovsky fixative and 1 % osmic acid, and dehydrated in ethanol series, embedded in Quetol 812 (Nissin E M Co.). Ultrathin sections were prepared by ultramicrotome (Reichelt, Ultracut OmU4), and stained with uranyl acetate and lead citrate. The samples were observed by using a TEM (Hitachi, H-700H) with an accerating voltage of 100 kV.

Results and Discussion

Post-mortem change in breaking strength during storage of rainbow trout muscle is shown in Fig. 22. Values of the breaking strength sharply decreased from 12 to 24 h after death, and then gradually decreased for 72 h-storage. This result is well coincided with the change of the breaking strength which was shown in Section 1. This suggests that rainbow trout muscle tenderizes within 24 h after death.

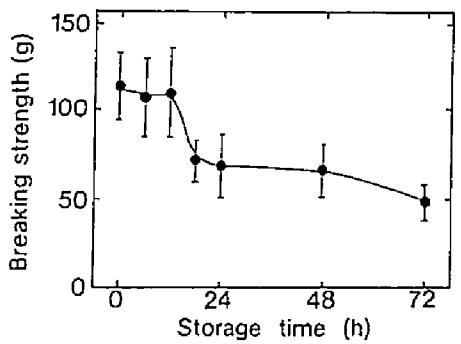

Fig. 22. Change in breaking strength of rainbow trout muscle during storage at 5 ^{o}C. Each value was expressed as an average of 10-14 determinations ± S.D.

Histological observations by using a TEM during storage are shown in Figs. 23-25. In the muscle of immediately after death (Fig. 23a), collagen fibrils (C) of the pericellular connective tissue were arranged orderly between muscle fibers. Z-discs (Z) of myofibrils in the muscle fibers were also evident (Fig. 23b). On the other hand, after storage for 24 h (Fig. 24), most of collagen fibrils of the pericellular connective tissue were disintegrated, and their fragments (C) were observed at places between muscle fibers (M). In the muscle which was stored for 72 h (Fig. 25a), pericellular collagen fibrils (C) were disintegrated more intensively. Z-discs (Z), however, were still remained evident (Fig. 25b).

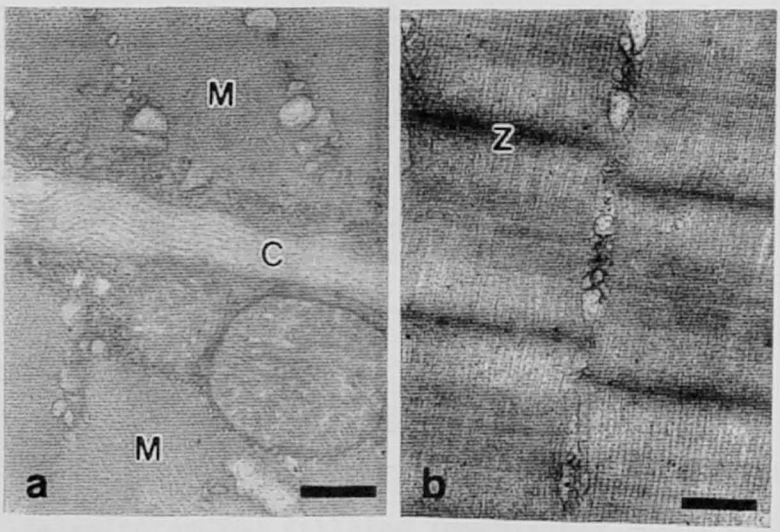

Fig. 23. Electron micrographs of rainbow trout muscle immediately after death. (a) collagen fibrils are arranged orderly between muscle fibers and (b) Z-discs of myofibrils are also intact. M: muscle fiber, C: collagen fibrils, Z: Z-disc. Bars represent 0.5 microns.

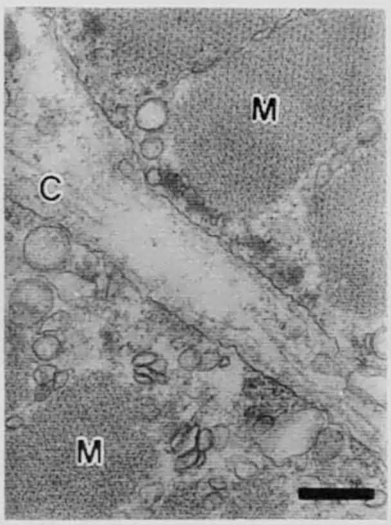

Fig. 24. Electron micrograph of rainbow trout muscle after storage for 24 h at 5 °C. Most of collagen fibrils are disintegrated and fragments of them are observed at places between muscle fibers. Bar represents 0.5 microns.

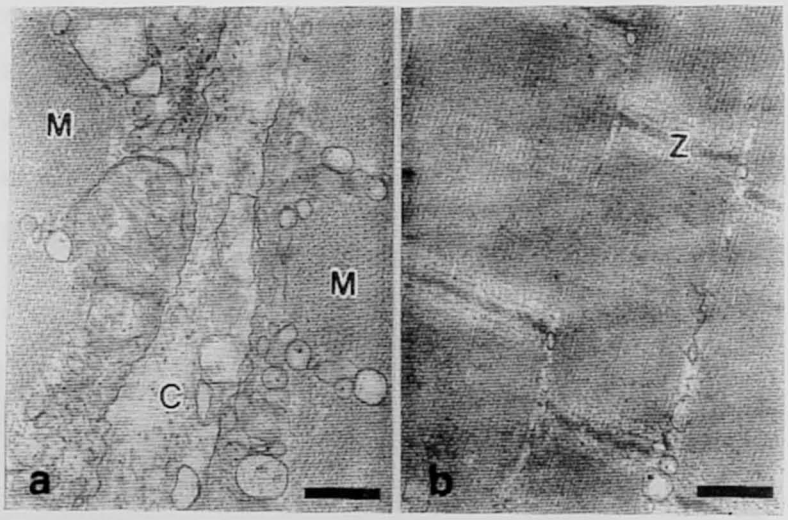

Fig. 25. Electron micrographs of rainbow trout muscle after storage for 72 h at 5 °C. (a) collagen fibrils disintegrate more intensively than 24 h-storage. (b) no evident breakdown of Z-disc was observed in spite of tenderizing of muscle. Bars represent 0.5 microns.

In the present study, collagen fibrils in the pericellular connective tissue showed evident disintegration during storage. This result shows that weakening of the pericellular connective tissue which was shown in Sections 1-3 of CHAPTER 2 is due to the disintegration of collagen fibrils. On the other hand, Z-discs of myofibrils showed no cutting during storage such as demonstrated in red sea bream muscle.[43] These results suggest that post-mortem tenderization which was recognized within a day is due to the disintegration of collagen fibrils rather than that of Z-discs.

It has also been reported the post-mortem change of the existing form of connectin in the early stage of storage.[29,31] However, no data could not be obtained as to the structure of connectin in the present study. Further histological study on connectin would be needed to clarify the relationship between tenderization of muscle and structural change of connectin.

Hallet and Bremner[46] have demonstrated that the gaping which is caused by the degradation of fine network of collagenous fibrils between muscle fiber and myocommata would be responsible for the tenderization of hoki (Macruronus novaezelandiae) muscle. However, as firmness of muscle has not been measured in the study, the time when tenderization of muscle occurred is not evident. The relationship between gaping and tenderization of muscle remains unclear. If the gaping has a close relationship to the tenderization of muscle, hoki muscle would have a possibility of causing later tenderization compared to other fishes[18,28,33] because no structural change has been observed in hoki muscle of 1 day storage.

Collagen, the most major constituents of the connective tissue, has been reported to play an important role for firmness of fish muscle.[50,51] In addition, as demonstrated in Section 1 and 2, the loss of physical strength and structural change of the pericellular connective tissue are responsible for the post-mortem tenderization of fish muscles. However, the reason why the weakening or structural change of the pericellular connective tissue occurred was not evident because of a low magnification of LM and SEM. In the present study, the structural change of collagen fibrils could be observed by higher magnified observations by using a TEM. This finding supports the idea that the disintegration of collagen fibrils leads to the loss of strength of the pericellular connective tissue, and consequently causes the post-mortem tenderization.

As the cause of the disintegration of collagen fibrils observed in the present study, the increase in physical strength by the contraction of muscle fibers could be suggested as previously shown in Section 2. In the case of rainbow trout (CHAPTER 1 Section 2), just after attaining full rigor of body, namely when fish muscle contracts fully, the muscle starts to tenderize. Accordingly, a hypothesis could be deduced as follows: the increase of physical strength which caused by the muscle contraction would disintegrate the collagen fibrils and thus result in the tenderization of muscle.

In fish muscle, type I and V collagen exist.[68,69] In addition, Sato et al.[67] have reported that type V collagen solubility changes during storage corresponding to the

tenderization of rainbow trout muscle, and the change would be occurred by cleavage of telopeptide and/or intermolecular cross-links. If the pericellular collagen fibrils are formed by type V collagen as mammalian and avian,[49,70] cleavage of type V collagen would cause the disintegration of pericellular collagen fibrils and consequently post-mortem tenderization.

CHAPTER 3

Post-mortem Change of Intramuscular Collagen

As shown in CHAPTER 2, it was clarified histologically that post-mortem tenderization of fish muscle was due to the degradation of pericellular collagen fibrils. Collagen is the most major constituent of connective tissue, and its content corresponds closely to muscle firmness of fishes,[50,51] turban shell,[71] and abalone.[72] In addition, collagen has close relationship to post-mortem tenderization. Montero and Borderias,[65] Montero and Mackie,[66] and Sato et al.[67] have reported the change of collagen solubility of collagen during storage corresponding to textural change of fish muscle.

In this CHAPTER, determination of hydroxyproline, which is the marker of collagen degradation, is performed, and correspondence between collagen fibril degradation and change of collagen solubility is studied.

Section 1. Determination of Free and Peptide Forms Hydroxyproline Contents

It was resolved that structural change of the pericellular collagen fibrils would cause post-mortem tenderization of fish muscle during storage (CHAPTER 2). The detection of the degradation of collagen molecule seemed to be required. This section describes some trials to determine free and peptide forms of hydroxyproline (Hyp) were made during chill storage of fish

muscle.

Materials and Methods

Materials

Yellowtail (Seriola quinqueradiata), tiger puffer (Takifugu rubripes), rainbow trout (Oncorhynchus mykiss) and sardine (Sardinops melanostictus) were obtained alive from a city market, decapitated and stored at 5 ^{o}C.

Breaking Strength

Breaking strength of muscle was measured as mentioned previously (CHAPTER 2 Section 3).

Determination of hydroxyproline in free and peptide forms

Fish muscle (2 g) was homogenized with 10 ml of 80 % ethanol. After centrifugation (3000 rpm, 5 min), the supernatant was stocked. Five milliliter of 80 % ethanol was added to the residue, and the solution was stirred by glass stick, centrifuged at 3000 rpm, 5 min. The supernatants were combined and adjusted to 20 ml by 80 % ethanol. One milliliter of the extract solution was evaporated and re-resolved in 1 ml of 0.1 N HCl. The solution was applied on SEP-PAK C18 (Waters) which was equilibrated by 60 % (v/v) acetonitrile, distilled water, and 0.1 N HCl. Ten microliter of the filtrate was pipetted in a glass tube (50 mm x 5 mm i.d.) and dried by vacuum. For determination of peptide form Hyp, sample was hydrolyzed with vapor HCl at 150 ^{o}C for 1 h. Derivatization with phenyl isothiocyanate (PITC) and separation

of PTC-amino acids were performed by the method of Bidlingmeyer et al.[73] with a slight modification.[74]

Results

Change in breaking strength

Breaking strength of each fish muscle, except for tiger puffer, decreased within a day (Table 3). Especially in the case of sardine, the value of the breaking strength decreased from immediately after death, and attained the lowest firmness only after 12 h-storage. Rainbow trout and yellowtail muscles tenderized slowly in comparison with sardine muscle. On the other hand, tiger puffer muscle did not tenderize even after 72 h-storage.

Table 3. Change in the breaking strength of fish muscle during chill storage

(g)

	Storage period (h)		
	0	24	72
Rainbow trout	118±21	68±18	51±9
Yellowtail	149±24	59±9	56±15
Sardine	126±9	37±6	29±9
Tiger puffer	174±34	209±32	165±24

Each value is expressed as the mean of 8-14 determinations±S.D.

Change in contents of free and peptide forms Hyp

Table 4 shows the change in contents of free and peptide forms Hyp. Free Hyp of rainbow trout muscle increased about 30 % from 0 to 24 h after death, and the content was almost constant from 24 to 72 h. On the other hand, peptide form Hyp content decreased from 0 to 24 h, and increased from 24 to 72 h.

In case of yellowtail muscle, free Hyp increased for 72 h continuously. The increasing ratio of free Hyp between 0 and 24 h was 40 %, 0 and 72 h was 70 %, respectively; the peptide form of Hyp could not be detected during 3 day-storage. In sardine muscle, the content of free Hyp was smaller than that of rainbow trout and yellowtail muscles, and showed no change during 72 h-storage. On the contrary, peptide form Hyp increased 34 % between 0 and 24 h, and was constant from 24 to 72 h. On the occasion of tiger puffer muscle, both of free and peptide forms Hyp could not be detected.

Table 4. Change in contents of free and peptide forms of hydroxyproline during storage

(nmol/g muscle)

		Storage period (h)		
		0	24	72
Rainbow trout	f	112.6±27.3	139.9±13.1	135.2±14.6
	p	134.2±25.5	108.1±24.6	126.8±7.2
Yellowtail	F	74.1±21.6	102.2±17.4	126.8±29.1
	P	N.D.	N.D.	N.D.
Sardine	F	10.8±0.3	10.5±0.5	9.9±2.9
	P	78.6±26.7	99.4±5.8	99.4±5.5
Tiger puffer	F	N.D.	N.D.	N.D.
	P	N.D.	N.D.	N.D.

"F" and "P" indicate free and peptide form of hydroxyproline content, respectively. Each value is expressed as the mean of 3 determinations ±S.D. N.D., not detected.

Discussion

In case of rainbow trout and yellowtail muscles, free form Hyp increased during storage. On the other hand, peptide form Hyp increased in sardine muscle. This difference would be due to the difference of sensitivity to proteases resulting from structural diversity of peptides including Hyp or collagen molecule. Possibly, these peptides derive from collagen because Hyp is an amino acid which is included in collagen specifically in muscle. Since the free and peptide forms of Hyp contents, which were detected in the present study were about 1 to 10 % of total Hyp in fish muscle,[75] the change in Hyp content would be because of degradation of limited portion of collagen molecule such as telopeptide. Collagen molecules have a possibility to undergo a limited cleavage in telopeptide by several proteases such as pepsin, trypsin, thermolysin, and cathepsin L.[71,76] On the other hand, it has been reported that type V collagen is suffered from some cleavage during storage corresponding to the post-mortem tenderization.[77] Therefore, the increased free or peptide forms would be derived from type V collagen. This type of collagen can be degraded by type IV and V collagenases,[78] although Sato *et al*.[77] have reported that degradation products of type V collagen were not detected after 24h-storage of rainbow trout muscle.

Tiger puffer muscle showed no tenderization, and both of free and peptide forms of Hyp could not be detected (Table 4), suggesting that collagenolytic activity in tiger puffer muscle is relatively low during storage. Pericellular collagen fibrils of tiger puffer muscle have two to three times as thick as those of

other fishes.[79] Adachi et al.[80] reported that thickness of collagen fibrils was corresponded to the proportion of type I and type V, and that the more type I is contained, the more is thickness of collagen fibrils. From this point of view, the pericellular collagen fibrils in tiger puffer muscle would include higher proportion of type I, and lower that of type V collagen. No detection of free and peptide forms hydroxyproline contents indicates the higher proportion of type I collagen which is stable during storage.[67]

Section 2. Comparison of Muscle Structure and Collagen Solubility between Sardine and Tiger Puffer

It is clarified that post-mortem tenderization of rainbow trout muscle is due to the disintegration of pericellular collagen fibrils (CHAPTER 2 Section 4). In addition, cleavage of covalent bond in type V collagen could be speculated for rainbow trout muscle according to the report by Sato et al.[67]

In this study, to investigate the hypothesis about the mechanism of post-mortem tenderization which has been reported in rainbow trout muscle (CHAPTER 2 Section 4), sardine muscle, which shows notable tenderization, and tiger puffer muscle, which seldom shows tenderization after death, were examined as to change in muscle structure and collagen solubility[67] during chill storage.

Materials and Methods

Materials

Live sardine (20 cm, 100 g) and tiger puffer (32 cm, 900 g) were obtained. After decapitation, each fish was stored at 5 ^{o}C in polyethylene bags.

Breaking strength

Breaking strength was measured as shown in Section 3 of CHAPTER 2.

Light microscopy

Muscle structure after compression was observed by using a

LM as previously reported in Section 1 of CHAPTER 2.

Transmission electron microscopy

Fine structure of muscle was observed as shown in previously (CHAPTER 2 Section 4).

Solubility of collagen

Solubilities of type I and V collagens were measured according to the method reported by Sato et al.[67]

Results

Breaking strength

Table 5 shows the result of measuring breaking strength. Sardine muscle tenderized markedly after 24 h-storage, but tiger puffer muscle did not as shown in Section 1 of CHAPTER 3.

Table 5. Change of breaking strength of fish muscle during storage

(g)

	Storage period (h)	
	0	24
Tiger puffer	373±49	486±90
Sardine	127±19	33±2

Each value is expressed as the mean of 8-14 determinations±S.D.

Light microscopy

LM observations were done about compressed muscles (Figs. 26 and 27). In transverse section, tiger puffer muscle showed no structural change even after 24 h-storage (Fig. 26). In sardine muscle, however, detachment of muscle fibers was recognized after 24 h-storage (Fig. 27). This shows the weakening of pericellular connective tissue during storage as previously described in other fishes (CHAPTER 2 Section 1 and 2).

In longitudinal sections, tiger puffer muscle had no differences during storage (Fig. 28). In sardine muscle (Fig. 29), detachment of muscle fibers was observed, but disintegration of muscle fibers was not observed even in the tenderized muscle.

Transmission electron microscopy

Disintegration of pericellular collagen fibrils was observed in sardine muscle after 24 h-storage (Fig. 30). This corresponded to the decrease in the breaking strength (Table 5), and coincided with the result of rainbow trout (CHAPTER 2 Section 4). On the contrary, no structural change was observed in pericellular collagen fibrils of tiger puffer muscle after 24 h-storage (Fig. 31). These results show that the stability of pericellular collagen fibrils keeps the firmness of tiger puffer muscle during storage.

Figs. 32 and 33 show the collagen fibrils of myocommata of sardine and tiger puffer muscles. Surprisingly, collagen fibrils were not disintegrated in the stored sardine muscle (Fig. 32B). Namely, disintegration of collagen fibrils after death is specific in pericellular connective tissue in muscle.

The structure of myofibrils is shown in Figs. 34 and 35. As the same in rainbow trout (CHAPTER 2 Section 4), myofibrils were not cut in sardine and tiger puffer muscles during storage. Additionally, Z-disc structure was not disintegrated even after compression (Figs. 36 and 37). According to these results, it is concluded that Z-disc of myofibril has little effect on post-mortem tenderization in early period of storage.

Solubility of type I and V collagens

Solubilities of type I and V collagens were summarized in Figs. 38 and 39. Significant difference was not recognized during storage in tiger puffer muscle (Fig. 38). In sardine muscle, however, content of type V collagen was reduced to one-half of that of fresh muscle in spite of no significant change in type I collagen content (Fig. 39). In type V collagen, contents of insoluble and pepsin-solubilized collagen were decreased. These results suggest that tenderized muscle shows the increase in type V collagen solubility and that solubility change of type V collagen does not occur in the muscle which shows no tenderization.

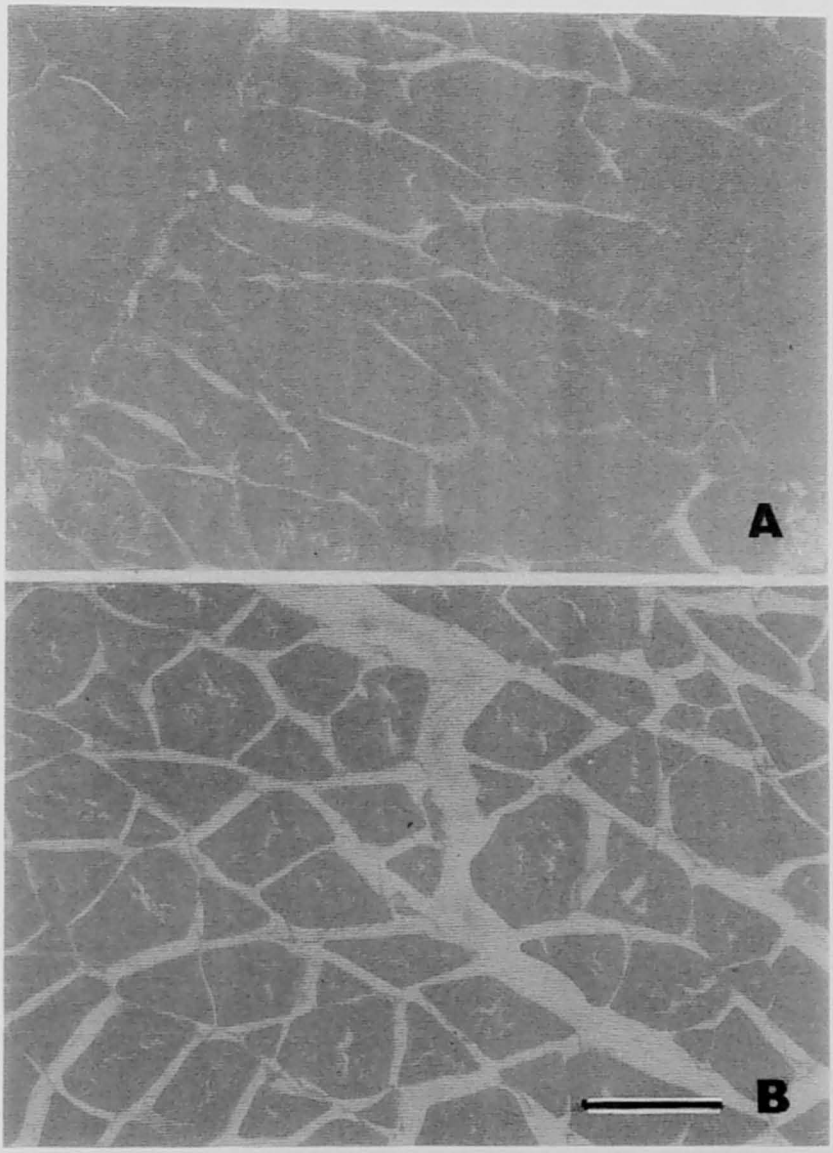

Fig. 26. Transverse sections of compressed sardine muscle. (A) Immediately, and (B) 1 day-storage after death, respectively. In the storage muscle (B), muscle fibers are detached each other, and it is shown that pericellular connective tissue weakened during chill storage. Bar=100 microns.

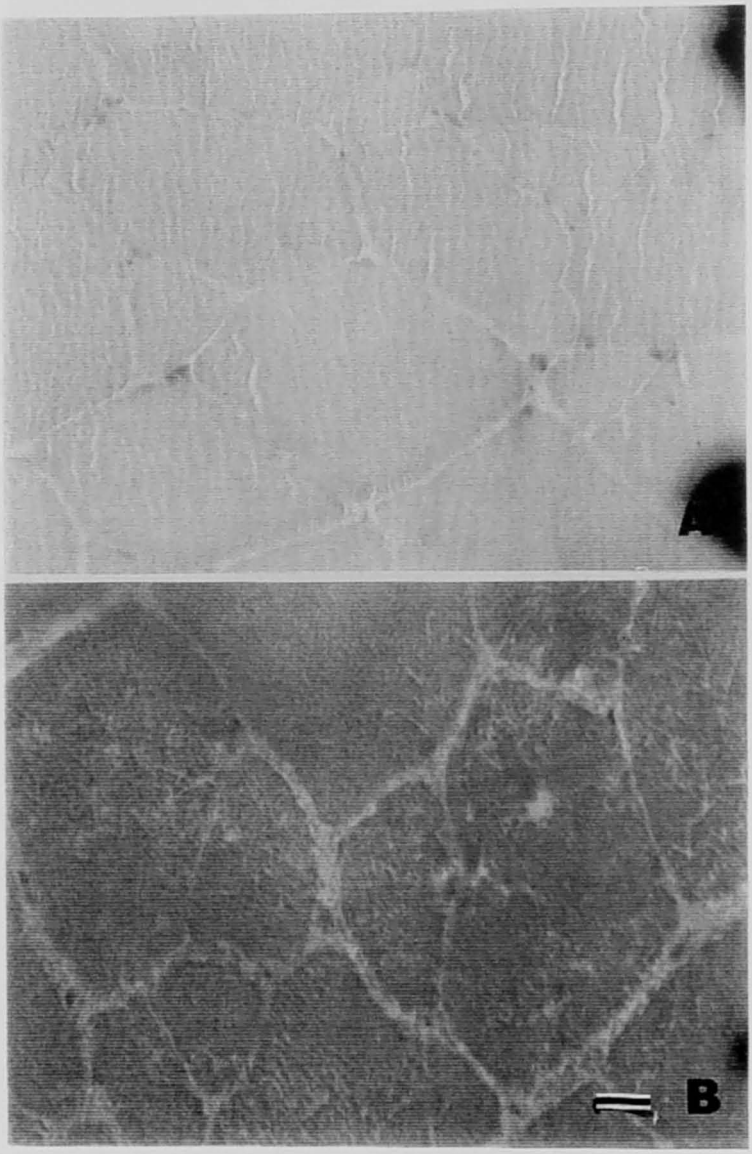

Fig. 27. Transverse sections of compressed tiger puffer muscle. (A) Immediately, and (B) 1 day-storage after death. No structural change is observed after storage. Bar=10 microns.

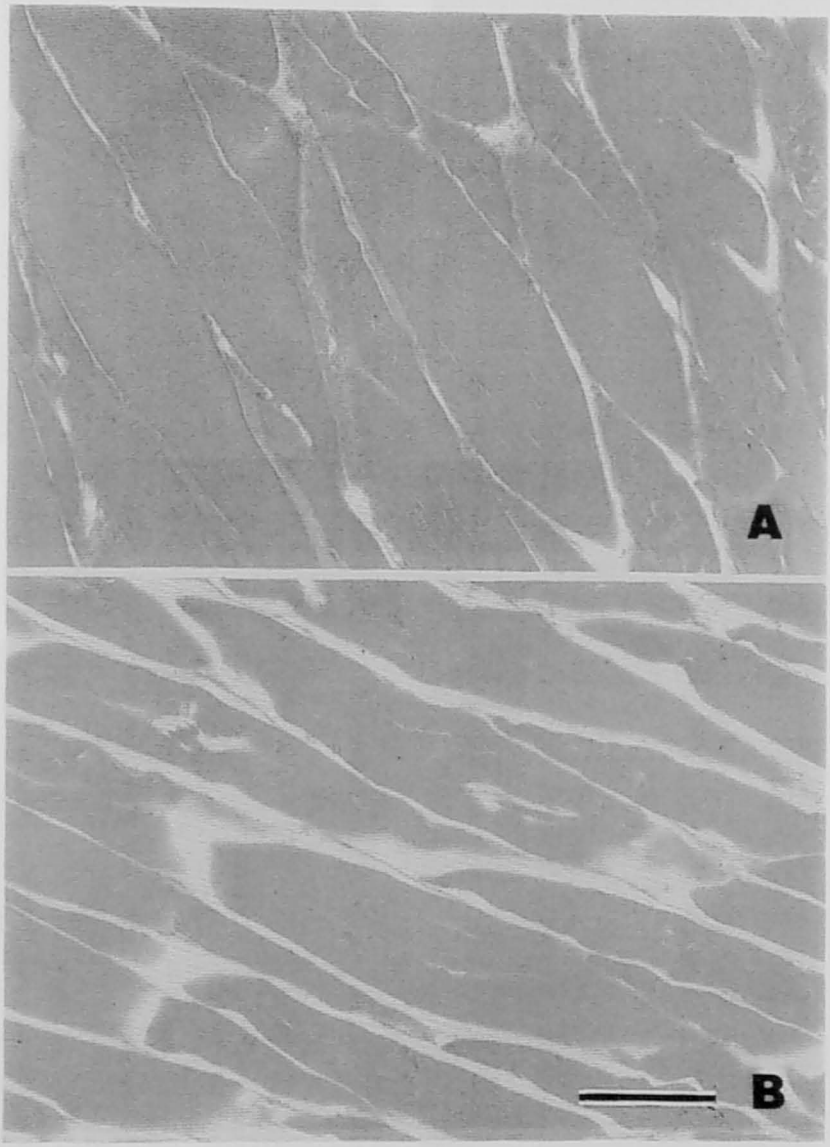

Fig. 28. Longitudinal sections of compressed sardine muscle. (A) Immediately, and (B) 1 day-storage after death. Note that muscle fibers are not cut by the compressing force. Bar=100 microns.

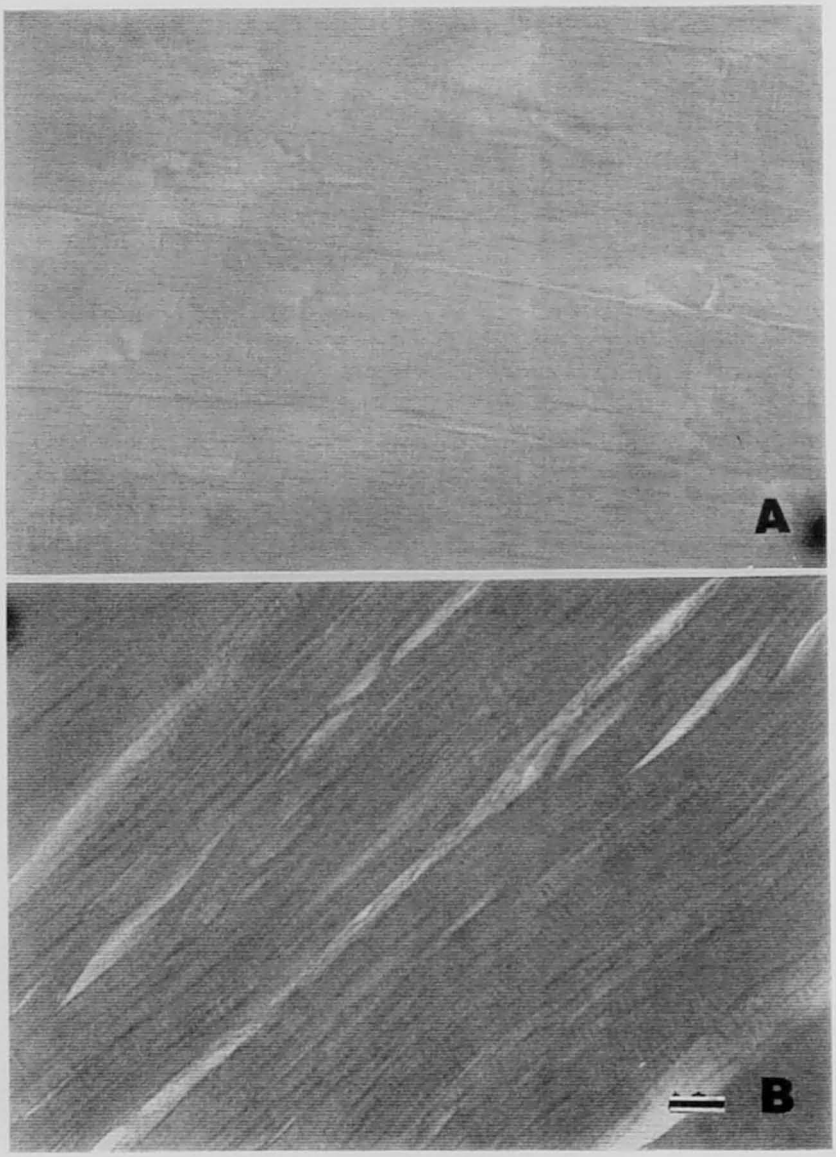

Fig. 29. Longitudinal sections of compressed tiger puffer muscle. (A) Immediately, and (B) 1 day-storage after death. Bar=10 microns.

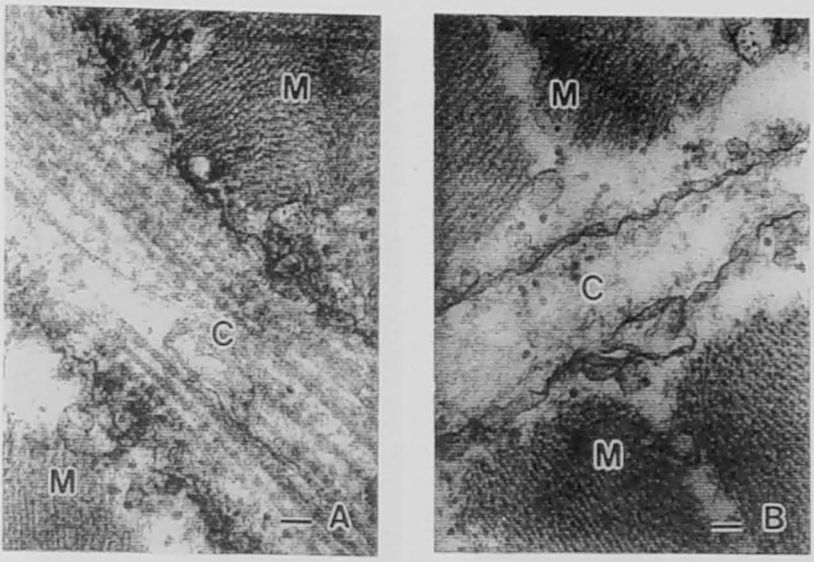

Fig. 30. Fine structure of pericellular connective tissue of sardine muscle. (A) Immediately, and (B) 1 day-storage after death. Collagen fibrils are disintegrated after chill storage. Bars=100 nm.

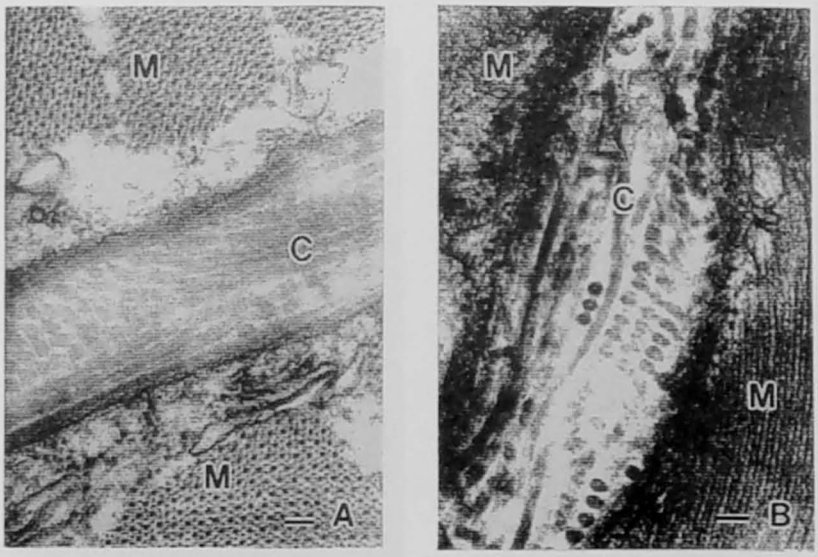

Fig. 31. Fine structure of pericellular connective tissue of tiger puffer muscle. (A) Immediately, and (B) 1 day-storage after death. Collagen fibrils does not show any structural change even after 1 day-storage. Bars=100 nm.

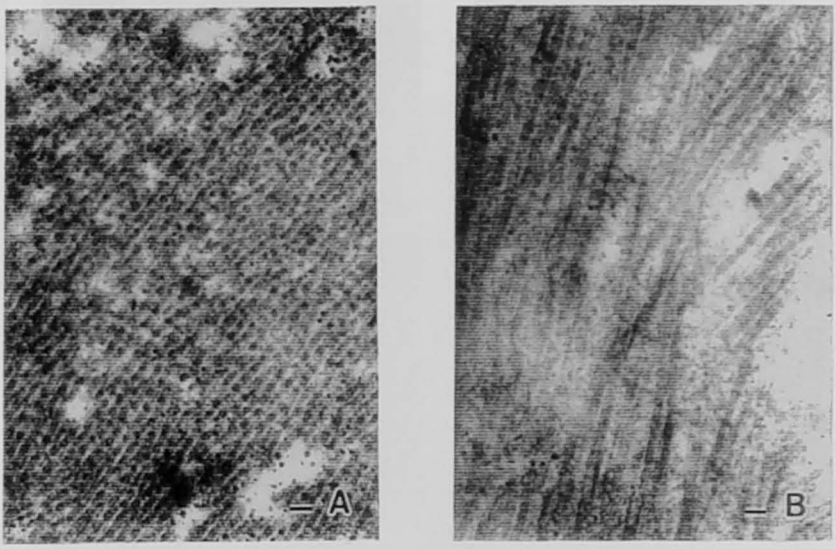

Fig. 32. Collagen fibrils of myocommata of sardine muscle. (A) Immediately, and (B) 1 day-storage after death. No disintegration is observed even after 1 day-storage. Bars=100 nm.

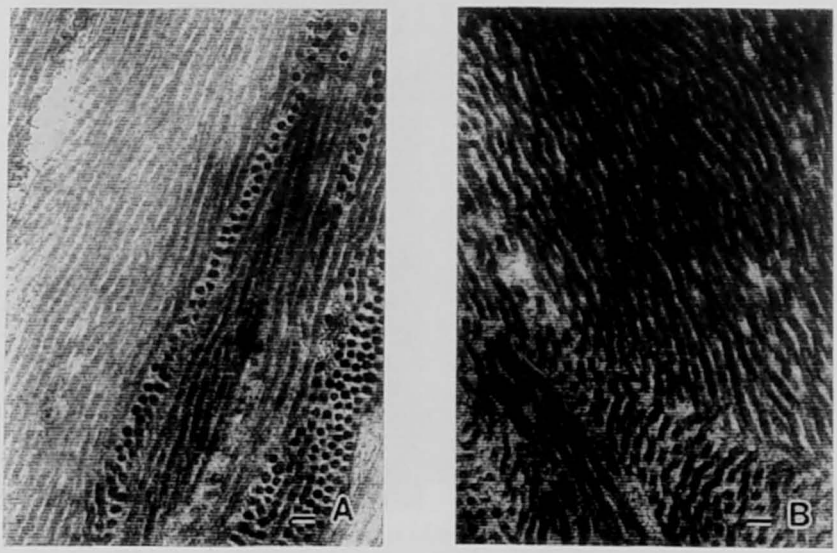

Fig. 33. Collagen fibrils of myocommata of tiger puffer muscle. (A) Immediately, and (B) 1 day-storage after death. Bars=100 nm.

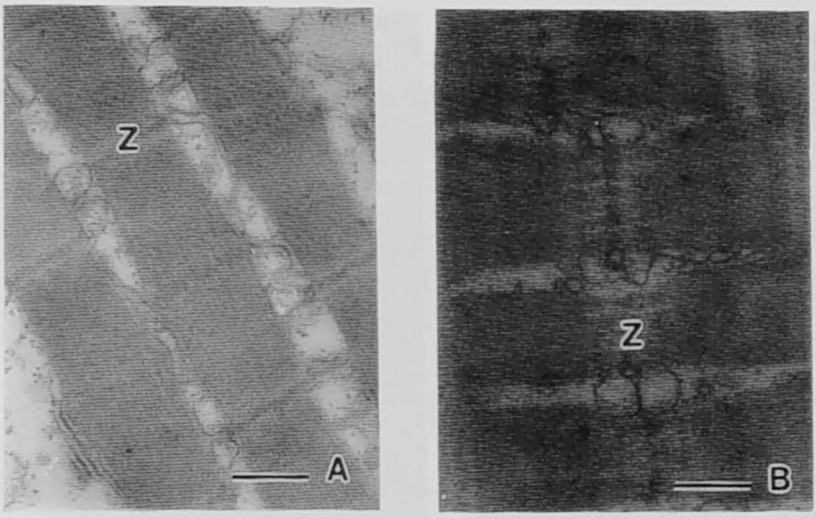

Fig. 34. Myofibrils of sardine muscle. (A) Immediately, and (B) 1 day-storage. Myofibrils are not disintegrated at Z-disc structure. Bars=500 nm.

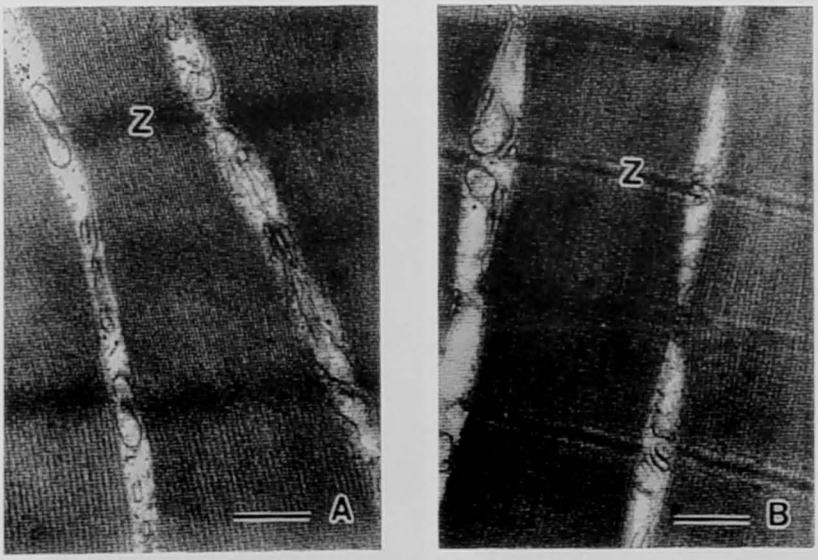

Fig. 35. Myofibrils of tiger puffer muscle. (A) Immediately, and (B) 1 day-storage. Bars=500 nm.

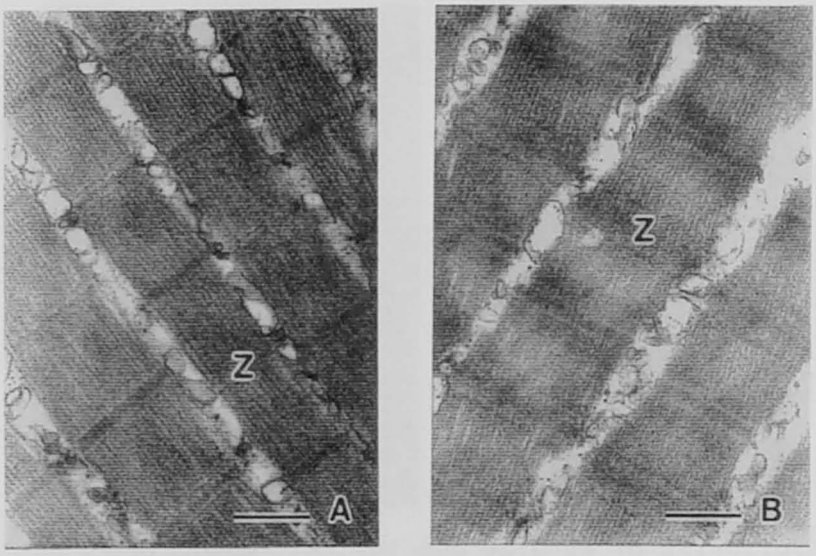

Fig. 36. Myofibrils of sardine muscle after compression. Cutting or disintegration of myofibrils are not observed. (A) Immediately, and (B) 1 day-storage after death. Bars=500 nm.

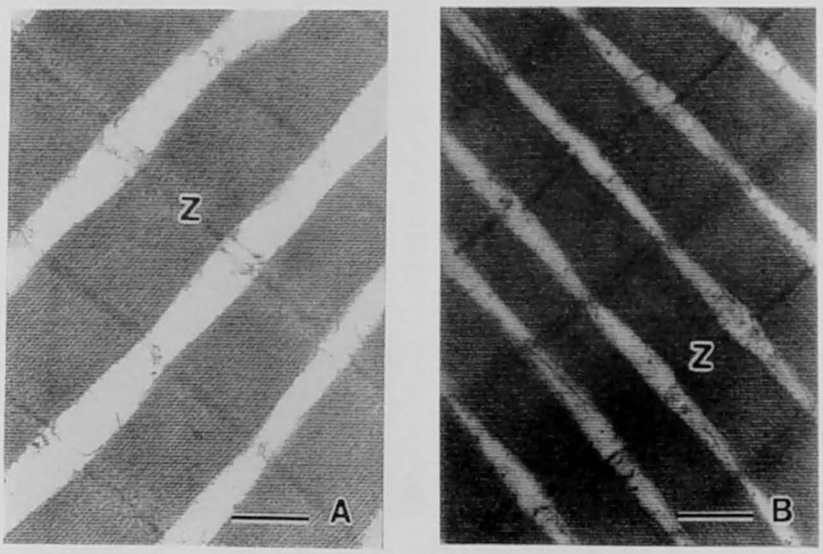

Fig. 37. Myofibrils of tiger puffer muscle after compression. (A) Immediately, and (B) 1 day-storage after death. Bars=500 nm.

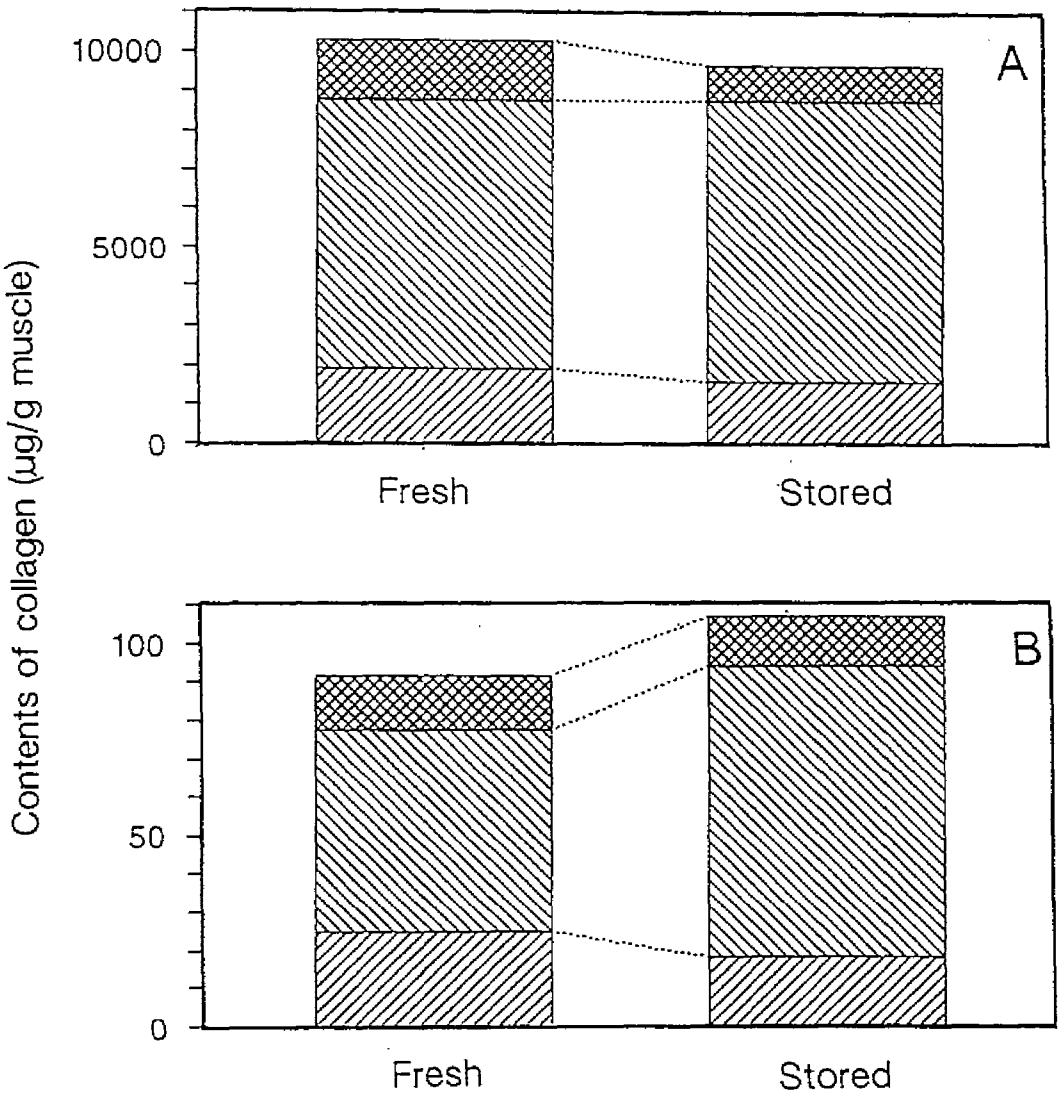

Fig. 38. Solubility of type I (A) and V (B) collagens of tiger puffer muscle. No significant differences are detected in both types of collagens during storage. ▨; acid-soluble collagen, ▧; pepsin-solubilized collagen, and ▩; insoluble collagen.

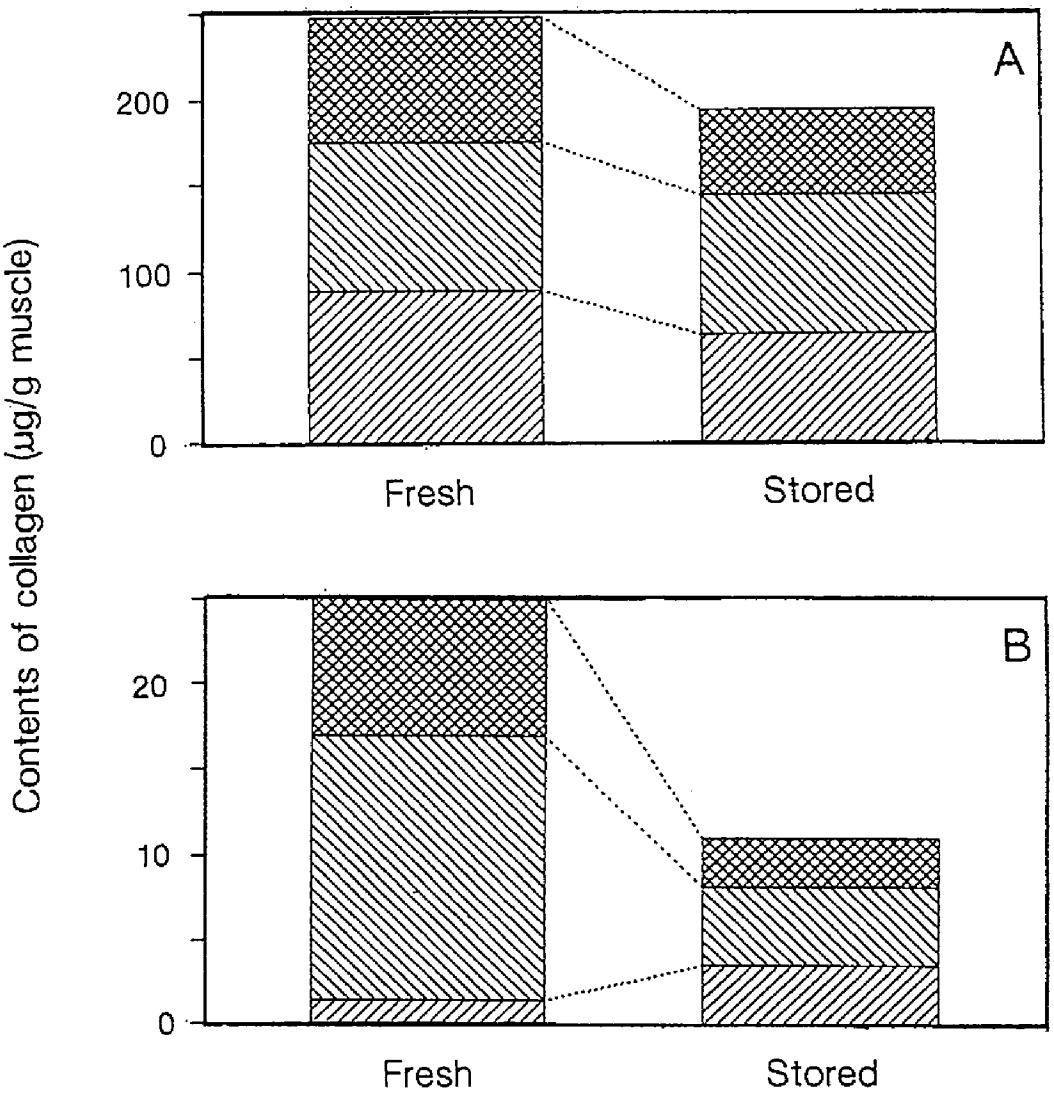

Fig. 39. Solubility of type I (A) and V (B) collagens of sardine muscle. Note that the content of type V collagen is decreased significantly after 1 day-storage. ▨; acid-soluble collagen, ▧; pepsin-solubilized collagen, and ▩; insoluble collagen.

Discussion

In the present study, disintegration of pericellular collagen fibrils and change in type V collagen solubility during storage were examined in sardine and tiger puffer muscles. According to the results which were obtained in this study, tenderized muscle showed disintegration of pericellular collagen fibrils and increase in type V collagen solubility, but non-tenderizing muscle did not. This supports strongly the hypothesis that change in type V collagen cause the disintegration of pericellular collagen fibrils and consequently post-mortem tenderization as shown by Sato et al.[67]

Montero and Borderias[65] have reported that heat soluble collagen increases corresponding to decrease of shear strength value during chill storage in trout (Salmo irideus) muscle. Montero and Mackie[66] have shown an increase of proteolytic activity in parallel to increase of heat soluble collagen in cod (Gadus morhua). In these studies, solubilities of each type of collagen have not been studied yet, but tendency of increasing solubility of collagen is coincided with the results of the present study.

Collagen is stabilized by covalent cross-links which are formed in telopeptide region,[81] and the increase of type V collagen solubility in this study would be due to the cleavage of the covalent bond. According to the result that proteolytic activity increases during storage,[66] the change in collagen stability would be due to action of proteases. Collagen is degradaded by various proteases such as collagenase, elastase,

pepsin, trypsin, and cathepsin L.[71,76] Steiner et al.[63] have shown that the existence of lysosome in high density in connective tissue of coho salmon (Oncorhynchus kisutch) and steelhead trout (Salmo gairdneri). Yamashita and Konagaya[64,76] have suggested that cathepsin L, one of lysosomal proteases, cleaves collagen at non-helical region, and cathepsin L localizes in phagocyte-like cells which exist in pericellular connective tissue in chum salmon (Oncorhynchus keta) muscle. They suggest that proteolytic breakdown of collagen by cathepsin L causes the softening of chum salmon muscle during storage.

Collagen fibrils which constitute myocommata did not disintegrate even in the sardine muscle (Fig. 32). The thickness of pericellular collagen fibrils is 20-30 nm, and that of myocommata is 30-40 nm. On the contrary, in case of tiger puffer muscle, the thickness of both of them is approximately 40 nm (Fig. 33). Adachi et al.[80] have shown that the more type I collagen is contained, the thicker is the collagen fibril diameter. Accordingly, the pericellular collagen fibrils of sardine muscle would have less content of type I collagen, and those of tiger puffer muscle have relatively high content of type I collagen which is stable during storage. Possibly, this results from the difference in stability of the pericellular collagen fibrils during storage.

It has been reported that breaking of myofibrils or degradation of alpha-connectin would cause post-mortem tenderization.[29,31,32] In the present study, however, the breaking of myofibrils and muscle fibers after compression were

not observed even after 24 h-storage, suggesting that myofibrils and alpha-connectin have little effect on change in firmness in early period of chill storage.

CHAPTER 4

Distribution of Type I and V Collagens in Rainbow Trout Muscle

The disintegration of collagen fibrils which localizes in pericellular connective tissue causes the post-mortem tenderization of fish muscle (CHAPTER 2). It has been clarified that collagen fibrils in pericellular connective tissue consist of type V collagen in mammalian and avian muscles by immnohistochemical techniques.[49,70] Type I and type V collagens have been reported so far[68,69] in fish muscle. However, there are no informations concerning the distribution of the two types of collagen molecules in fish muscle.

The objectives of the present study are to elucidate the distribution of type I and V collagens in fish muscle by immunohistochemical techniques and clarify the molecular species which consist of disintegrated collagen fibrils.

Materials and Methods

Fixation

Live rainbow trout was decapitated and its dorsal muscle was cut into small pieces (3 x 3 x 5 mm). These pieces were fixed in 0.8 % paraformaldehyde and 0.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 3 h at 5 ^{o}C.

Immunofluorescence microscopy

After fixation, the samples were dehydrated in ethanol

series, and embedded in paraffin at 62 ^{o}C. Paraffin sections of 7 microns in thickness were prepared by microtome (Nakamura Works Co.). The sections were deparaffined, and prepared by non-fat milk (Block Ace, Dainippon Pharmaceutical Co.) for 30 min to block the non-specific binding sites. The sections were reacted with primary antibody (100 µg/ml) for 1 h. After washing by phosphate-buffered saline (PBS, pH 7.2) for 30 min, the sections were incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Zymet) diluted at 1:100 with PBS for 30 min. After washing by PBS for 30 min, the sections were mounted in glycerin-PBS and coverslipped. The sections were examined and photographed by using a fluorescence microscope (BH2-UCD, OLYMPUS).

Immunoelectron microscopy

After fixation, the samples were cut into smaller pieces (1 x 1 x 3 mm), dehydrated with ethanol series, and embedded in epoxyresine (Epok 812, Ohken Co.). Ultrathin sections were prepared by ultramicrotome (MT-6000, LKB). The sections were mounted on carbon-coated formval films on nickel grids, etched by 10 % H_2O_2 in methanol for 60 min, and floated sequentially on drops of PBS (10 min), non-fat milk (Block Ace, Dainippon Pharmaceutical Co.), PBS (10 min x 3), primary antibody (overnight at 5 ^{o}C), PBS (10 min x 3), gold-conjugate goat anti-rabbit IgG (Zymet, 20 nm in diameter, 5 h at 5 ^{o}C). The grids were stained with uranylacetate and leadcitrate, and then observed by using a TEM (H-800, Hitachi) with an accerating voltage of 100 kV.

Results

Immunofluorescence microscopy

Figures 40 and 41 show the distribution of type I and V collagens respectively by immunofluorescence microscopy. Myocommata was stained with anti-type I collagen antibody seriously (Fig. 40A, arrow), and pericellular connective tissue was also stained at places (arrowheads). On the other hand, non-immune rabbit serum did not react to myocommata (Fig. 40B, arrow). Anti-type V collagen antibody stained pericellular connective tissue (Fig. 41A, arrows), but did not stain myocommata (Fig. 41A, arrowhead). These results suggest that type I collagen exists mainly in myocommata, and type V collagen exists in pericellular connective tissue.

Immunoelectron microscopy

Type I collagen was present in the collagen fibrils of myocommata (Fig. 42A). In myocommata, except for collagen fibrils, fibroblast-like cells (arrow) and non-collagenous structure (arrowhead) were observed (Fig. 42A), but no immunolabelling was observed on them. In the higher magnified observation (Fig. 42B), many gold particles existed on the collagen fibrils, but the particles were scarcely observed on muscle fiber (M). The signal intensity in pericellular connective tissue was inferior to that in myocommata (Fig. 42C). On the other hand, type V collagen localized in pericellular connective tissue (Fig. 43A). Faint signal was also observed on myocommata (Fig. 43B).

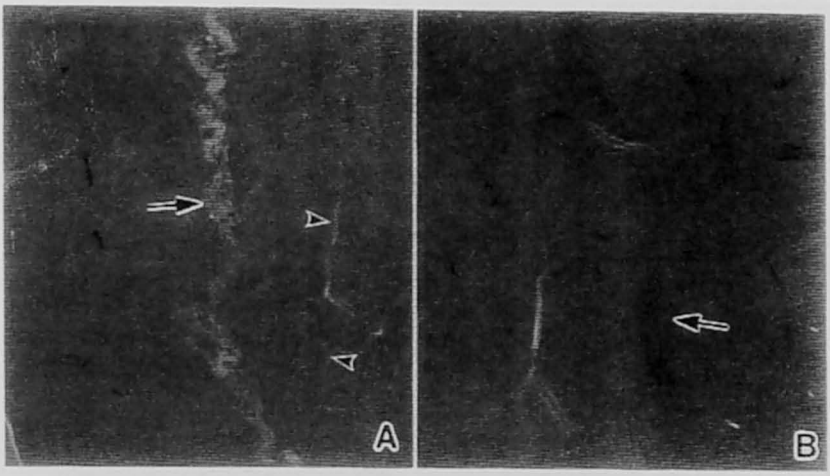

Fig. 40. Immunofluorescence microscopy by using anti-type I collagen antibody (A). Myocommata is stained seriously (arrow). In addition, pericellular connective tissue is also stained at places (arrowheads). (B) Control staining by using non-immune rabbit serum. Myocommata is not stained (arrow).

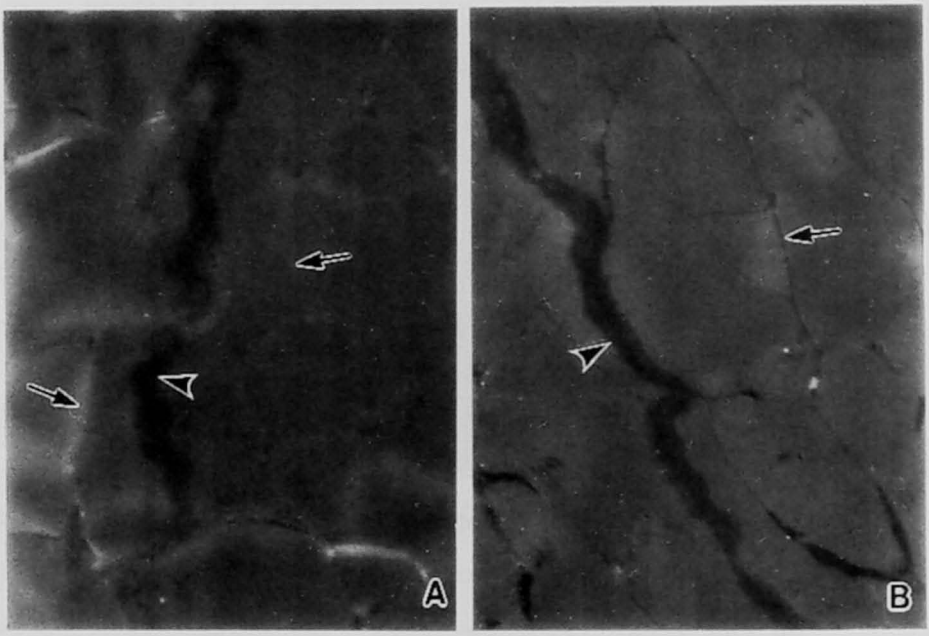

Fig. 41. Immunofluorescence microscopy by using anti-type V collagen antibody (A). Myocommata is not stained (arrowhead). On the other hand, pericellular connective tissue is crossreacted to the antibody (arrows). (B) Control staining by using non-immune rabbit serum.

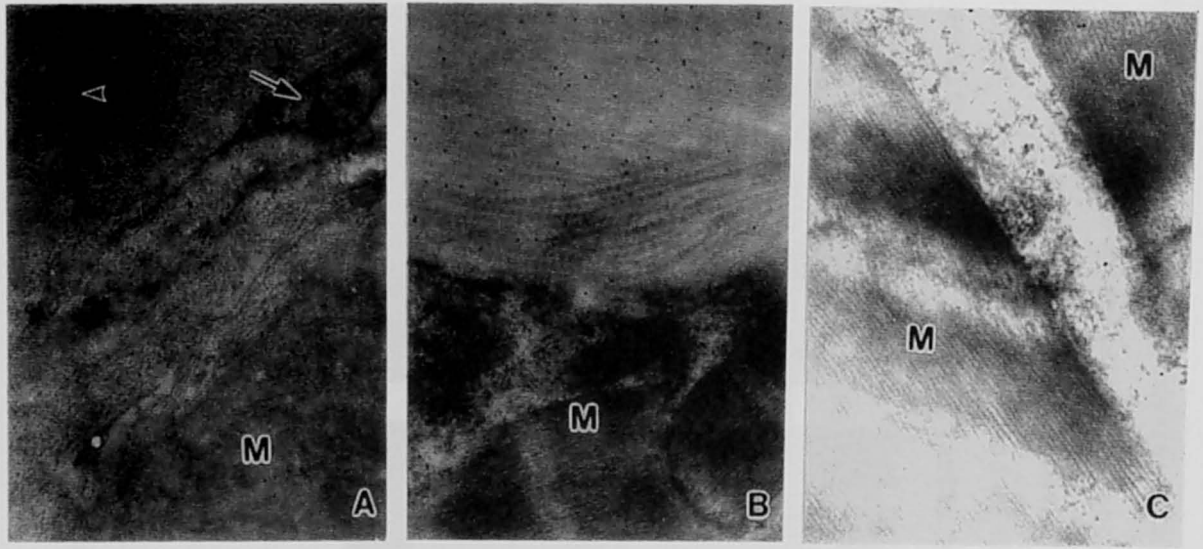

Fig. 42. Immunoelectron microscopy by using anti-type I collagen antibody. In myocommata, fibroblast-like cell (arrow) and non-collagenous structure (arrowhead) are exist. Type I collagen distributes in the collagen fibrils of myocommata (B). In the pericelluar connective tissue (C), the cross-reactivity is lower than that in myocommata.

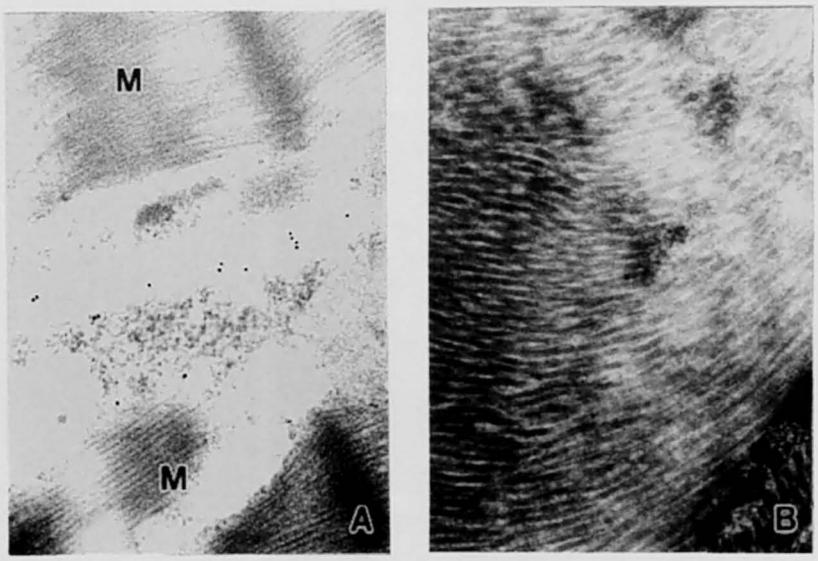

Fig. 43. Immunoelectron microscopy by using anti-type V collagen antibody. Higher cross-reactivity is observed in the pericellular connective tissue (A) than myocommata (B).

Statistical analysis

According to the results obtained by immunoelectron microscopy, distribution density of gold particles were summarized in Table 6. The cross-reactivity of antibody against type I collagen to myocommata was as about 8 times as that to pericellular connective tissue. On the other hand, antibody against type V collagen crossreacted with pericellular connective tissue specifically, and the reactivity was as seven times as that to myocommata. These differences were statistically significant, indicating that type V collagen principally localizes in the pericellular connective tissue.

Table 6. Density of gold particles (particles/μm^2)

	Pericellular connective tissue	Myocommata	Myofiber
Type I	1.72±0.89	13.63±1.81	0.67±0.36
Type V	7.02±2.99	1.00±0.61	0.57±0.27

Each value is evaluated by 8-10 photographs (avarage±S.D.).

Discussion

As described in CHAPTER 2, the tenderization of fish muscle during storage is caused by weakening the pericellular connective tissue, which results from the disintegration of collagen fibrils. According to the results of the present study, it was clarified that the disintegrated collagen fibrils were mainly formed from type.V collagen. This finding fact suggests that some changes which occurred in type V collagen molecule produce the disintegration of pericelluar collagen fibrils. It has been demonstrated that solubility of type V collagen increases during chill storage.[67] This was also mentioned in Section 2 of CHAPTER 3. The increase in of the solubility would be produced by cleavage of intermolecular cross-links, and/or non-helical region.[67] Possibly, the degradation of a limited portion of type V collagen molecule results in the disintegration of collagen fibrils.

The molecular species-specific distribution of collagen in muscle has been reported in mammalian and avian.[49,70] In those studies, type I collagen was found to distribute in epimysium and perimysium, and type V in pericellular connective tissue. In case of shrimp muscle, minor component AR-(II) exists in pericellular connective tissue.[82] The results of the present study agree well with those of these studies.

There is a possibility that type I collagen also localizes in the pericellular connective tissue because the cross-reactivity of anti-type I collagen antibody to the pericellular connective tissue is higher than that to myofibrils, which would

be due to the non-specific reaction (Table 6). Birk et al.[83,84] and Doane et al.[85] have reported that type I and V collagens make hybrid fibrils. From the results of the present study, however, it is unknown whether the pericellular collagen fibrils are hybrid fibrils.

Increasing the proportion of type V collagen progressively allows to decrease in the diameter of heterotypic type I + V fibrils.[80,83-85] In the present study, the diameter of collagen fibrils in myocommata was found to be 40-50 nm, two times bigger than that of pericellular collagen fibrils. The difference in fibril diameter would also be due to the proportion of type V collagen.

Birk et al.[83] have suggested that type V collagen is covered with type I collagen, and the type V is exposed by degradation of the type I in corneal stroma of chick embryo. It has been also shown that helical domain of type V collagen is buried in type I collagen, and only the NH_2-terminal domain is exposed on the surface of collagen fibrils by using a monoclonal antibody which is directed against amino-terminal domain.[85] The crossreaction of the anti-type V collagen antibody to myocommata of the present study would be due to the same arrangement of collagen molecules. To clarify the reason why the collagen fibrils in myocommata are stable during storage (CHAPTER 3 Section 2), a study on molecular arrangement in the collagen fibrils is needed.

SUMMARY AND CONCLUSIONS

CHAPTER 1

Section 1:

The breaking strength of muscle was measured by a puncture test to evaluate quantitatively change in fish muscle firmness during storage The correlation between sensory and puncture tests for evaluating muscle firmness was also studied according to the rank correlation coefficient. The breaking strength of rainbow trout muscles at 0, 12, 18, 24, 48, and 72 h after death was measured, and sensory evaluation for the firmness of the same muscle was performed. The rank correlation coefficient between the two firmness orders determined by the sensory and the puncture tests was extremely high (0.961), strongly suggesting that the puncture test was valid to evaluate the change in muscle firmness during storage.

Section 2:

To clarify the correspondence between the textural change of muscle and rigor mortis, changes in rigor index and breaking strength of muscle were examined on plaice, parrot bass, yellowtail, carp, red sea-bream, striped grunt, tiger puffer, and rainbow trout. Upon storage at 5 ^{o}C, most fishes reached maximal rigor within 24 h after death. The maximal rigor continued within at least 72 h after death and no resolution of rigor was observed within this period. Meanwhile, the breaking strength of muscle of all the fishes tested except for tiger puffer decreased sharply within 24 h after death and then decreased gradually, although

the values were markedly diverse among the fish species. These findings suggested that fish muscle tenderization proceeded independently with the rigor regardless of fish species.

CHAPTER 2

Section 1:

A histological study using a newly devised compression test was carried out to clarify the mechanism of the post-mortem tenderization of rainbow trout (Oncorhynchus mykiss) muscle. The integrity of the pericellular connective tissue structure is suggested to be very important for the firmness of fish muscle. During chill storage, the gradual disintegration of the pericellular connective tissue structure was clearly demonstrated by the light microscopic observations after compressing, while little change was observed without it. It is, thus, suggested that the post-mortem tenderization of fish muscle would be closely related to the gradual disintegration of the pericellular connective tissue structure after death.

Section 2:

To determine the structural change involved in the post-mortem tenderization of fish muscle, histological study was performed for yellowtail, flatfish, red sea bream, and tiger puffer. After decapitation, each fish was stored at 5 ^{o}C and change in breaking strength was measured. Histological observation was performed by using a light microscope for the stored muscles after compression at 100 g/cm^2 for 10 sec. Muscle

tenderization was demonstrated for yellowtail, flatfish, and red sea bream, but not for tiger puffer. Detachment of muscle fibers by compression was observed dependent the storage time for yellowtail and flatfish. In case of red sea bream, compression condition employed in the present study was so strong that the detachment of muscle fibers was demonstrated even immediately after death. In case of tiger puffer, in contrast, the detachment of muscle fibers after compression was hardly observed at 72 h after death. These results suggest that the weakening of the pericellular connective tissue detected by compression causes tenderization of these fish muscle.

Section 3:

Collagen fibrillar network was observed by using cell-maceration/SEM method combined with measuring breaking strength of rainbow trout, yellowtail, and tiger puffer muscles. Rainbow trout and yellowtail muscles tenderized during storage, but tiger puffer muscle showed no tenderization even after 72 h-storage. According to the observations of collagen fibrillar network, thickness of pericellular connective tissue was thinner and density of collagenous fibrils was lower during 24 h-storage compared to fresh muscles in rainbow trout and yellowtail. On the other hand, no structural change was observed for 72 h-storage in tiger puffer muscle. These results show that the structural change in collagen fibrillar network was corresponded to the post-mortem tenderization. This agrees well with the results obtained by the LM and TEM observations.

Section 4:

Change in fine structure of rainbow trout muscle during

storage at 5 ^{o}C was examined using a transmission electron microscope with correlating the results to the change in the breaking strength of muscle. The value of breaking strength decreased within 24 h-storage after death. At the same time, collagen fibrils in the pericellular connective tissue were disintegrated, while no evident breakdown of Z-disc was observed. According to these results, it was suggested that post-mortem tenderization of fish muscle during chill storage was due to the disintegration of collagen fibrils in the pericellular connective tissue rather than the weakening of Z-discs.

CHAPTER 3

Section 1:

Determination of free and peptide forms hydroxyproline contents was performed to detect the degradation of collagen in rainbow trout, yellowtail, sardine, and tiger puffer muscle. In rainbow trout and yellowtail muscles, hydroxyproline contents in free form increased after one day storage. Hydroxyproline in peptide form increased in sardine muscle. On the other hand, in tiger puffer, neither free nor peptide forms of hydroxyproline increased. The results of the present study would support a hypothesis that post-mortem tenderization of fish muscle was caused by the degradation of collagen fibrils.

Section 2:

Changes of firmness, muscle structure, and collagen solubility were studied in sardine and tiger puffer muscles

during chill storage. In case of sardine muscle which showed notable tenderization after 1 day storage, weakening of pericellular connective tissue, disintegration of pericellular collagen fibrils, and increase of type V collagen solubility were recognized. In contrast, tiger puffer muscle showed no tenderization, and additionally, no differences in muscle structure and collagen solubility were detected during one day chill storage compared with the muscle of immediately after death. These results suggested that fish muscle tenderization would result from the weakening of the pericellular connective tissue caused by the change of type V collagen, and that post-mortem tenderization would not occur in tiger puffer muscle because there were no changes observed in type V collagen.

CHAPTER 4

Distribution of type I and V collagens in fish muscle was studied by immunohistological techniques using anti-type I and V collagen polyclonal antibodies. Type I collagen localized mainly in myocommata, and partially in pericellular connective tissue. On the other hand, type V collagen scarcely localized in myocommata, but existed in pericellular connective tissue with a specificity. These results indicated that collagen fibrils which caused the weakening of the pericellular connective tissue during storage mainly consist of type V collagen.

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