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INFLUENCE OF BLEEDING ON POST-MORTEM TENDERIZATION OF FISH MUSCLE DURING CHILLED STORAGE

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Abstract

The influence of bleeding on post-mortem tenderization of fish muscle during storage was studied. Six fish species were used for the present study. Among them, delay of post-mortem tenderization of muscle by bleeding was shown in the pelagic fishes: yellowtail, horse mackerel, and striped jack. On the other hand, bleeding had no influence on the muscle firmness of the bottom fishes: red sea bream, flatfish, and rudder-fish. According to transmission electron microscopy, degradation of pericellular collagen fibrils was delayed in bled yellowtail and horse mackerel. In the case of striped jack, though, collagen fibril degradation could not be observed; slower weakening of the pericellular connective tissue was detected by compression tests on the bled sample. On the contrary, no structural difference was observed between the tested and the control samples in the bottom fishes during storage. According to these results, the delay of muscle tenderization of pelagic fishes would be due to the outflow of a collagenolytic factor contained in blood.

Key Words: Collagen, connective tissue, bleeding, fish, muscle, firmness, hydroxyproline.

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Introduction

Firmness of raw fish muscle is an important index of freshness. But, fish muscle firmness decreases rapidly after death during chilled storage [2, 3, 4, 5, 6, 7, 13, 16, 17, 18, 21, 23, 24]. Because the tenderization of muscle means a deterioration in quality, understanding the muscle tenderization mechanism and developing a method for preventing it are important for fisheries.

It has been reported that the killing procedure of fish influences the tenderization rate [8, 16, 18]. Leaving the fish in the air, dipping it in cold sea water, and instant killing were compared with regard to the change of muscle firmness, and instant killing was the best for maintenance of muscle firmness in the case of yellowtail and jack mackerel [16, 18]. In addition, killing by electrical shock is as effective as the killing by cutting of the spinal cord with regard to changes in the texture of rainbow trout muscle during storage on ice [8]. In Japan, when fish is killed by a blow to the head, one set of gill arches and tail are cut to bleed the fish. It is generally accepted that bleeding can maintain muscle firmness during storage, but there are no studies on this effect.

Firmness of raw fish muscle has a significant relationship with the content of collagen [14, 19]. In addition, post-mortem tenderization of fish muscle is reported to be caused by weakening of the pericellular connective tissue and the disintegration of collagen fibrils [4, 5, 6, 7]. Disintegration of collagen fibrils has also been observed in the gaping fish muscle (gaping, the deterioration of fish muscle quality as a food, is a phenomena that occurs in the stored fish muscle) [10, 11, 12]. Collagen is an important factor for fish muscle firmness, but there are no reports about the influence of the killing procedure on the fish muscle collagen during storage.

In the present study, we investigated the influence of bleeding on muscle firmness, and made a histological observation on the morphology of collagen fibrils of the pericellular connective tissue.

Materials and Methods

Samples

Yellowtail (39-50 cm, 1.5-2.7 kg), horse mackerel

(17-20 cm, 100-140 g), striped jack (32-33 cm, 740-850 g), red sea bream (30-36 cm, 750-1400 g), flatfish (30-35 cm, 580-950 g), and rudder-fish (23-24 cm, 450-500 g) were purchased from a fish store in Nara, Japan. All fish were cultured fish and alive when purchased. Bleeding was done as follows: the fish were spiked through their brains, and one set of gill arches in each was cut for bleeding (tested sample). In the control samples, only a spike was put through the brain, but the fish were not bled. For each species, both the tested and the control groups consisted of three individuals. When the bleeding was done, outflow blood was collected in a test tube and the volume was measured. After the fish were killed, they were packed in polyethylene bags and stored at 5°C.

Evaluation of muscle firmness

To investigate the post-mortem change of muscle firmness, the breaking strength of muscle was measured as previously reported [5]. Briefly, a slice (10 mm in thickness) was excised perpendicular to the orientation of muscle fibers from the dorsal muscle of stored fish. A cylindrical plunger (3 mm in diameter) was pierced into the slice parallel to the orientation of muscle fibers at a speed of 60 mm/min, and the maximum force which was recorded by a rheometer (RT-1002A, Fudoh, Tokyo, Japan) was regarded as the breaking strength. The breaking strength was measured every three hours until 24 hours after death. Each value was expressed as an average of 5-10 determinations.

Light microscopy

Muscle cubes (5 x 5 x 5 mm) were excised from the dorsal muscle by a blade at three hours interval, and were immersed in 5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and fixed. The fixed samples were dehydrated by ethanol series followed by paraffin embedding. Paraffin sections were prepared by a microtome and they were stained by the Elastica van Gieson stain method. Stained sections were observed with a light microscope (LM, BX50, Olympus, Tokyo, Japan), and the number of blood cells was measured.

In the case of striped jack muscle, to know which cellular portion in the muscle tissue would become weak corresponding to the post-mortem tenderization, light microscopic observation with a compression test [4] was carried out. The compression test was done by compressing a 10 mm muscle cube at 100 g/cm^2 for 10 seconds by a cylindrical plunger (40 mm in diameter) parallel to the orientation of muscle fibers. The compressed muscle cubes were fixed and embedded in the paraffin, and its structure was observed by LM.

Transmission electron microscopy

From the fixed muscle cube, a smaller piece (1 x 1 x 5 mm) was cut and dehydrated in an ethanol series. It was embedded in Epon 812 (Ohken Company, Tokyo, Japan), and ultrathin sections were prepared on an ultramicrotome (MT-6000, DuPont, Wilmington, DE, USA). The sections were stained with uranyl acetate and lead citrate, and observed in a H-800 Hitachi (Tokyo, Japan) transmission electron microscope (TEM) operated at an accelerating voltage of 100 kV.

Determination of total hydroxyproline content in the muscle extract

A piece of muscle (2 g) was excised and homogenized with 20 ml of 80% ethanol. The homogenate was centrifuged at 10,000 *g* for 20 minutes. The supernatant was adjusted to 30 ml by 80% ethanol. These extraction procedures were performed every three hours after the death of the fish. One hundred and fifty microliters of the extract was pipetted in a test tube (6 x 30 mm), and dried in a centrifugal concentrator (CC-181, TOMY, Tokyo, Japan). The precipitate was hydrolyzed with 6 N HCl at 150°C for 1 hour. The hydrolyzate was dried in a centrifugal concentrator; phenyl isothiocyanate derivatives were prepared, the phenylthiocarbamyl-amino acids were separated, and the hydroxyproline (Hyp) content was measured by high-performance liquid chromatography (L-7000, Hitachi, Japan) according to the method of Sato *et al.* [22].

Results

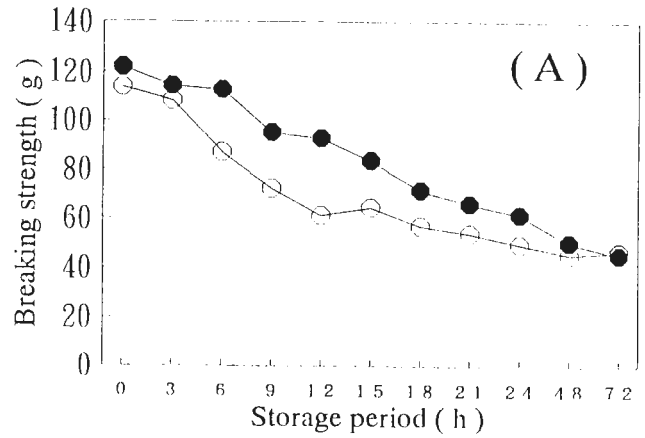
Change of muscle firmness

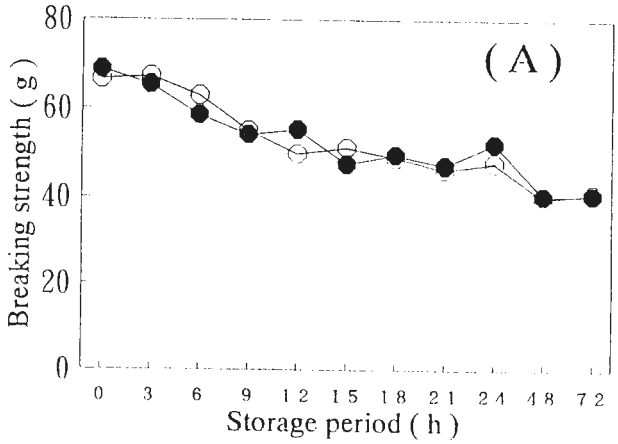
The results of breaking strength are shown in Figures 1 and 2. Figure 1 shows the results of the pelagic fishes: yellowtail, horse mackerel, and striped jack. In the controls of these fish species, muscle firmness decreased continuously during 12 hours of storage. On the contrary, in the experimental (bled) samples, muscle tenderization was delayed for 3 hours in horse mackerel, 6 hours in yellowtail, and 9 hours in striped jack, respectively. These results show that bleeding can delay the tenderization of pelagic fish muscle.

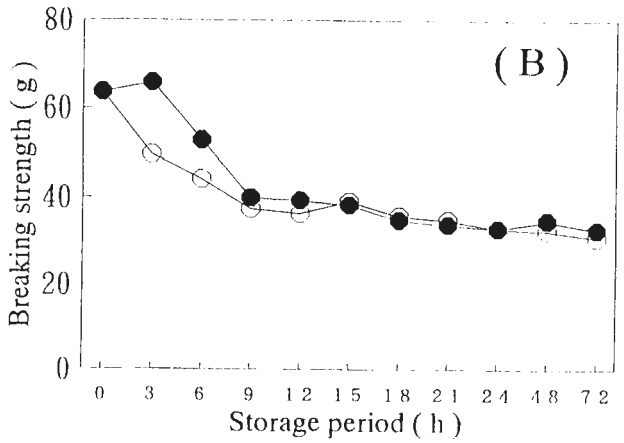
Figure 2 shows the results of the bottom fishes: red sea bream, flatfish, and rudder-fish. In these cases, both the control and the tested samples showed almost the same tenderizing tendency during 24 hour storage. These results show that bleeding has no effect on muscle tenderization rate during storage in the case of bottom fishes. Hence, the effect of bleeding on muscle firmness is specific for pelagic fishes.

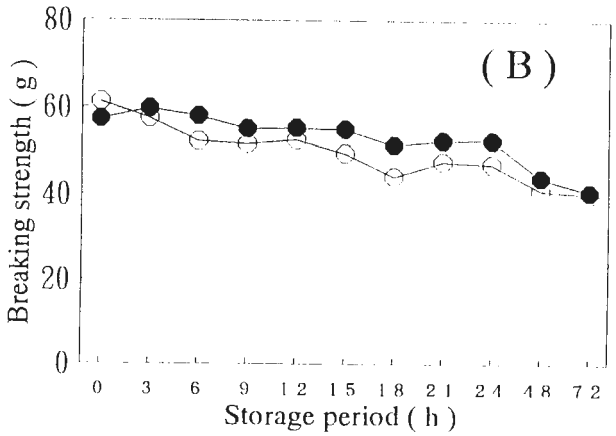
Outflow blood volume

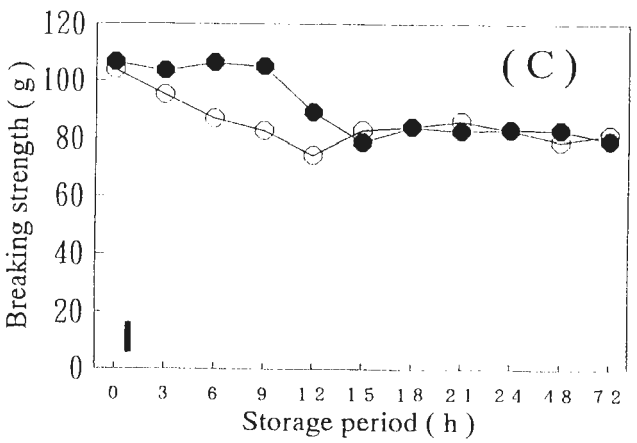
Table 1 shows the volumes of the blood samples which were collected at bleeding. In the pelagic fishes, the volume was 10.3-14.1 ml/kg, much higher than that of the bottom fishes (5.6-8.3 ml/kg). Itazawa *et al.* [15]

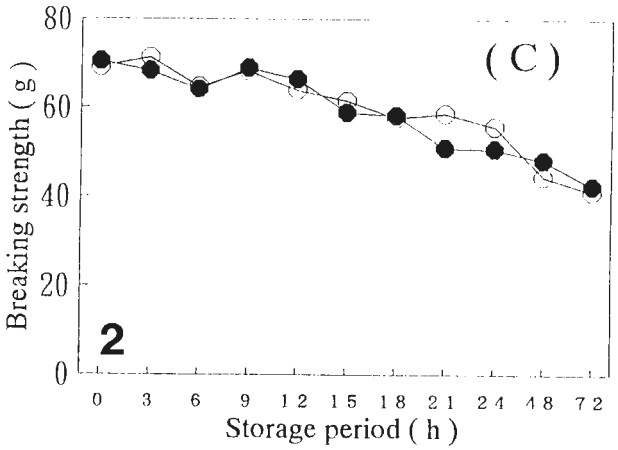












Figures 1 (at left) and 2 (at right). Change of muscle firmness during chilled storage: (**1A**) yellowtail, (**1B**) horse mackerel, (**1C**) striped jack, (**2A**) red sea bream, (**2B**) flatfish, and (**2C**) rudder-fish. Solid circles: tested (bled) fish; hollow circles: control. The breaking strength value was expressed as the average of 7-10 determinations. In each fish specie, the experimental (bled) and the control group consisted of three individuals.

Table 1. Volumes of outflow blood (ml/kg).

Species	Blood volume
Striped jack	14.1
Yellowtail	12.8
Horse mackerel	10.3
Red sea bream	8.3
Rudder-fish	6.4
Flatfish	5.6

Table 2. Numbers of blood cells in muscle (cells/mm^2).

Species	Tested (A)	Control (B)	Outflow rate (%)*
Striped jack	9.8	20.3	57.0
Yellowtail	13.6	27.3	50.2
Horse mackerel	7.3	20.3	64.0
Red sea bream	6.5	11.4	43.0
Rudder-fish	8.1	14.6	44.5
Flatfish	2.4	11.4	78.9

*Outflow rate = {(B - A) / B} x 100%

Table 3. Average diameter of collagen fibrils (nm).

Species	Diameter
Striped jack	17.5
Yellowtail	20.0
Horse mackerel	24.3
Red sea bream	24.0
Rudder-fish	34.3
Flatfish	20.5

reported that the total blood volumes of red sea bream and yellowtail were 53-62, and 33-75 ml/kg, respectively, and on average not significantly different. Nevertheless, in the present study, outflow blood volume in the yellowtail was one and a half times as much as that of the red sea bream. This could be because this fish bleeds easier due to the diameter of its blood vessels and/or because of a difference in the blood content of the internal organs. The specific effect of bleeding on muscle firmness in the pelagic fishes would then be due to the outflow of a muscle tenderizing factor, which is present in blood.

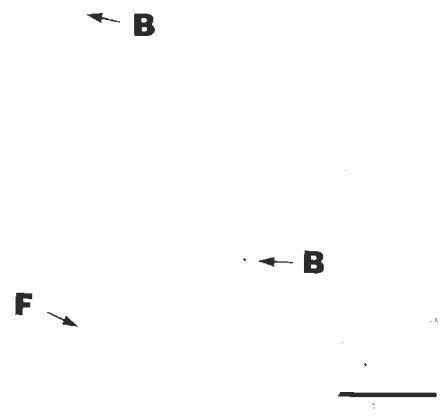


Figure 3. Light microscopy of yellowtail muscle. B: blood cell; F: fibroblast-like cell. No difference was observed between the tested (bled) muscle and the control except for the number of blood cells. Bar = 50 μm.

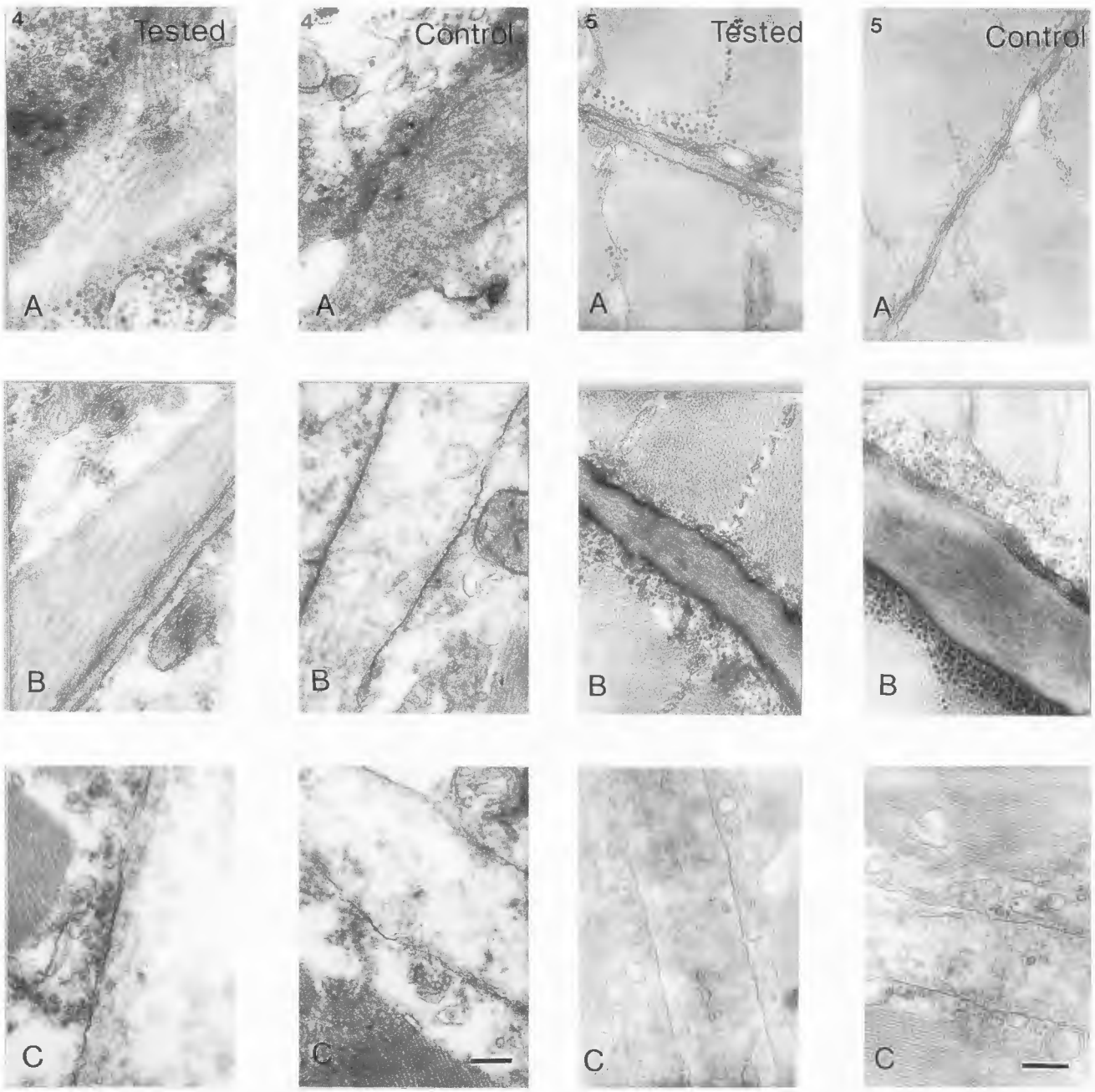
Table 4. Hydroxyproline contents in the extract (nmol/g muscle)

Species	Fresh	72 hour-stored
Striped jack	30.4	31.8
Yellowtail	66.0	65.4
Horse mackerel	55.9	52.1
Red sea bream	43.0	46.0
Rudder-fish	45.8	47.4
Flatfish	23.2	26.8

Histological observations

Yellowtail muscle was observed by LM (Fig. 3). Both a fibroblast-like cell (F) and a blood cell (B) are shown. These structures were virtually the same as in other fishes (data not shown). According to these observations, the number of blood cells was counted (Table 2). The blood cell number of the bled sample was about one-half of that of the control. In the case of the flatfish, the number of blood cells in the bled fish was only about one-fifth of that in the control. These results suggest that more than half the volume of blood in muscle was removed by bleeding. However, except for the blood cell number, no structural differences were observed between the bled and the control samples in conventional LM observations.

Muscle fine structure was observed by TEM (Figs. 4 and 5). Observations were made on the collagen fibrils in the pericellular connective tissue, which has a close relationship to the post-mortem tenderization of



Figures 4 (at left) and 5 (at right). Change of fine structure of pericellular collagen fibrils in yellowtail (**Figure 4**) and in striped jack (**Figure 5**) muscle muscle during chilled storage: (**A**) fresh, (**B**) 6 hour-storage (**Figure 4**) and 9 hour-storage (**Figure 5**), and (C) 24 hour-storage. Bars = 200 nm. **Figure 4.** Especially at 6 hour-storage, collagen fibrils were clearly observed in the tested (bled) fish (**left panel**), but the fibrils were disintegrated and/or disappeared in the control (**right panel**). **Figure 5.** No structural difference was observed between the tested (bled) fish (**left panel**) and the control (**right panel**).

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muscle [5, 7]. In the case of yellowtail (Fig. 4), the fibrillar structure of collagen was clearly observed in the fresh muscle. In the case of the control sample at 6 hours after death, which was already decreased in firmness, almost all collagen fibrils had disintegrated. On the other hand, in the case of the bled sample, collagen fibrils maintained their structure, even after a 6 hour storage. After 24 hours of storage, the collagen fibrils of the bled sample had also disintegrated. Virtually the same results were obtained in horse mackerel (data not

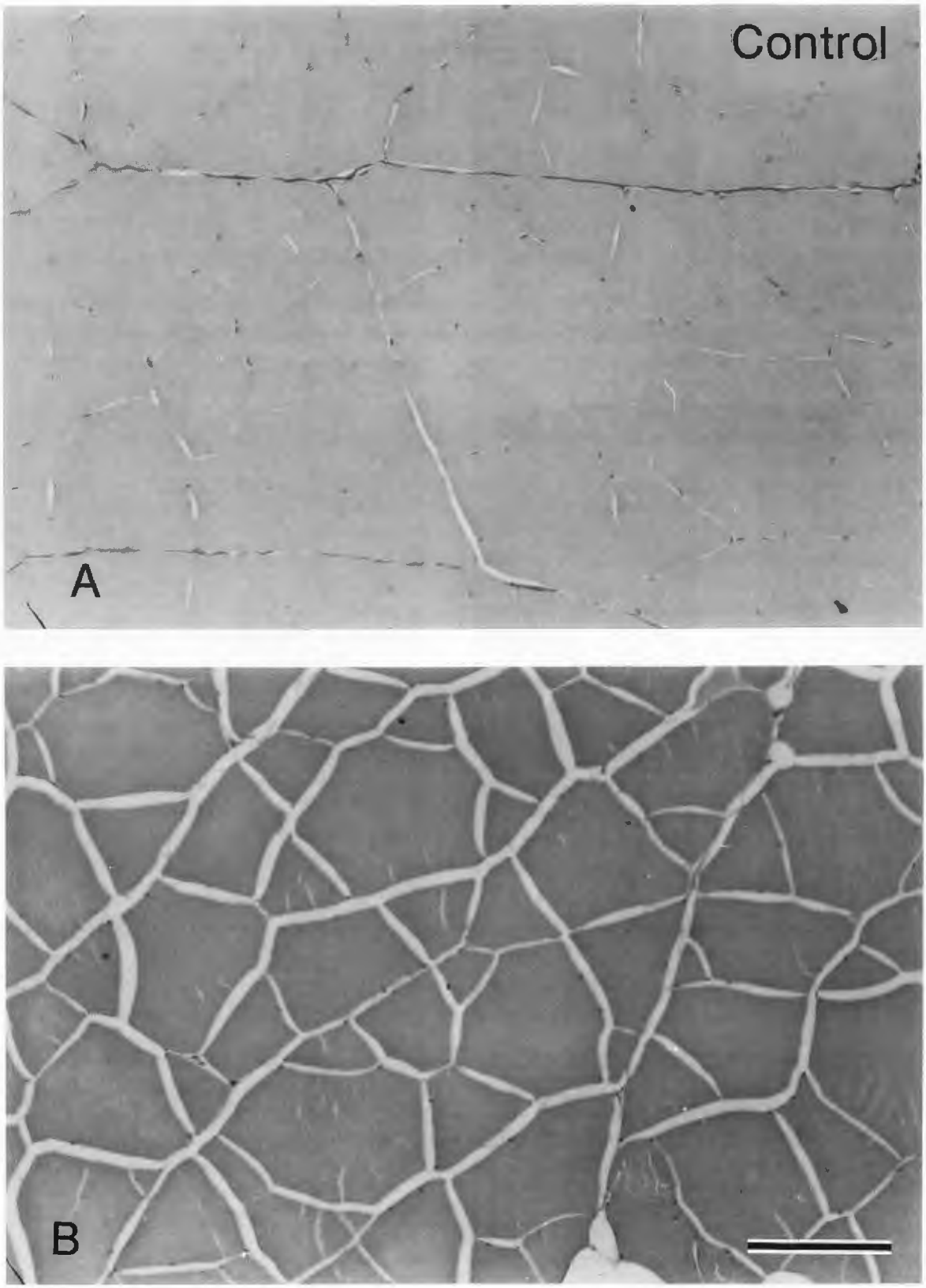
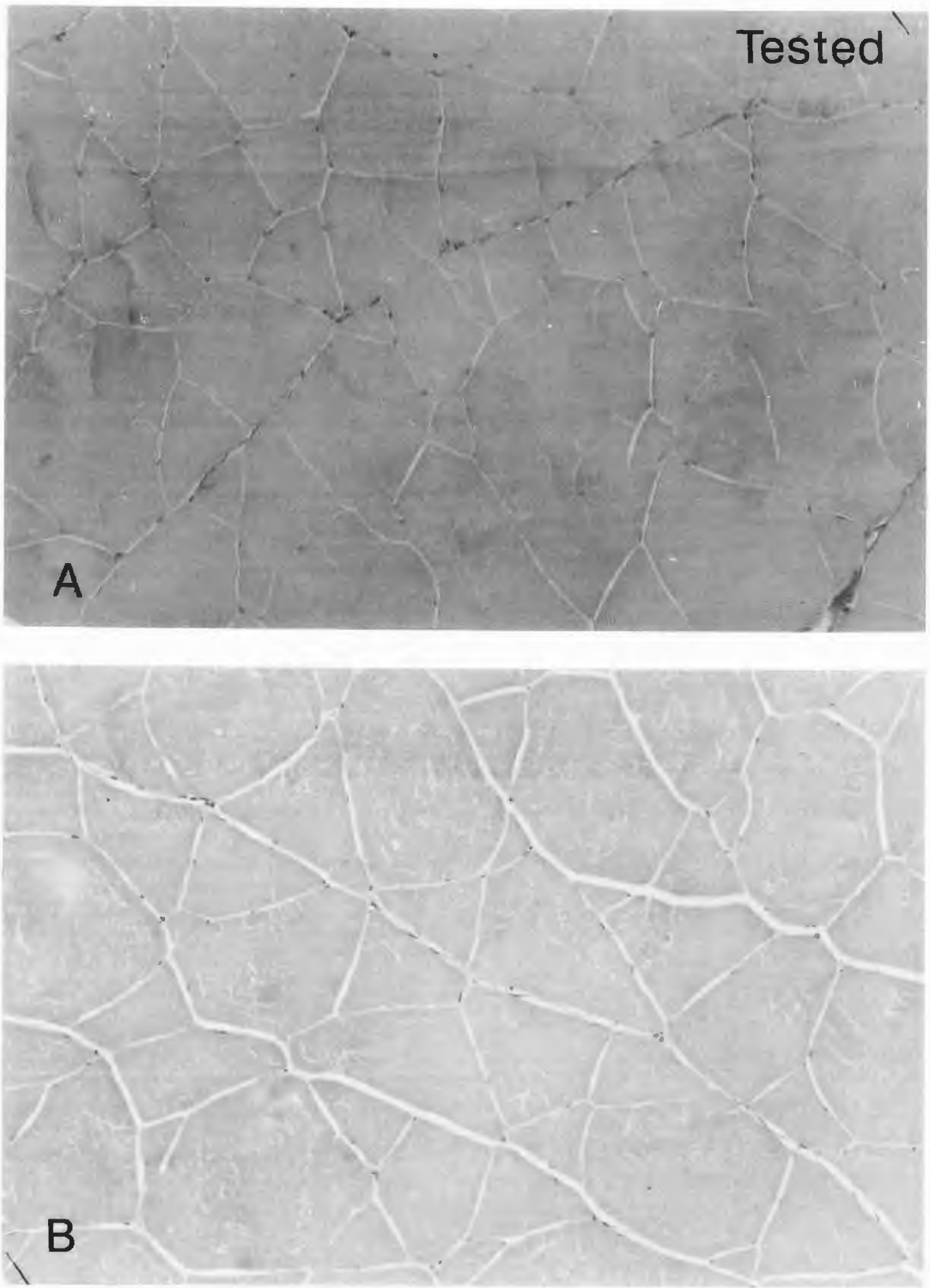


Figure 6 (above and facing page). Light microscopy of the compressed muscle of striped jack: (**A**) fresh, and (**B**) 9 hour-storage. The distance between muscle fibers is larger in the control (**above**) than the tested (bled) fish (**facing**



page) at 9 hour-storage. This result showed that the mechanical strength of pericellular connective tissue became relatively weak and that a larger space was caused by compression in the control sample. Bar = 100 μm.

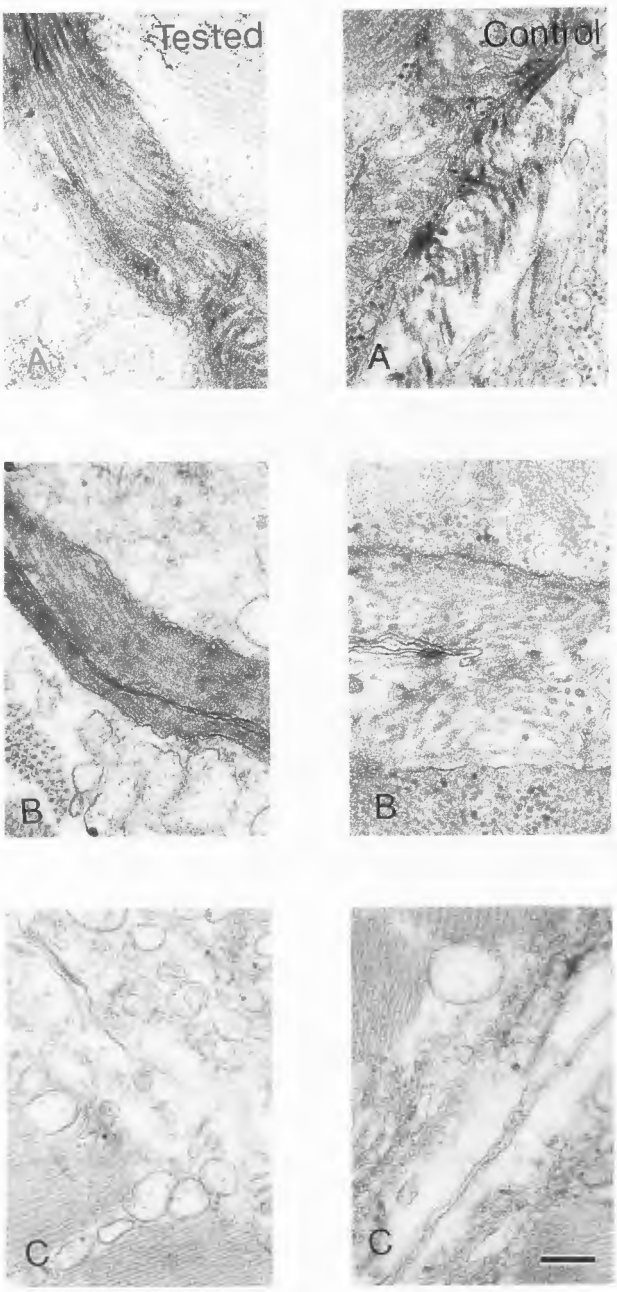


Figure 7. Change of fine structure of pericellular collagen fibrils in control (**left panel**) and tested (**right panel**) red sea bream muscle during chilled storage: (**A**) fresh, (**B**) 6 hour-storage, and (**C**) 24 hour-storage. No effect of bleeding was observed in the muscle fine structure. Bar = 200 nm.

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shown). These results show that collagen fibril disintegration is delayed by bleeding, and this could be the reason why bled muscle is tenderized relatively slowly. In the case of striped jack, collagen fibril disintegration was not observed after 9 hours of storage in spite of the difference in muscle firmness at that time (Fig. 5). Then, the compression test was tried on striped jack muscle (Fig. 6). The distance between muscle fibers was larger in the control than the tested. This result showed that the mechanical strength of pericellular connective tissue became relatively less and a larger space was caused by compression in the control sample. The pericellular connective tissue of the striped jack is very narrow at places (Fig. 5), and it is difficult to observe the pericellular collagen fibril structure. A weakening of pericellular connective tissue in the control sample at 9 hour-storage could occur in such a narrow region.

The results of red sea bream are shown in Figure 7. In both groups, collagen fibril structure was clearly observed until after 6 hours of storage, and it disintegrated with the tenderization of muscle after 6 hours. The disintegration rate of collagen fibrils was not different between the two groups. The same phenomenon was observed in flatfish and rudder-fish (data not shown). This would explain why the tenderization rate was not changed by bleeding.

The diameter of collagen fibril was measured from the TEM observations (Table 3). The sequence of thickness shows a tendency for bottom fish collagen fibrils to be thicker than those of pelagic fish. The variation in collagen fibril thickness could be related to collagen fibril stability.

Change of Hyp content in the muscle extract

Hyp contents in the extract at 0 and 3 days storage are shown in Table 4. No significant changes during storage were detected in any of the fish species. Postmortem disintegration of collagen fibril has been reported to be due to the degradation of type V collagen molecule [21]. The absence of changes in Hyp content may be explained by the fact that degradation of the collagen molecule would occur in a region that does not contain Hyp.

Discussion

In the present study, delay of muscle tenderization and collagen fibril disintegration were caused by bleeding specifically in the pelagic fishes, but not in the bottom fishes. The delay could be caused by outflow of a collagenolytic factor present in the blood. Although there was no difference in outflow blood volume of muscle between the pelagic and the bottom fishes, the rate of collagen disintegration was different. This means that a collagenolytic factor present in the pelagic fish blood is specifically released by bleeding.

The diameter of pericellular collagen fibrils was smaller in the pelagic fishes than in the bottom fishes, according to TEM observations (Table 3). In fish muscle collagen, genetically different molecular species,

type I and type V, have been reported [20]. Type I and type V collagens form heterotypic fibrils, and a higher proportion of type V collagen results in the formation of thinner fibrils [1, 9]. According to these reports, pelagic fish pericellular collagen fibrils (except for flatfish) had a higher content of type V collagen. Type V collagen degradation has been reported to be the cause of post-mortem tenderization of fish muscle because the degradation was parallel to the change of muscle firmness during storage [21]. In other words, muscle which contains much type V collagen has a tendency to be tenderized during storage. The delay of collagen fibril disintegration in the pelagic fishes by bleeding would be due to the outflow of some factor which degrades the type V collagen.

Yamashita and Konagaya reported that softening of chum salmon muscle during spawning migration was caused by cathepsin L which existed in phagocyte-like cells around muscle fibers [25, 26]. In the present study, however, TEM did not show involvement of phagocyte-like cells in the disintegration of pericellular collagen fibrils. A presumed collagenolytic factor would be localized everywhere in the pericellular connective tissue, not only in the phagocyte-like cells. In the serum, many kinds of proteases exist, and it could be speculated that some of these enzymes could participate in collagen degradation.

The Hyp content of the extract is higher in the pelagic fishes than in the bottom fishes (Table 4). A peptide which contains Hyp would be the degradation product of collagen. A larger amount of such a peptide then would mean that collagen degradation activity is relatively high. According to this hypothesis, a higher activity would cause the earlier tenderization of the pelagic fish muscle, and the post-mortem tenderization would be delayed because of the release of the collagenolytic factor. To prove this hypothesis, the factor would have to be purified, and its localization in muscle studied by immunoelectron microscopy.

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Discussion with Reviewers

L. Edelmann: Did you consider investigating post-mortem changes by cryomethods, including X-ray microanalysis?

Author: We indeed have considered using these methods, but at the moment we do not have the necessary equipment in our laboratory. We are now trying to measure the pH in the extracellular region by using fluorescein methods. This would be a very important factor in relation to activities of some enzymes such as cathepsins or collagenase.

L. Edelmann: Did you consider additional bleeding methods to remove more blood immediately after killing of the fish (e.g., by centrifuging)?

Author: No, we did not, because the method which we adopted in the present study is the most common and the easiest one to use in practice. If we would adopt new methods, such as centrifugation, for example, there is a possibility that other factors would influence the post-mortem change of muscle. Therefore, we take care not to do anything which is not common in fishery.