Influence of blood deposition phenomenon on quality of ordinary muscle in yellowtail *(Seriola quinqueradiata)* during storage

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Chapter I General Introduction

1.1 Circulation of flesh fish

Japan is an island nation with a population of 128 million, a land area of 377801 km², a coastline of 29751 km, and an EEZ of approximately 4.05 million km², the sixth largest in the world and about twelve times larger than the national land area. A combination of warm and cold currents flows along the coasts, creating one of the most abundant fishing grounds in the world. (MAFF, 2008)

In recent years, various fish species are distributed as live fish. The main consumption form of these live fish is sashimi. Generally, in the evaluation of the quality of marine products, the most important evaluation items are taste and color, but in the case of sashimi, physical properties are also important in addition to this (Inoue et al., 2000).

Wild fish are harvested by a variety of methods, such as hand-gathering, spearfishing, netting, angling and trapping. Struggling is an inevitable process in the wild fish harvested. In Japan, Purse-seine fishery and trawl fishery were mostly commercial captured fishery. Purse-seines are used in the open ocean, and target dense schools of single-species pelagic fish like tuna and mackerel. A vertical net ‘curtain’ is used to surround the school of fish, the bottom of which is then draw together to enclose the fish, rather like tightening the cords of a drawstring purse. On the other hand, the pelagic trawls and bottom trawls were also used frequently capture method in commercial. Pelagic trawls are generally towed by one or two boats. The target of fish was mid- and surface water fish, such as herring, hoki and mackerel. The path of the boats and trawls

are adjusted according acoustic technology to locate the position and depth of the target fish. Fish that have been Purse-seines and trawled are subject to more stress from fighting the net for hours (Antonio et al., 2011). Furthermore, the fish in the ship transport process was exposed to the condition: suffocation in air or live chilling on ice (Olsen et al., 2006).

In 2016, global fishery reached a peak of about 171 million tons, and farming fishery accounted for 47% of total fishery. (Fig. 1-1)

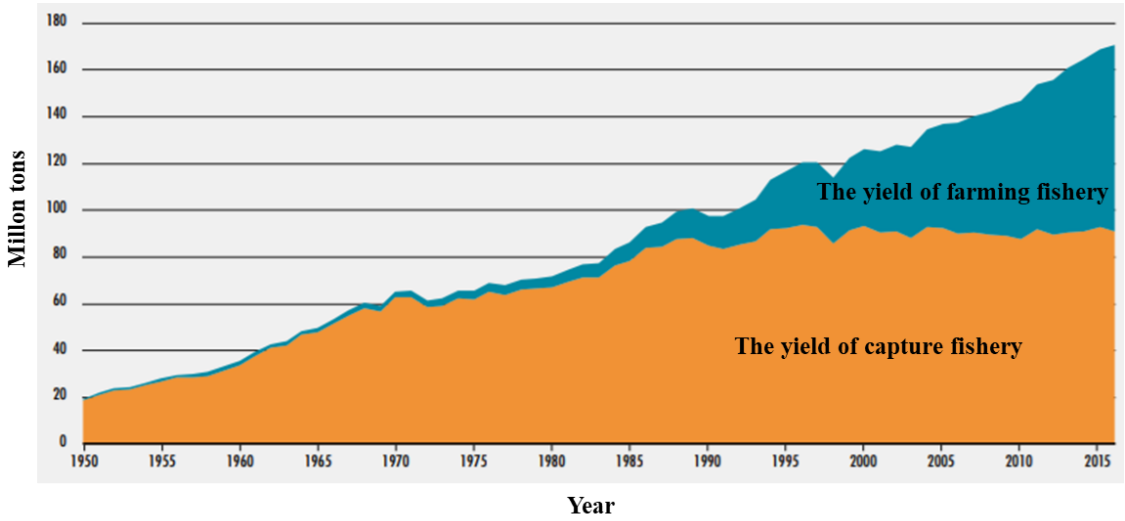

Fig. 1-1 The yield of capture and farming fishery around the world during year 2016. (Modified from FAO, 2018)

In 2016, the global capture fishery production was 90.9 million tons, and the total amount of farming fishery in the world was 79.3 million tons. (Table 1-1)

Table 1-1 The yield of capture and farming fishery production

Year	2011	2012	2013	2014	2015	2016
Yield						
Capture fishery						
Inland	10.7	11.2	11.2	11.3	11.4	11.6
Marine	81.5	78.4	79.4	79.9	81.2	79.3
Total capture	**92.2**	**89.5**	**90.6**	**91.2**	**92.7**	**90.9**
Farming fishery						
Inland	38.6	42.0	44.8	46.9	48.6	51.4
Marine	23.2	24.4	25.4	26.8	27.5	28.7
Total farming	**61.8**	**66.4**	**70.2**	**73.7**	**76.1**	**80.0**
Total capture and farming fishery	**154.0**	**156.0**	**160.7**	**164.9**	**168.7**	**170.9**

(Modified from FAO, 2018)

Fish and fishery products are among the most traded food products in the world today. In 2016, approximately 35% of global fish production entered international trade for human consumption or non-edible forms.

1.1.1 Capture fishery

Capture fisheries resources are highly diverse. FAO landing statistics refer to about 2 500 species or group of species most of which are finfish. By far the most numerous fish species, and those most important to aquaculture and fisheries, are teleosts or bony fish, which in the sea extend from small "grazing" species such as anchovy to large active predatory fish such as tuna. A similarly wide range is also found in freshwater, with the most important species from a production point of view belonging to the carp family. These account for over half the total of inland waters fisheries production. *(FAO, 2019)*

1.1.2 Aquaculture fishery

Aquatic fish farming includes a variety of systems in inland, coastal and marine areas that use and produce a variety of fish species. Aquaculture can be a very productive use of resources, with the amount of food produced per hectare considerably higher than with arable farming or livestock rearing. Aquafeed resources production is one of the fastest expanding agricultural industries in the world, with growth rates in excess of 30 percent per year. Resource availability and use have allowed a more than three times faster sector growth compared with terrestrial farm animal meat production. *(FAO, 2019)*

1.2 The major categories of fishery in Japan

Japanese marine fisheries are divided into three categories: distant-water fisheries (operated mainly on the high seas, as well as under bilateral agreements in the EEZs of foreign countries); offshore fisheries (operated mainly in the domestic EEZ, as well as under bilateral agreements in the EEZs of neighbouring countries); and coastal fisheries (operated mainly in waters adjacent to fishing villages).

1.2.1 Costal fishery

Costal fishery refers to marine fisheries engaged in the territorial sea of 12 nautical miles at sea. Japan mainly use small fishing boats or rafts and use angling, scoop netting, gill nets, boat seine, payao fishing, set net fishing and ground nets for fishing

operations. *(Pearson, 1922)*

1.2.2 Offshore fishery

More than half of Japan's catch is supported by offshore fisheries operating within 200 nautical miles (EEZ) of Japan.

1.2.3 Distant-water fishery

The workplaces (fishing grounds) fishery of distant-water fishery are the world's oceans, such as the Pacific Ocean, the Atlantic Ocean and the Indian Ocean. The fishing method is the “long-line fishery”, which takes tuna, the “bonito single fishing fishery” that uses bonito fishing, and “squid fishing” that catches squid in New Zealand. There are “Fishing Fishery”, “Troll Fishing” and “Overseas Rolling Net Fishing”.

1.2.4 Inland aquaculture fishery

Inland fisheries refer to fisheries and aquaculture that are generally conducted on inland waters such as rivers and lakes. The inland water surface is not only a place for fishery production targeting a variety of freshwater seafood, but also fishing. It is mainly classified into warm water freshwater fish and cold-water freshwater fish. There are a wide variety of hot-water freshwater fish such as carp, goldfish, catfish and eel, but cold-water freshwater fish mainly refers to land-sealed salmonids (amago and rainbow trout).

1.2.5 Mariculture

Generally, fish, shellfish, seaweed, etc. are cultivated using seawater on the coast. This includes pearl culture. The most fish species in marine aquaculture are yellowtail, amber, Thailand, flounder, and striped horse mackerel. The marine farm is less than a 30-minute boat ride from the fishing pier.

1.3 Evaluation for flesh quality

Freshness of fish is very important in terms of distribution and processing, and a wide variety of freshness determination methods are considered. However, none of these methods is complete, and it is important to understand the principle of the freshness judgment index and to adopt the most appropriate method depending on the situation. Freshness determination methods are roughly classified into sensory methods, physiological methods, chemical methods, physical methods, histological methods, microbiological methods and the like *(FAO,2019)*.

sensory test: For the purpose of acceptance, a sensory test that makes an evaluation based on human sense is most suitable. Sensory tests are advantageous in that they do not require extensive equipment, and some test items, such as odors, may far exceed the sensitivity of instrumental analysis. A well-trained group of inspectors will give sufficiently reliable results. Appearance (gloss and color of body surface, presence or absence of eyebrows, eye color, eyebrow color, transparency and color of meat, etc.), odor (such as rotten odor, presence or absence of abnormal odor), hardness (such as stiffness after death), etc. Is the inspection item*(Gill, 1995)*.

Physiological method: As a physiological method, a freshness determination method using spinal reflexes is applied in the market and the like. It is used to determine the state of high freshness immediately after immediate killing, using the phenomenon that when the dabs of stamens of stamens statements are pricked with a hand key, the phlegm works through the spinal cord reflex.

Chemical method: In chemical methods, indicators utilizing intracellular energy metabolism are used. The K value is based on the degradation process of ATP and is an index showing the activity. In chemical methods, there are also indicators based on the involvement of microorganisms. For example, polyamines are produced by the decarboxylation of microorganisms by the elimination of the carboxyl group of free amino acids. The amount of histamine, cadaverine and putrescine produced can be used as a freshness index and a decay index. Volatile base nitrogen derived from ammonia, trimethylamine, dimethylamine and the like is frequently produced by microorganisms and is a rot index *(Saito et al., 1959; Gill, 1995)*.

Physical method: Physical methods include measurement using a texture measuring device, an electrical sensor, a near infrared spectrometer, and a nuclear magnetic resonance apparatus *(Okazaki, 2012)*.

1.4 Muscle proteins of Fish

There are three main groups of fish-muscle tissue protein *(Sotelo et al., 2000)*, structural proteins, sarcoplasmic proteins and connective-tissue proteins. The structure proteins (myosin, actin, tropormyosin, and actomyosin) consisting 70-80% of the total

proteins are soluble only in salt solutions of high ionic strength. The sarcoplasmic proteins, forming approximately 25-30% of the total proteins depending on the fish species, are generally soluble in water or buffers of low ionic strength. Connective-tissue proteins constitute only 3% of the protein in Teleostei and about 10% in elasmobranchii *(Ahn et al., 2013)*.

Fish muscle and tissues are composed of structures like myofibrillar *(Hayes &Flower, 2013)* and collagen proteins *(Xu et al., 2015)*. Myofibrillar proteins occupy the main components which are crucial for maintaining the integrity of texture and mechanical properties of the muscle *(Wang, artinez & Olsen, 2009)*. A transmission electron microscope was used to observe the delay in degradation of pericellular collagen fibrils in six species of bled fishes (yellowtail, horse mackerel, striped jack, red sea bream, flatfish and rudder-fish) *(Ando et al., 1999)*. Their results show that bleeding can delay collagen fibril degradation and muscle softening of pelagic fish, but had no influence on demersal.

Therefore, we believe that blood has an effect on both myofibrils and collagen fibers. We mainly study whether blood sedimentation has accelerated decomposition of these two proteins.

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Chapter II Effect of blood deposition phenomenon on flesh quality of yellowtail *(Seriola quinqueradiata)* during storage

2.1 Introduction

Blood volume in most elasmobranchs and teleost fish is normally 3-7% of the body weight *(Brill et al., 1998; Williams and Wortby, 2002)*. It is widely accepted that in the fish industry, it is necessary to handle bleeding, because the taste of meat can be preserved and improved when the fish is bled *(Ando, Nishiyabu, Tsukamasa & Makinodan, 1999)*. According to *Vanezis & Trujillo (1996)*, blood hypostasis in cadavers was particularly observed during the first 12 h and decreased thereafter. The same phenomenon can be observed in fish, but there is almost no research published on this. When the fish is dead, blood pressure drops rapidly and there is no driving force to support the vascular system for the blood to stay in the arteries, arterioles and capillaries of the dorsal muscle *(Olsen et al., 2006)*. Additionally, gravity pulls the stagnant blood to the lowest points of the fish body and blood migrates into muscle tissues during storage. Therefore, this study aims to investigate the influence of blood deposited in the fish muscle on flesh quality.

Normal biological processes such as cell death, proliferation, migration, invasion and protein turnover are regulated by proteolytic enzymes from five catalytic proteases (aspartic, cysteine, metallo, serine and threonine proteases) *(Mohamed &Sloane, 2006; Roshy, Sloane &Moin, 2003)*. Protein hydrolysates have been gaining greater interest in recent years *(Villamil et al., 2017)*. Cysteine protease such as Cathepsin B and

Cathepsin L which are located in cell membranes and endosomal or lysosomal vesicles are suspected of causing the softening of post-mortem muscle of fish *(Sriket, 2014)*. Cathepsins B, H, and L have been found to cause muscle softening in tilapia *(Sherekar, Gore &Ninjoor, 1988)*, mackerel *(Jiang, Lee &Chen, 1994)*, and in chum salmon. Cathepsin L is a predominant proteinase responsible for autolysis of post-mortem degradation in mackerel *(Aoki &Ueno, 1997)* and arrowtooth flounder *(Visessanguan, Menino, Kim &An, 2001)*. Ahimbisibwe et al. *(2010)* illustrated that cathepsin L was present in the blood of carp, amberjack and red sea bream. Moreover, cysteine proteases in white blood cells from the spleen migrate into fish muscle tissues by blood circulation and influence flesh softening *(Ogata, 2015)*. However, cysteine proteases in muscle have been studied extensively, but the content of cathepsins in fish blood was not been clearly studied. Therefore, the influence of enzyme from fish blood may be a critical issue in fish softening.

In general, aquaculture and fishery industry are important parts of the economy of many countries around the world *(Villamil, Váquiro &Solanilla, 2017)*, especially in Japan, where 86% of the Japanese domestic fishery production are destined for human consumption *(FAO, 2009)*. In a country with a traditional fish eating culture, particularly with an appreciation for raw fish, fish used as raw material must be fresh *(FAO, 2009; Terayamai, Ohshima, Ushio &Yamanaka, 2002)*. Compared with other Asian countries such as China and Korea, Japan is surely a country which most strictly observes customary behavior. Indeed, it is a traditional habit in Japan that when individual species are captured, the fishes are placed with the left side down during

transport, storage and selling. This unilateral placement custom is regarded as an unwritten rule of the industry. As a consequence, we consider that this placement custom may have an influence on the poor flesh quality of the fish resulting from the blood deposition.

In captured fishery (purse-seine fishery and dragnet fishery), it is impossible to immediately and completely remove blood. Therefore, suffocation in air or live chilling on ice are the common killing methods *(Olsen et al., 2006)*. The commercial value of purse-seine fishery and dragnet fishery is less than angling fishery and the price varies greatly when fish enters market circulation. In our study, we examined the influence of blood deposition on the flesh quality of yellowtail during storage. We measured the breaking strength and color a* value of ordinary muscle. SDS-PAGE and western-blot were used to evaluate protein deterioration. Furthermore, cathepsin B and B+L activities in muscle and blood components were also measured.

2.2 *Experiment method*

2.2.1 sample fish and slaughter method

Cultured yellowtail (*Seriola quinqueradiata*) were used as the fish sample. Samples of fish [body weigh 4.45 ± 0.67 kg; body length 66.5 ± 2.3 cm (n =11)] were purchased from fish farmers in Nagasaki prefecture's fisheries cooperative association and transported to the laboratory after spinal-cord destruction with blood removal (SCD) and suffocation in air without blood removal (SA). The fish were stored at 10 ℃ and arranged with the left side of the fish down. Four groups: the upper-side of the SCD

group (SCD-upper), the under-side of the SCD group (SCD-under), the upper-side of the SA group (SA-upper) and the under-side of the SA group (SA-under), were sampled at 4, 24, 48 and 96 h after storage.

2.2.2 Measurement of color a*

The color of 1-cm thick slice of dorsal ordinary muscle was measured using a color chromameter (Minolta CR-200b, Konica-Minolta, Tokyo, Japan). Color variations were obtained as a* for green-red chromaticity (green -60 to red +60). Measurements were recorded in quintuplicates per sample at each sampling time *(Mora et al., 2007)*.

2.2.3 Heme protein extraction and determination

One g muscle samples were immediately frozen in liquid nitrogen, and then homogenized with 9ml buffer (10Mm Tris, 1mM EDTA·2Na, 80mM KCl; pH 8.0) following *Kranen et al. (1999)*. Extraction samples were immersed in buffer (0.7mM KCN, 0.6mM $K_3Fe(CN)_6$, 1mM KH_2PO_4, 1ml TritonX-100; pH 7.0~7.4) for 10min. The supernatants were pooled and determined following *Mark and Herbert (2002).* Total Heme protein concentration was calculated from absorbance at 540nm and 700nm using the following correction for sample turbidity:

$$[H]/(mg/g) = (A540-A700) * 17000/11300 * D * d * V/M * 1000$$

where[H]: heme protein concentration(mg/g); A_{540}, A_{700}: absorbance at 540nm and 700nm; 117000: (g/mol) molecular mass of heme protein; 11300: (g/mol) molar extinction coefficient at 540nm; D: dilution factor; d: light path(1cm); V: muscle extract

volume (liters); M: wet weight (grams) of muscle sample.

2.2.4 Measurement of the breaking strength

The dorsal muscle was cut at a thickness of 10 mm, vertically to the orientation of muscle fibers. We used a cylindrical plunger (diameter 3 mm) to measure firmness. The plunger was inserted into the sliced block, parallel to the orientation of muscle fiber, at a speed of 1 mm/s using a Rheoner model RE-3305s rheometer (Yamaden Co., Tokyo, Japan). The maximum force needed to penetrate the muscle was regarded as the breaking strength (N) *(Mora, Hamada, Okamoto, Tateishi &Tachibana, 2007)*.

2.2.5 Blood cell separation

Blood was drawn from the posterior spinal artery of the fish tail by using a heparinized syringe while the fish was still alive. The blood collected was kept and diluted 1: 9 with 1.35% phosphate buffered saline (PBS).

Heparinized fish blood was extensively washed with 1.35% PBS and centrifuged with discontinuous gradients of lymphocyte separation medium (LSM), resulting in the simultaneous separation of white blood cell, red blood cell and plasma *(Derek, Gillian &David, 1998)*. White blood cells and red blood cells were separated from blood washed in 1.35% PBS three times, respectively. The sample was immediately distributed into a specimen tube containing 1.35% PBS. White blood cells and red blood cells were counted with a Burker-Turk hemocytometer to calculate dilution times.

2.2.6 Protein concentration

Protein concentration was determined and rectified following the method of *Lowry et al. (1951)* using bovine serum albumin as the standard.

2.2.7 Muscle pH and Cathepsin B and B+L activity

In the assay on enzyme activity at room temperature, the pH of 1- cm thick slices of dorsal ordinary muscle were measured using a Testo 205 meter (Testo, Japan). One gram of dorsal ordinary muscle was homogenized with 2 ml of 0.05 M Tris-HCl buffer and then centrifuged. The supernatant was used for measurement of the enzyme activity. Crude enzymes of white blood cells, red blood cells and plasma were extracted and homogenized with 2 ml of 1.35% PBS using a sonicator (Wall industries, Exeter, NH, USA) and the final enzyme preparation was stored at -20 ℃. Cathepsin activity was measured following *Yoshida et al. (2009)*. Cathepsin activities were assayed using Z-Arg-Arg-MCA and Z-Phe-Arg-MCA as substrates (1mM Z-Arg-Arg-MCA and Z-Phe-Arg-MCA in dimethyl sulfoxide). The hydrolysis of Z-Arg-Arg-MCA (PunChem CID: 439707) was indicative of the activity of Cathepsin B, whereas that of Z-Phe-Arg-MCA (PunChem CID: 87282815) represented the activity of cathepsin B+L. The substrate was diluted to 0.25 mM with distilled water before use. The reaction mixture consisting of 200 µl of 0.4 M acetate buffer (pH 5.5~ 6.9) containing 5 mM EDTA,100 µl of 20 mM cysteine and 20 µl of the enzyme solution was adjusted to a total volume of 0.8 ml. The mixture was preincubated at 37 ℃ for 1 min, and re-incubated for 8 min under the same condition after adding 200 µl of 0.25 µM substrate. The reaction was terminated

by adding 1.5 ml of 0.1 M sodium acetate buffer (pH 4.3) containing 0.1 M sodium monochloroacetate. Fluorescence intensity of the released 7- amino- 4-methylcoumarin (AMC) liberated by enzyme hydrolysis was measured by RF-1500 spectrofluorophotometer (Shimadzu Co., Kyoto, Japan) at an excitation wavelength of 380 nm and an emission wavelength of 460 nm.

2.2.8 SDS/polyacrylamide gel electrophoresis and western-blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed following *Leammli (1970)* and modified by Yoshida *(Yoshida et al., 2009)* using a 7.5% to 15% gradient separating gel slab. The muscle sample (0.025 g) was added in 1 ml sample solution (4% SDS, 20% glycerol, 125 mM Tris-HCL (pH 8.0), 0.002% bromophenol blue, 8 M urea, and 10% 2-mercaptoethanol). The homogenate mixture was boiled for 10 min and analyzed by SDS-PAGE. The gel was stained with 0.1% Coomassie brillant blue R-250 in 50% methanol and 10% acetic acid, and destained with 50% methanol and 7.5% acetic acid. After running the poly-acrylamide gel, we transferred the proteins to a PVDF membrane in order to perform an immuno-detection assay *(Lohoefer et al., 2014)*. Mouse anti- myosin heavy chain antibody (1:1000, Developmental Studies Hybridoma Bank, Iowa, America) and rabbit anti-mouse antibody (1:5000, GE healthcare, Chicago, IL, USA) were used in the immunoblot assay. Protein quantification was performed by comparing the band intensities of samples between different groups.

2.2.9 Statistical analyses

All data analyses were performed in the R statistical environment (v.1.1.442). The values of the continuous variables were compared using the nonparametric Mann–Whitney *U*-test. Critical values less than $P < 0.05$ ($^{\#}$), $P < 0.01$($^{\#\#}$), $P < 0.001$($^{\#\#\#}$) were considered statistically significant.

2.3 *Results*

2.3.1 Change in color a*

The change in color a* of the under and upper-sides of the SA and the SCD groups are shown in Fig. 2-1. The color a* of both SA groups was significantly more intense than that of both SCD groups during the 96 h storage. The SA-under was significantly higher than the SA-upper during the 4 - 96 h storage. (Fig. 2-1).

2.3.2 Change in heme protein concentration

The main factor responsible for color change in white fish was residual blood, with color intensity depending on the content and chemical state of hemoglobin (Olsen et al., 2006). Since there was a difference in color a* between SA-upper and SA-under, the total heme levels in the SA-upper and the SA-under ordinary muscles of yellowtail were measured. The total heme content was slightly higher in the SA-under (1.69 ± 0.62 mg / g) than in the SA-upper (1.16 ± 0.31 mg / g) at 4 h storage, and the heme level was consistent with the result of a* at 4 h storage. In our study, the total heme level was also constantly at a higher level in the SA-under than in the

SA-upper during 96 h (Fig. 2-2).

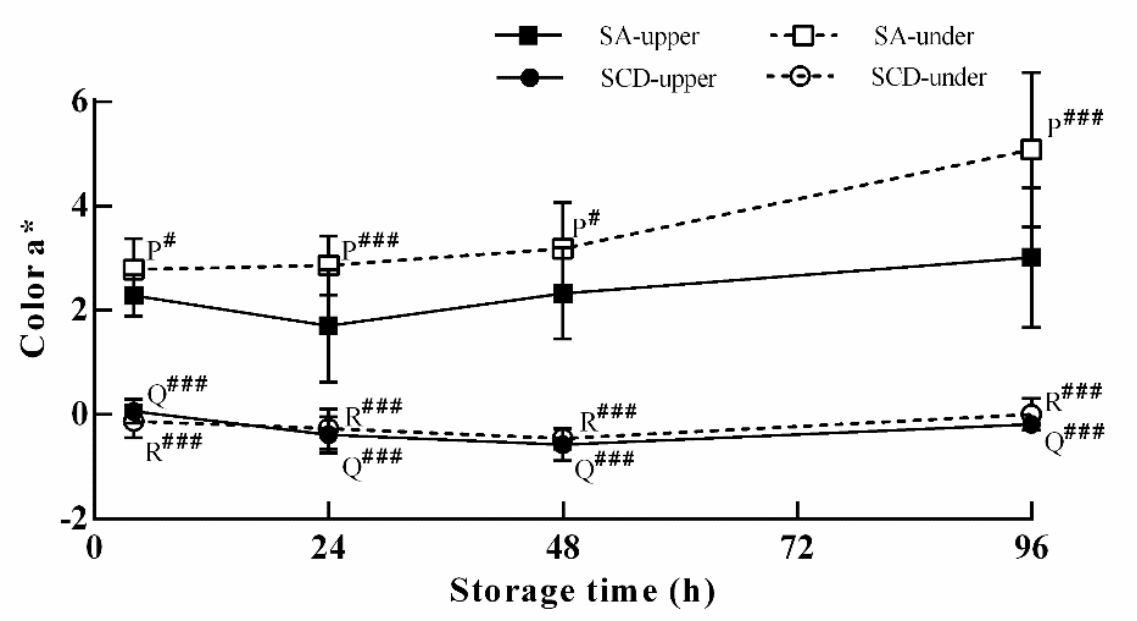

Figure 2-1 The effect of killing method and blood deposition on the color a* of ordinary muscle during storage at 10 ℃. Data are mean ± standard deviation (n =4). $P^{\#}$ ($p<0.05$), $P^{\#\#\#}$ ($p<0.001$) significant difference between the SA-upper and the SA-under at same storage time, $Q^{\#\#\#}$ ($p<0.001$) significant difference between the SCD-upper and the SA-upper at same storage time, $R^{\#\#\#}$ ($p<0.001$) significant difference between the SCD-under and the SA-under at same storage time.

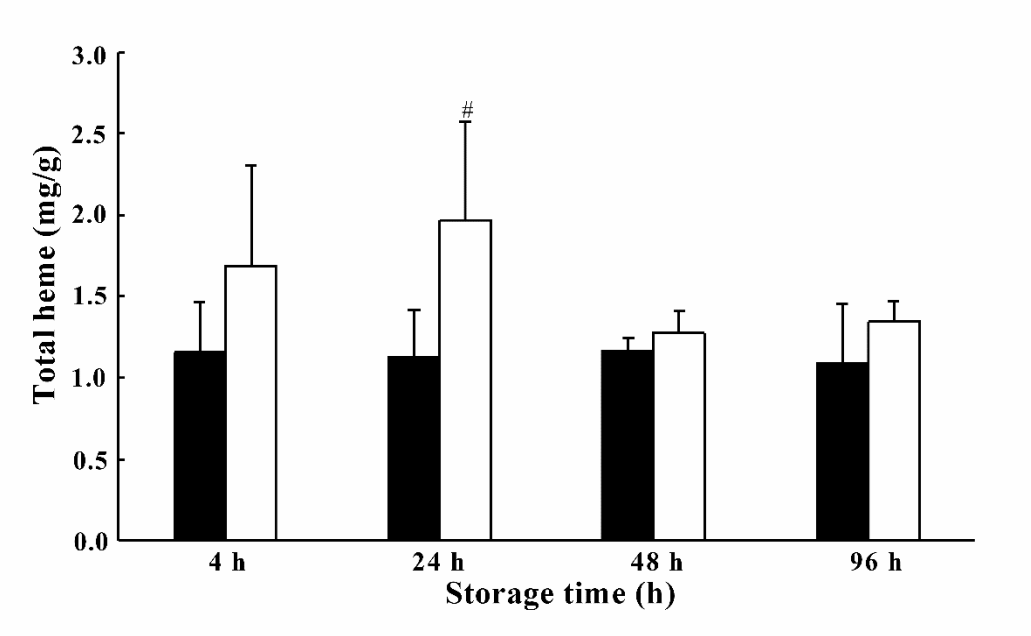

Figure 2-2 The effect of blood deposition on the total heme content of ordinary muscle during storage at 10 ℃. Black bar represents upper-sides. White bar represents under-sides. Data are mean ± standard deviation (n =3). $^{\#}$ ($p<0.05$) significant difference between the SA-upper and the SA-under at 24 h storage.

2.3.3 Change of breaking strengths

The breaking strengths of all groups (1.01 ± 0.11 N in the SCD-upper, 1.06 ± 0.10 N in the SCD-under, 0.79 ± 0.17 N in the SA-upper and 0.72 ± 0.14 N in the SA-under at 4 h, respectively) rapidly decreased during the 24 h storage, and thereafter gradually decreased and reached minimum values of 0.49 ± 0.14 N in the SCD-upper, 0.39 ± 0.08 N in the SCD-under, 0.35 ± 0.04 N in the SA-upper and 0.29 ± 0.05 N in the SA-under at 96 h, respectively. In all experimental groups, the breaking strength was constantly at a lower value in the SA-under than in the other three groups during 96 h storage. As shown in Fig. 2-3, the breaking strengths of the SCD-upper and the SCD-under groups were always higher than those in both SA groups. In the SA group, the value of the SA-upper was higher than the SA-under during the 96 h storage.

2.3.4 SDS-PAGE and western-blot in SCD and SA group

Following the analysis of protein degradation, we determined the amount of myosin heavy chain in the SA and SCD groups at protein level using SDS-PAGE and Western-blot analysis. Coomassie staining revealed a significantly lower intensity of intact MHC (200 KDa) in the SA group than in the SCD group after 4 h storage, and a strong intensity of 20 KDa band in the SA-under after 4 h storage. No other obvious changes were observed (Fig. 2-4 A). Western-blot analysis using antibody against MHC confirmed the results from the SDS-PAGE. The anti-MHC activity was strongly seen at the 200KDa band in both the SCD and SA groups. But the bands at 20-120 KDa of the SCD group were almost not observable. In the SA group, the high intensity of anti

- MHC activity bands were observed at 200 KDa in both the upper and under-sides. At 4 h storage, the bands around 50-120 KDa of the SA groups could be observed (Fig. 2-4 B). The degradation in the SA and SCD groups indicated that blood might have influenced intact MHC. In order to clarify the blood influence on MHC of the upper and under-sides, we observed the bands of the SA groups from 24 h to 96 h storage (Fig. 2-4 C and D). Coomassie staining showed that the intact MHC at 200 KDa had a slight decrease in the SA-upper as compared to the SA-under during storage. The high-density bands between 20-120 KDa were strongly observed in both the upper and the under-sides at 96 h storage. Western-blotting revealed strong anti-MHC activity bands between 50-120 KDa in the under-sides than in the upper-sides at all storage times. Moreover, the thick bands between 30-50 KDa were observed in the SA-under at 48 h, and in the SA-upper and the SA-under at 96 h of storage (Fig. 2-4 D). These results indicate that the under-sides have a stronger degradation than the upper-sides which may have been influenced by the existence of blood during storage.

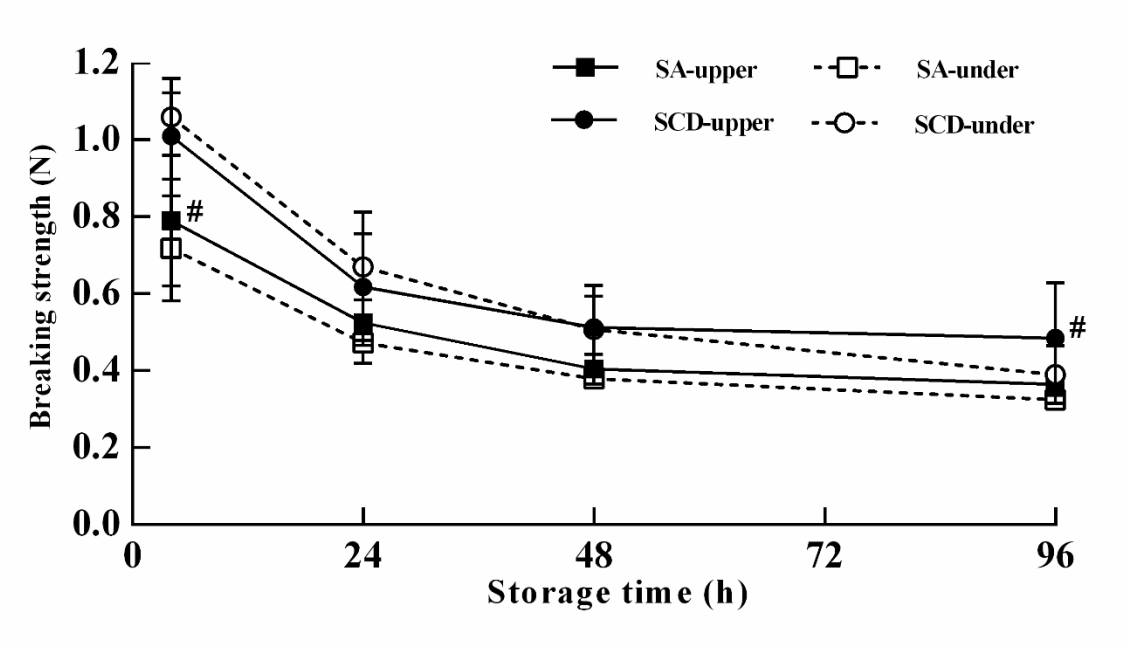

Figure 2-3 The effect of killing method and blood deposition on the breaking strength of ordinary muscle during storage at 10 ℃. Data are mean ± standard deviation (n =4). # ($p<0.05$) significant difference between the SA-under and the SA-upper at 4 h, the SCD-upper and the SCD-under at 96 h.

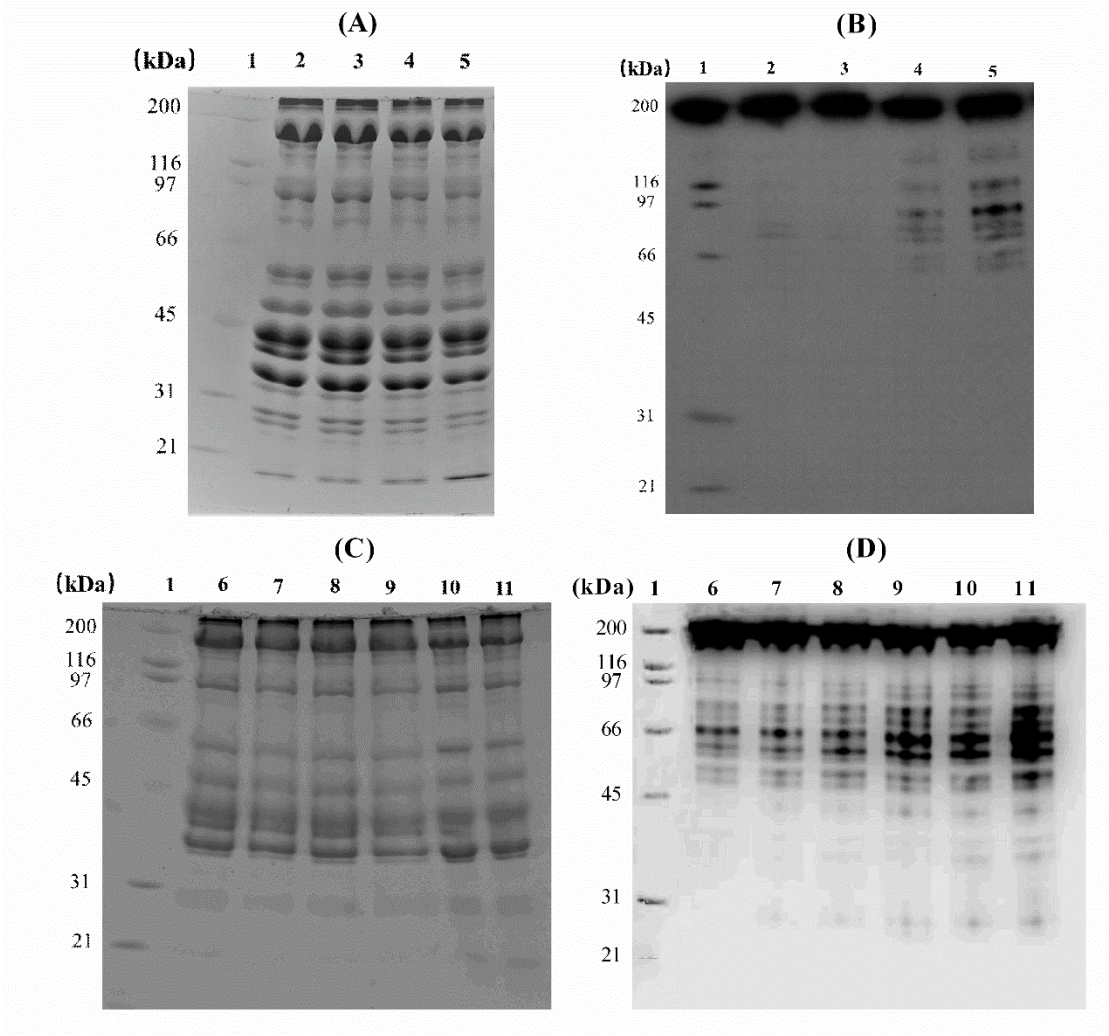

Figure 2-4 SDS-PAGE (A and C) and western-blot results (B and D) developed with anti-MHC IgG of dorsal ordinary muscle of yellowtail during storage at 10 ℃. Each muscle sample was solubilized in the SDS-urea solution and applied on SDS-PAGE using 7.5-15% gradient polyacrylamide gel. The samples in A and B were stored for 4 h, C and D were stored for 24- 96 h. Lane 1: MW maker; Lane 2: SCD-upper for 4 h storage; Lane 3: SCD-under for 4 h storage; Lane 4: SA-upper for 4 h storage; Lane 5: SA-under for 4 h storage. Lane 6: SA-upper for 24 h storage; Lane 7: SA-under group for 24 h storage; Lane 8: SA-upper for 48 h storage; Lane 9: SA-under for 48 h storage; Lane 10: SA-upper for 96 h storage; Lane 11: SA-under for 96 h storage.

2.3.5 Contents of Cathepsin B and B + L in muscle and blood of SA and SCD groups

During the 96 h storage, the initial pH value was 6.9 ± 0.2 in SCD-upper, 6.9 ± 0.1 in the SCD-under, 6.1 ± 0.2 in the SA-upper and the SA-under of the ordinary muscle. The pH in all groups decreased as the storage time was prolonged. The pH in the SA-under was lower than in the other three groups during the 24 to 96 h storage (data not shown). This value determined the reaction conditions needed for the enzyme activity. We chose different pH reaction conditions in measuring the enzyme activity at pH 6.0 ~ 6.9 after 4 h storage based on the pH of fish muscle. By using pH 6.0 ~ 6.9, we assumed that in each reaction the pH of those enzymes was consistent with the storage time of fish muscle during storage.

Measurements of cathepsin B and cathepsin B + L activities in the ordinary muscle of the yellowtail after storage for 4 h revealed significantly higher values in the SA groups than in the SCD groups (Fig. 2-5). The significant difference of enzyme activities in muscle between the bled and non-bled groups indicate that the blood remaining in the muscle had an influence on fish muscle. Therefore, we suspect that the high level of cathepsin B and B+L came from blood and influenced the fish muscle, especially the under-side part.

we measured the Cathepsin B and B+L activities of the blood components of yellowtail peripheral blood. A microscope was used to confirm that the purity of separated white and red blood cells reached 95%. As shown in Table 2-1, cathepsin B and B+L activities were significantly higher in white blood cells than in the red blood

cells and plasma. Therefore, we considered that activities of Cathepsin B and B+L basically came from the white blood cells.

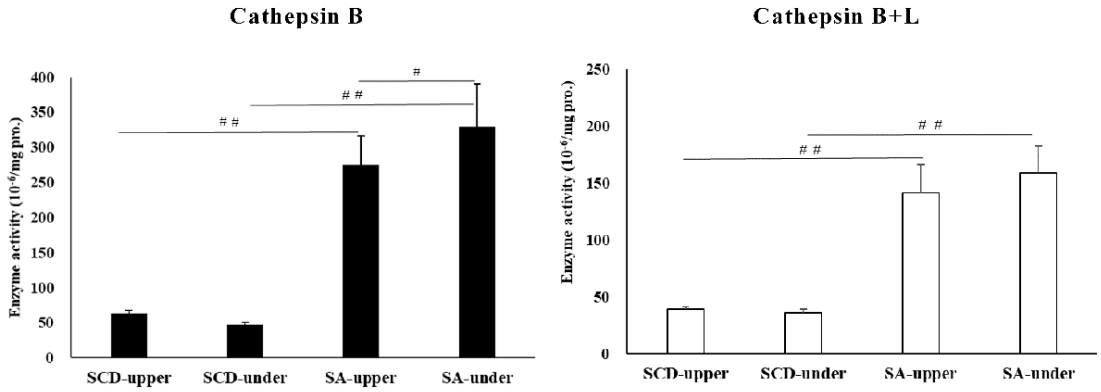

Figure 2-5 Levels of Cathepsin B and Cathepsin B+L activities of ordinary muscle of yellowtail at 4 h storage. Data are mean ± standard deviation (n =4). $^{\#}$ ($p<0.05$) significant difference between the SA-upper and the SA-under for Cathepsin B, $^{\#\#}$ ($p<0.01$) significant difference between the SCD-upper and the SA-upper, the SCD-under and the SA-under for Cathepsin B. The SCD-upper and the SA-upper, the SCD-under and the SA-under for Cathepsin B+L.

Table 1 Cathepsin B and Cathepsin B+L activities of white blood cell, red blood cell and plasma from peripheral blood of yellowtail

Blood components	Specific activities ($\times 10^{-6}$ U/mg pro.)	
	Cathepsin B	Cathepsin B+L
White blood cell	1911 ± 751	1844 ± 913
Red blood cell	2.57 ± 0.56 ##	2.89 ± 0.34 ##
Plasma	38.2 ± 32.9 ##	43.5 ± 37.4 ##

Data are mean ± standard deviation (n =3). ## ($p<0.01$) significant difference between white blood cell group and red blood cell group, white blood cell group and

2.4 *Disscussion*

Combining the above with the result of the color a*, we considered that the increase in the red coloration of under-sides was due to blood. Furthermore, the a* and heme values of the SA group were higher in under-sides than in upper-sides, and this phenomenon suggests that blood deposited in a downward direction as a result of the direction of the fish placement. We called this phenomenon gravitational blood deposition. In previous studies, bleeding was considered a useful method to delay the progress of rigor-mortis, which consequently delay muscle softening. Bleeding also maintains the high commercial value of fresh fish *(Mochizuki, Ueno, Satoh & Hida, 1998)*. The organoleptic rating was used to illustrate that the bled sample was highly evaluated for its bright red color and did not have a fishy smell *(Terayama & Yamanaka, 2000)*. Without the use of antioxidant, the ordinary muscle without the bleeding

procedure was more pro-oxidative than the ordinary muscle that was bled during the early stage of ice storage *(Sohn et al., 2007)*. So far, the custom of removing blood from fish blood after landing has become a rule to attain high flesh quality. But, the mechanism of bleeding which influences fish muscle texture and quality have not been clarified.

It was reported that the breaking strength value of the bled group was higher than spiked and non-bled groups from 6 to 18 h storage in yellowtail (Ando et al., 1999). Moreover, the breaking force of the dorsal muscle of bled skipjack was higher than that of the non-bled group during 114 h storage on ice *(Terayama et al., 2002).* Our study yielded the same result, in where the breaking strength of the SCD group was higher than that of the SA group throughout 4 to 96 h storage. Our study also showed that the breaking strength of the SA-under group decreased the fastest among the other groups. These results indicate that blood had an effect on muscle structure, resulting in the deterioration of fish flesh quality, especially in the SA-under group.

Fish muscle and tissues are composed of structures like myofibrillar (Hayes &Flower, 2013) and collagen proteins (Xu et al., 2015). Myofibrillar proteins occupy the main components which are crucial for maintaining the integrity of texture and mechanical properties of the muscle (Wang, artinez & Olsen, 2009). A transmission electron microscope was used to observe the delay in degradation of pericellular collagen fibrils in six species of bled fishes (yellowtail, horse mackerel, striped jack, red sea bream, flatfish and rudder-fish) (Ando et al., 1999). Their results show that bleeding can delay collagen fibril degradation and muscle softening of pelagic fish, but had no influence

on demersal. The degradation of MHC of bled cod muscle in 120- 200 KDa became visible after 5 days due to the influence of Cathepsin D during storage at 6 ℃, but no band was observed below 75 KDa *(Wang et al., 2009)*. The same results in the SCD groups was observed in our study. Furthermore, we also studied SA groups, in where we found that the anti-MHC activity bands within 20-200 KDa were strongly visible during 4-96 h storage. Post-mortem changes led to fragmentation and weakening of myofibrils, including the weakening of Z-disk, and myosin and actin degradation which were induced by Calpains and Cathepsins at the early and later stages of storage *(Ahmed, Donkor, Street &Vasiljevic, 2015).* Hence, our current results showed that the decrease in the breaking strength of fish muscle and the degradation of MHC in SA groups might have been induced by proteolytic enzymes from blood.

The cysteine proteases of cathepsin B and B + L are known as thiol proteases, and the protein and synthetic matrix can be hydrolyzed under an acid pH condition *(Ueno, Ikeda &Aoki, 1990)*. The high activities of cathepsin B, D and L were detected in chum salmon muscle, which was suspected to be the reason for muscle softening *(Yamashita et al., 1990)*. Researchers have described different procedures for isolating marine cysteine or thiol proteinases from the muscle of marine fishes *(Yamashita et al., 1990; Yoshida et al., 2015).* However, there are few studies on proteolytic enzymes in the blood of fish. Therefore, we measured the Cathepsin B and B+L activities of the blood components of yellowtail peripheral blood. A microscope was used to confirm that the purity of separated white and red blood cells reached 95%. As shown in Table 1, cathepsin B and B+L activities were significantly higher in white blood cells than in

the red blood cells and plasma. Therefore, we considered that activities of Cathepsin B and B+L basically came from the white blood cells. The cathepsin B and H in various tissues and peripheral blood cells of rats were studied using sensitive immunoassays *(Kominami, Tsukahara, Bando &Katunuma, 1985)*. In our research, Cathepsin B and cathepsin B+L activities were significantly higher in the dorsal muscle of the SA group than in the SCD group, which means the cathepsin B and B+L activities were higher in the non-bled group (SA groups) than in the bled group (SCD groups). Those results suggest that blood cell (mainly the white blood cells with high cathepsin B and B+L activities) deposition along with the migration of hydrolysis of enzyme activities affect the muscle of the under-sides, consequently decreasing the flesh quality. Therefore, gravitational blood deposition might cause undesirable protein deterioration. Deterioration of muscle quality should be given more attention.

The results of the color a*, where the under-side had more color intensity than upper-side breaking strength, where the under-side was always lower than upper-side, and blood along with the cathepsin B and B +L from white blood cells having a tendency to deposit downward, all suggest the presence of blood mortem adversely affects the quality of fish muscle. In addition, the higher MHC degradation in the under-side during the 96 h storage of ordinary muscle of yellowtail indicates that the degradation of structural proteins was accelerated in the deposited blood. Further studies are needed to determine all types of protein proteolytic enzymes in blood and their effect on connective tissue degradation. It is also important to study more effective methods of removing and inhibiting enzymes in the blood.

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Chapter III Effect of blood deposition phenomenon on α-actinin and freshness changes of yellowtail (*Seriola quinqueradiata)* during storage

3.1 Introduction

It is generally believed that fish muscles softening may occur during the preservation, and those muscle softens are related to the extracellular matrix and the decomposition of myofibrils (Ando et al., 1995; Kimiya et al., 2005; Shigemura et al., 2004; Sherekar et al., 1988; Tachibana et al., 2001). In order to prevent fish muscle softening, different slaughter method (medulla oblongata assassination, spinal-cord destruction and blood removal) and storage temperature (storage at 5~10℃) were used to prevent the softening and cold rigidity (Mishima et al., 2005; Ando et al., 1999; Terayama et al., 2000; Mochizuki et al., 1998).

After large amount of fish were caught, it is impossible to do the blood removal treatment. Compared with other Asia countries such as China and Korea, Japan is surely the most strictly observance of customary behavior country. Indeed, there is a traditional habit in Japan that when the fish was captured, the fishes were placed on the same side for transport, storage and selling. This unilateral placement custom is regarded as an industry unwritten rule which be complied. In our previous studies, the a* and heme values of the SA group were higher in under-sides than in upper-sides, and this phenomenon suggests that blood deposited in a downward direction as a result of the direction of the fish placement, and blood along with the cathepsin B and B +L from

white blood cells having a tendency to deposit downward, all suggest the presence of blood mortem adversely affects the quality of fish muscle (Jiang et al., 2019).

Therefore, this chapter was focused on effect of blood deposition phenomenon on α-actinin and freshness changes of yellowtail during storage, we use TEM to observe the ultastructure of Mf and investigate the effects of blood deposition phenomenon on freshness decresed.

3.2 Experiment method

3.2.1 sample fish and slaughter method

Cultured yellowtail (*Seriola quinqueradiata*) were used as the fish sample. Samples of fish [body weigh 3.19 ± 1.60 kg; body length 59.78 ± 10.43 cm (n =12)] were purchased from fish farmers in Nagasaki prefecture's fisheries cooperative association and transported to the laboratory after spinal-cord destruction with blood removal (SCD) and suffocation in air without blood removal (SA). The fish were stored at 10 ℃ and arranged with the left side of the fish down. Four groups: the upper-side of the SCD group (SCD-upper), the under-side of the SCD group (SCD-under), the upper-side of the SA group (SA-upper) and the under-side of the SA group (SA-under), were sampled at 4, 24, 48 and 96 h after storage.

3.2.2 Measurement of ATP related compounds

ATP-related compounds were extracted from 1 g each upper- and under-side of the SA and SCD group using perchlorate acid in accordance with the metod of Ehira,

Uchiyama, Uda, and Matsumiya (1970) (Figure 3-1). The pH of each extracted sample was adjusted to 6.4.

ATP and its related compounds, adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine monophosphate (IMP), inosine (HxR), and hypoxanthine (Hx), were determined by high-performance liquid chromatography (HPLC) auto analysis system (JASCO Co., Tokyo, Japan) as reported previously (Tsuchimoto et al., 1985). The freshness of the muscle was judged from the K-value calculated from the following equation:

$$\text{K-value (\%)} = \frac{\text{HxR} + \text{Hx}}{\text{ATP} + \text{ADP} + \text{AMP} + \text{IMP} + \text{HxR} + \text{Hx}} \times 100\%$$

For the group with the freshness of less than 40%, the rate of increase K-valuewas expressed by calculating the slope of the regression line (Yuan et al., 2019).

3.2.3 SDS/polyacrylamide gel electrophoresis and western-blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed following Leammli (1970) using a 7.5% separating gel slab. The muscle sample (0.05 g) was added in 1 ml sample solution (4% SDS, 125 mM Tris-HCL (pH 6.8), 0.002% bromophenol blue, 8 M urea, and 10% 2-mercaptoethanol). The homogenate mixture was boiled for 10 min and analyzed by SDS-PAGE. The gel was stained with 0.1% Coomassie brillant blue R-250 in 50% methanol and 10% acetic acid, and destained with 50% methanol and 7.5% acetic acid. After running the poly-acrylamide gel, we transferred the proteins to a PVDF membrane in order to perform

an immuno-detection assay (Tachibana et al., 2001). Rat anti- α-actinin IgG antibody and goat anti-rat IgG antibody (Organon Teknica company, Japan) were used in the immunoblot assay. Protein quantification was performed by comparing the band intensities of samples between different groups.

3.2.4 Contents of Cathepsin B and B + L in muscle of SA groups

In the assay on enzyme activity at room temperature, the pH of 1- cm thick slices of dorsal ordinary muscle were measured using a Testo 205 meter (Testo, Japan). One gram of dorsal ordinary muscle was homogenized with 2 ml of 0.05 M Tris-HCl buffer and then centrifuged. The supernatant was used for measurement of the enzyme activity. Cathepsin activity was measured following *Yoshida et al. (2009)*. Cathepsin activities were assayed using Z-Arg-Arg-MCA and Z-Phe-Arg-MCA as substrates (1mM Z-Arg-Arg-MCA and Z-Phe-Arg-MCA in dimethyl sulfoxide). The hydrolysis of Z-Arg-Arg-MCA (PunChem CID: 439707) was indicative of the activity of Cathepsin B, whereas that of Z-Phe-Arg-MCA (PunChem CID: 87282815) represented the activity of cathepsin B+L. The substrate was diluted to 0.25 mM with distilled water before use. The reaction mixture consisting of 200 µl of 0.4 M acetate buffer (pH 5.5~ 6.9) containing 5 mM EDTA,100 µl of 20 mM cysteine and 20 µl of the enzyme solution was adjusted to a total volume of 0.8 ml. The mixture was preincubated at 37 ℃ for 1 min, and re-incubated for 8 min under the same condition after adding 200 µl of 0.25 µM substrate. The reaction was terminated by adding 1.5 ml of 0.1 M sodium acetate buffer (pH 4.3) containing 0.1 M sodium monochloroacetate. Fluorescence intensity of the released 7- amino- 4-methylcoumarin (AMC) liberated by enzyme hydrolysis was

measured by RF-1500 spectrofluorophotometer (Shimadzu Co., Kyoto, Japan) at an excitation wavelength of 380 nm and an emission wavelength of 460 nm.

3.2.5 Preparation of samples for transmission electron microscope (TEM) observation

Muscle fiber samples of Upper- and under-side were collected from SA and SCD group on ice, prefixed immediately for 60 min at 2% glutaraldehyde buffered at pH 7.2 with 0.15 M phosphate buffer with ice bath according to the previous report (Xiao et al., 2010). After washing three times in 0.15 M phosphate buffer for 15 min, the postfix for 60 min subsequently in 2% osmium tetroxide buffered at pH 7.2. Samples were washed three times again in 0.15 M phosphate buffer for 15 min, dehydrated in a series of acetone up to 100% (in order of 60%, 70%, 90% twice, 99%, 100% twice, each for 15 min), cleared in a series of Propylene oxides (in order of Propylene oxides : acetone = 1:1 for 15 min, only Propylene oxides for 15 min), penetrated in a series of epoxy resin I containing dodecenyl succinic anhydride and methyl nadic anhydride (in order of epoxy resin I : propylene oxide = 1:1 for 30 min, 3:1 for 60 min, and only epoxy resin I at 40℃ for 60 min), and further penetrated in epoxy resin II added tridimethyl aminomethyl phenol to epoxy resin I at at 40℃ for 120 min, and then embedded in epoxy resin II. These muscle fiber samples in epoxy resin II were polymerized at 60℃ for 3 days (Figure 3-2).

After trimmed embedded fiber trips with glass knife, the cross and longitudinal ultra-thin sections were cut on an RMC-MT-6000-XL ultramicrotome with diamond knife. The sections were stained with 10% uranyl acetate in absolute methanol, followed by

0.2% lead citrate solution in distilled water and observed under JEM-100S electron microscope operated at 80 kV.

3.2.6 Statistical analyses

Data are presented as mean value ± SD. Differences before and after freeze-thawing were assessed by Student's *t* test. Critical values less than $P < 0.05$ (*), $P < 0.01$(**), $P < 0.001$(***) were considered statistically significant.

3.3Results

3.3.1 Changes in K-value of upper-side and under-side in SA and SCD during storage

The parameters for regression slopes of K-value change over time (0-40%) between upper- and under-side of SA group during storage at 10℃ are shown in Table 3-1. The rates of increase in K-values for the upper- and under-side of SA group were 13.91 ± 2.20%/24 h and 11.69 ± 0.98%/24 h (mean slope), respectively. Furthermore, the rates of increase in K-values were higher in SA group than in SCD group, the upper- and under-side of SCD group were 8.20 ± 0.71%/24 h and 8.82 ± 2.44%/24 h (mean slope), respectively.

Table 3-1 Parameters of regression lines of K-value (0-40%) during storage at 10℃

Group		Slope (ΔK_{10}%/24h)	Intercept	r		N	Mean slope (ΔK_{10}%/24h) ± SD
SA	upper	11.80	1.84	0.999	*	3	11.69 ±0.98
		10.36	0.20	0.999	*	3	
		11.90	0.26	0.999	*	3	
		12.72	-0.13	0.997	*	3	
		16.35	-0.51	0.998	*	3	
	under	12.02	1.27	0.996	*	3	13.91 ±2.20
		12.08	1.86	0.980		3	
		15.18	-0.20	0.998	*	3	
SCD	upper	7.92	-0.43	0.975	*	4	8.20 ±0.71
		7.58	-1.46	0.994	*	4	
		9.21	-2.44	0.999	*	4	
		8.11	-2.83	0.995		3	
		7.58	-0.86	0.984	*	4	
	under	12.47	-2.68	0.954		3	8.82 ±2.44
		7.64	-2.03	0.985	*	4	
		7.59	-1.21	1.000	**	3	

SA: Suffocation in air without blood removal.

SCD: Spinal-cord destruction with blood removal.

Each value is the mean ± SD of four fish.

One and two asterisks show $p<0.05$ and $p<0.01$ of the significant level, respectively.

3.3.2 Contents of Cathepsin B and B + L in muscle of SA groups

Measurements of cathepsin B and cathepsin B + L activities in in the ordinary muscle of the yellowtail after storage for 4 h revealed significantly higher values at 4 h than 24 h and 48 h. Compared the cathepsin B and B+L activities in the upper-side with the under-side of SA group, cathepsin B has the significant highest activities in the under-side of SA group at 4 h and 24 h. Moreover, the cathepsin B +L was remained higher activities in under- than upper- during storage (Table 3-2).

3.3.3 SDS-PAGE and western-blot in SCD and SA group

we determined the amount of α-actinin in the SA and SCD groups at protein level using SDS-PAGE and Western-blot analysis. Coomassie staining revealed no significantly difference in the intensity of α-actinin (110 KDa) in the SA group and the SCD group after 48 h storage (Fig. 3-3 A).

Western-blot analysis using antibody against α-actinin confirmed the results from the SDS-PAGE. The anti-α-actinin activity was strongly seen at the 110KDa band in both the SCD and SA groups. At 4 h storage, the bands around 45-97 KDa both of the SA and SCD groups could be observed. Moreover, the thick bands below 31 KDa were observed only in the SA-under at 24 and 48 h (Fig. 3-3 B). These results indicate that the under-sides have a stronger degradation than the upper-sides which may have been influenced by the existence of blood during storage.

Table 3-2 Cathepsin B and Cathepsin B+L activity of SA group in yellowtail ordinary muscle during storage at 10 ℃

Substrate	Group	Specific activity($\times 10^{-6}$ U/mg pro.)		
		4 h	24 h	48 h
Cathepsin B	SA-upper	281 ± 36*	167 ± 24*	179 ± 60
	SA-under	327 ± 50	189 ± 33	216 ± 49
Cathepsin B+L	SA-upper	148 ± 25	123 ± 49	160 ± 96
	SA-under	199 ± 84	154 ± 73	179 ± 89

Z-Arg-Arg-MCA was used as substrate for Cathepsin B.

Z-pHe-Arg-MCA was used as substrate for Cathepsin B+L.

SA: Suffocation in air without blood removal.

Each value is the mean ± SD of four fish.

*: $P < 0.05$, significant difference in cathepsin B of SA-upper and SA-under at 4 h and 24 h.

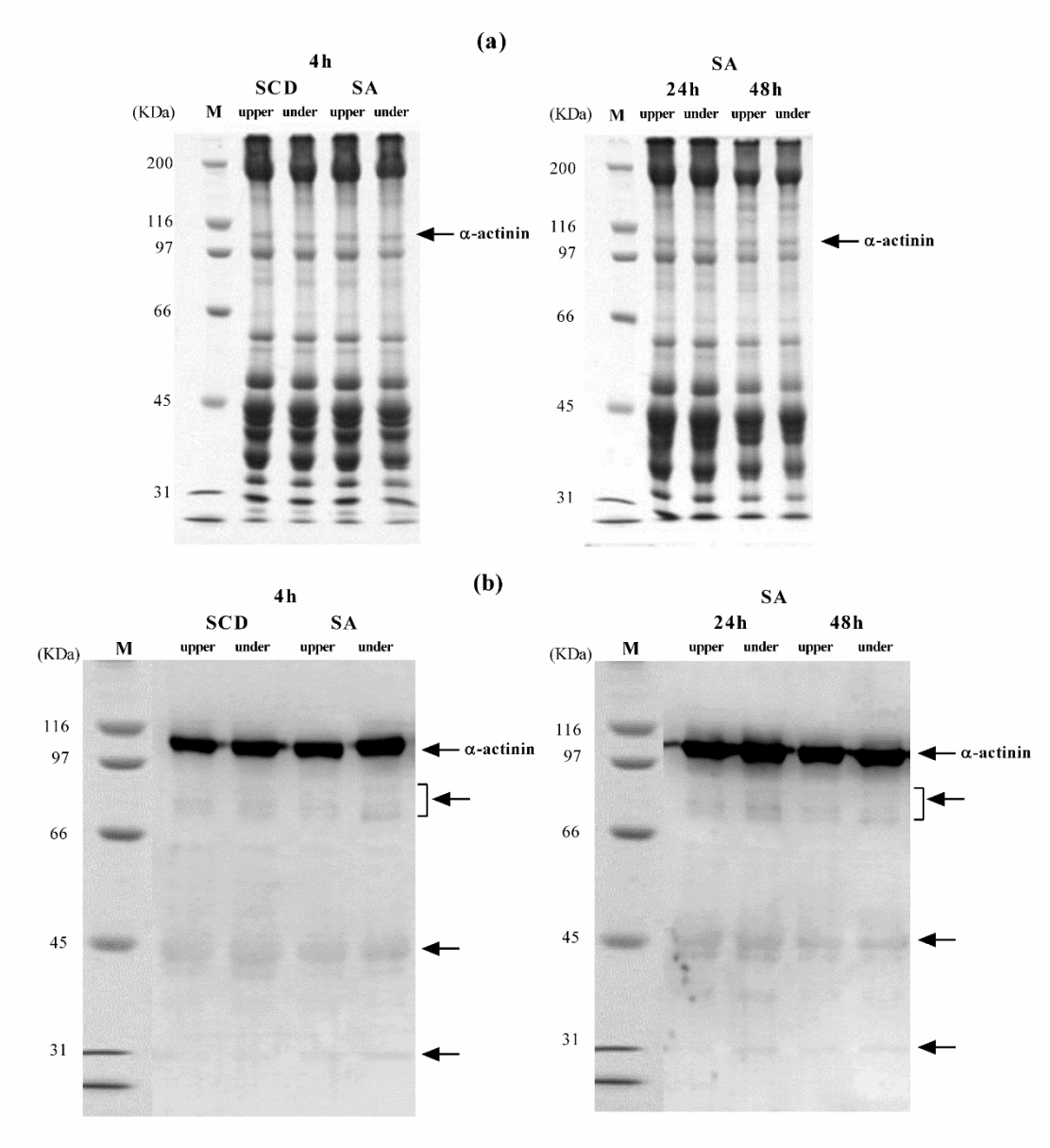

Figure 3-3 SDS-PAGE (a) of dorsal ordinary muscle of yellowtail during storage at 10 ℃ and its western-blot (b) developed with anti-α-actinin IgG. Each muscle sample was solubilized in the SDS-urea solution and applied on SDS-PAGE using 7.5% polyacrylamide gel. Lane M: maker

3.3.4 Histological observation of Mf ultrastructurre

The TEM result of longitudinal sections for Mfs in upper- and under-side of SA and SCD group were shown in Figure 3-4.

In SCD group, the Mf both of upper- and under-side showed peculiar transvers striations of the skeletal muscles that arranged neatly by the thin and thick filaments. Z line zigzagged through the middle of I band, and the actin filament was connected to the convex portion of Z line. The ultrastrcture of sarcoplasmic reticulum (SR) was observed extending in the longitudinal direction between Mfs. The SR showed a structure of T-tubule surrounded by two terminal cisternae were clearly observed during storage.

In SA group, I band of SA-upper was partly decreased, and due to the concentration of the Mfs, the Z line could not be observed clearly after 4 h storage. However, the SRs of SA-upper were partially expanded, the contact structure of SRs was still maintained after 48 h storage. But, the collapsed structure of SRs was strongly observed in SA-under, Furthermore, the space between the Mfs were expanded after 4 h storage.

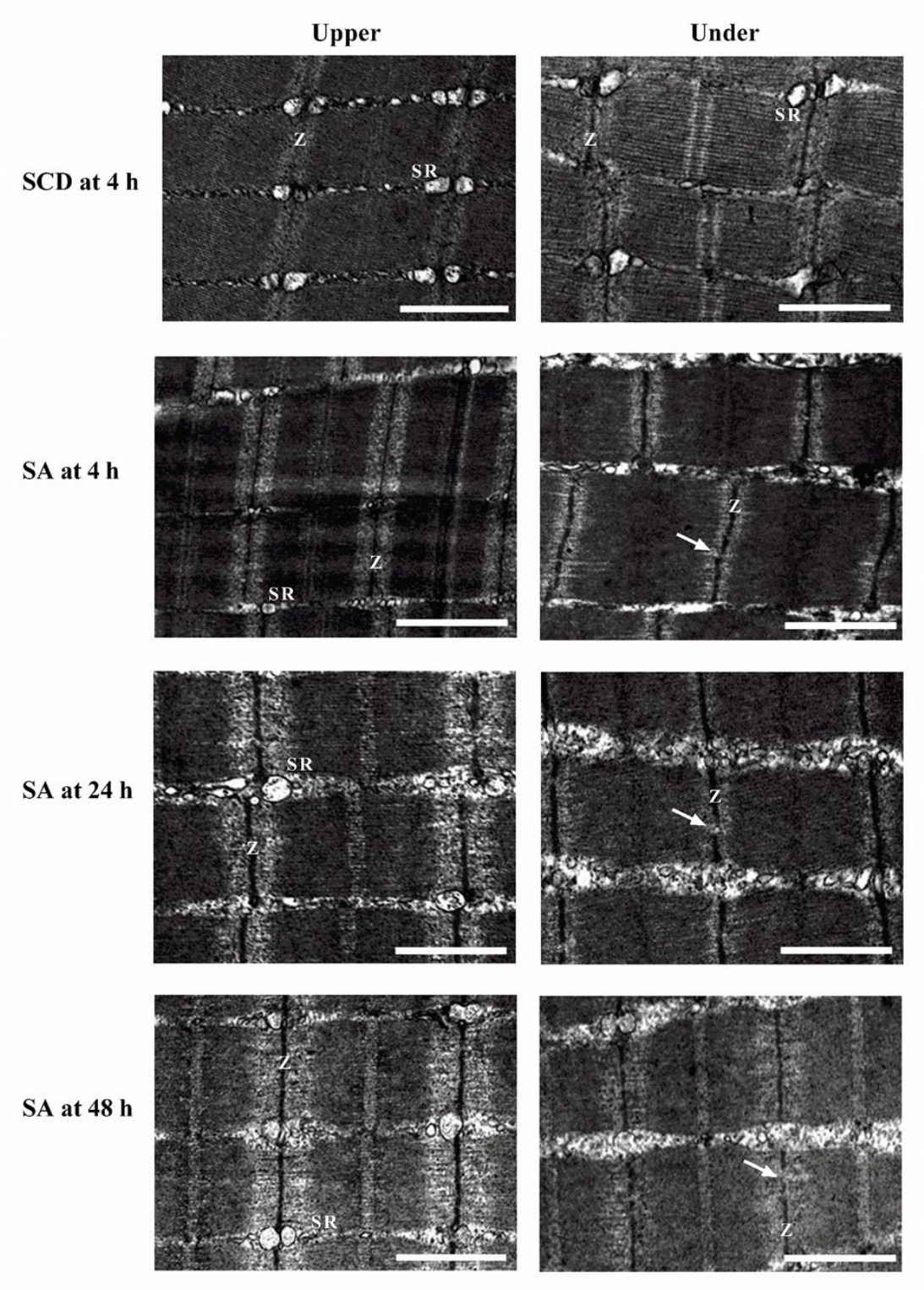

Figure 3-4 Ultrastucture of muscle fiber of dorsal ordinary muscle in yellowtail observed by transmission electron micrographs.

Z: Z line; SR: sarcoplasmic reticulum; Bars represent 2 μm.

3.4 Disscussion

In this study, we investigated the influence of blood deposition on fish muscle quality changes of ordinary muscle. The changes of K-value and α-actinin were measured, and the ultrastrcture of sarcoplasmic reticulum (SR) was observed by TEM.

The rates of increase in K-value of upper- and under-side from SCD group was no significant difference during 96 h storage. The cathepsin B and B+L activities at 4 h revealed significantly higher values than 24 h and 48 h. Compared the upper-side with the under-side of SA group, cathepsin B has the significant highest activities in the under-side of SA group at 4 h and 24 h. The deposited blood will cause the deposition of blood cells which protein enzymes come from blood cells were deposited from upper- to under-side, and influence the muscle quality of under-side (Jiang et al., 2019). In general, the proteinase which cause the softening of the muscle could degradation of the myofibrillar protein (Xiao et al., 2010) and collagen protein (Ando et al., 1995; Ando et al., 1991). Under the western-blot observation, we find out the degradation of α-actinin according to the blood deposition phenomenon. Ando et al. (1999) confirmed the degradation of the structure in collagen was influenced by blood. Furthermore, we observed the myofibrillar degradation was influenced by blood by using TEM. The collapsed structure of SRs was strongly observed in SA-under than in SA-upper during storage, and the space between the Mfs of SA-under were expanded after 4 h storage. So, we speculate that the degradation of Mfs and the structure destruction of SRs were both result from blood. Therefore, we confirmed the blood deposition phenomenon could accelerate the influence of muscle degradation during the storage.

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Chapter IV Effect of blood deposition on the degradation of the connective-tissue of the yellowtail *(Seriola quinqueradiata)* during storage

4.1Introduction

In forensic medicine, blood hypostasis in cadaver is defined as the setting of blood in the lower part of the body as a result of decreased blood flow *(Vanezis and Trujillo 1996)*. The same phenomenon can be observed in fish, but there is almost no research published on this. Blood pressure drops rapidly and there is no driving force to support the vascular system after the fish dies *(Olsen et al. 2006)*. Moreover, gravity pulls the stagnant blood to the lowest points of the fish body and blood migrates to the muscle tissues during storage. In our previous study, we observed that blood transfered to the low side of the fish after 4 h storage. This phenomenon, called blood deposition, caused the softening of fish muscle by structural protein (myosin heavy chain) degradation during storage *(Jiang et al. 2019)*.

There are three main groups of fish-muscle tissue proteins (structural proteins, sarcoplasmic proteins, and connective-tissue proteins). Connective-tissue proteins constitute about 3% of the protein in teleosts *(Ahn and Kim 2013; Sotelo et al. 2000)*. However, collagen fibril in the connective-tissue is the main load-bearing component within the tissue *(Ando et al. 1991)*. Hence, broken collagen fibrils may influence structural integrity and muscle quality. Structural change in the collagen fibrillar network corresponded to the post-mortem tenderization in rainbow trout and yellowtail

muscles during chilled storage *(Ando et al. 1995)*. Type V collagen were solubilized in softened sardine muscle with the concomitant weakening of connective-tissue during short-time chilled storage *(Sato et al. 1997)*. However, the quantity of type V collagen was only 5% or less of the whole collagen, whereas the amount of type I collagen was over 95% *(Sato et al. 1991)*. Both the type I and V collagens may have participated in the post-mortem softening in Japanese flounder *(Kimiya et al. 2005)* and yellowtail *(Shigemura et al. 2004)* during chilled storage. The soft texture of fish muscle may have been caused by the breakdown of the extracellular matrix, in particular collagen, and to a lesser extent by the proteolysis of the intracellular myofibrillar proteins *(Bremner 1999; Martinez et al. 2011)*. In our previous study, we found that ice crystals contributed to vulnerabilities in the extracellular matrix (collagen fibrils) *(Wang et al. 2017)*. Furthermore, blood also had influence on muscle quality by causing collagen fibril degradation. In pelagic fish, removal of blood could delay collagen fibril degradation, and muscle softening was observed by transmission electron microscopy (TEM) *(Ando et al. 1999)*.

This study was to determine the effect of blood deposition on flesh quality by histological observations (Light microscopy and SEM) of collagen fibrils in the pericellular connective tissue. Gelatin zymography was used to evaluate the gelatinolytic activities in muscle and blood, particularly, the effect of blood deposition on the flesh quality of the under-side fish muscle.

4.2Experiment method

4.2.1 sample fish and slaughter method

Cultured yellowtails *Seriola quinqueradiata* were used as sample fish. Fish samples (body weight 4.72 ± 0.89 kg; body length 66.51 ± 2.40 cm; $n = 7$) were purchased from fish farmers in Nagasaki Prefecture and transported to the laboratory for 30min after killing by spinal-cord destruction with blood removal (SCD) and suffocation in air without blood removal (SA). After arrived at lab, the fish were immediately preserved at 10 ℃ after arranging them on a fixed direction, so that the left side of fish was the under-side. Four sample groups were made: the upper-side of SCD group (SCD-upper), the under-side of SCD group (SCD-under), the upper-side of SA group (SA-upper) and the under-side of SA group (SA-under). According to our previous studies *(Jiang et al., 2019)*, we collected samples at 4 h and 96 h because the 4 h was the point at which blood begins to deposit, the 96 h was the point until which the deposited blood was preserved.

4.2.2 Measurement of color a*

The color of 1-cm thick slice of dorsal ordinary muscle was measured using a color chromameter (Minolta CR-200b, Konica-Minolta, Tokyo, Japan). Color variations were obtained as a* for green-red chromaticity (green -60 to red +60). Measurements were recorded in quintuplicates per sample at each sampling time *(Mora et al. 2007)*.

4.2.3 Preparation of sample for microscopic observations

To detect tissue softening due to blood deposition, a compression test was performed according to the method of Ando et al. *(1991)*. A muscle cube (10 × 10 × 10 mm) was each excised at 4 and 96 h from the ordinary muscle of the fish. The muscle cubes were compressed parallel to the orientation of muscle fibers at 100 g/cm2 for 10 s using a cylindrical plunger (40 mm diameter). After the compression test, the muscle cubes were fixed in formalin buffer solution (10 % formalin, 0.02 M sodium phosphate buffer, pH 7.2) and embedded in paraffin. Sliced sections of 4 μm-thickness were prepared and stained with Van Gieson for light microscopic (BX50, OLYMPUS, Japan) observation *(Kageyama and Watanabe 1978)*.

4.2.4 Preparation of samples for scanning electron microscopy (SEM)

The cell-maceration/SEM method *(Ohtani 1987)* was used to reveal the three-dimensional structure of the collagen fibril network of pericellular connective tissues. Muscle cubes were cut (2 × 2 × 8 mm) parallel to the orientation of the muscle fibers and pre-fixed in buffer (2.5% glutaraldehyde, 2.0% paraformaldehyde and 0.15 M phosphate buffer, pH 7.2). The muscle tissue was extracted with 2 M NaOH for 6 days at room temperature, and then post-fixed with 1 % tannic acid and 1 % asmium tetroxide for 2 h each. Samples were sequentially dehydrated in a series of graded methyl alcohol (50 - 99%, 15 min each), frozen and fractured with a cooled sharp blade under liquid nitrogen. The fractured samples were then transferred in 2-methyl-2-propanol and freeze-dried (Model JFD-320, JOEL, Tokyo, Japan). Finally, samples were sputter-

coated with a palladium alloy (30 mA, 45 s) using an auto fine coater (Model JFC-1600, JEOL). We observed the samples by SEM (Model JSM6380, JEOL) using an accelerating voltage of 10 kV.

4.2.5 Tissue and blood cell extraction

Blood was drawn from the posterior spinal artery at the fish tail by using a heparinized syringe while the fish was alive. Heparinized fish blood was extensively washed with 1.35 % phosphate buffered saline (PBS) and centrifuged with discontinuous gradients of lymphocyte separation medium (LSM) (Funakoshi company, Japan), resulting to the simultaneous separation of white blood cell, red blood cell and plasm *(Fisher et al. 1998)*. White blood cells and red blood cells were separated from the blood and washed in 1.35 % PBS for three times, respectively. Separated blood cells and plasma were homogenized with five volumes of cold 1.35 % PBS, and the blood was extracted with 50 volumes of cold 1.35 % PBS because of remarkably higher enzyme activities. All homogenates were centrifuged at 15000 × g for 10 min and the supernatant was used for zymographic analysis.

Each muscle (0.2 g) of a different storage time was homogenized in ten volumes of cold 1.35 % PBS, and the homogenate was centrifuged at 15000 × g for 10 min. The supernatant was used for zymographic analysis *(Kubota et al. 1998)*.

4.2.6 Gelatin zymography

Each supernatant from tissue and blood extracts was mixed with 1/1 volume of 0.125

M Tris-HCl buffer (pH 6.8) containing 4% SDS, 20% glycerol, 0.04% bromophenol blue, and applied to 10% gelatin-SDS-polyacrylamide gels *(Wu et al., 2009)*. The gels were electrophoresed at 4°C. After electrophoresis, the gel was immersed into 0.05 M Tris-maleic acid buffer (pH 6.5) and 0.05 M Tris-HCl (pH 7.4) containing 5 mM $CaCl_2$, 2.5% Triton X-100, and then shaken gently for 30 min at room temperature to renature proteins which was denatured by SDS. The gel was incubated for 16 h at 37°C in 0.05 M Tris-maleic acid buffer (pH 6.5) and 0.05 M Tris-HCl (pH 7.4) containing 50 mM $CaCl_2$, then stained with 0.1% coomassie brillant blue R-250 in 50% methanol and 10% acetic acid, and finally destained with 50% methanol and 7.5% acetic acid. Activity was detected as a transparent band not stained by CBB R-250 *(Kubota et al. 1998)*.

4.2.7 Crude collagen extraction

The crude collagen was extracted as described by sato et al. (1988) with slightly modified. The dorsal ordinary muscle of yellowtail (30 g) was homogenized in 10 volumes (v/w) of 0.1 M NaOH under 4 °C. The homogenate was centrifuged at 10000 g for 20 min. The residue was stirred in 20 volumes (v/w) of 0.1 M NaOH overnight and centrifuged at 10000 g for 20 min. The final residue was washed with distilled water and then dialyzed against distilled water overnight. The precipitate was used for SDS-PAGE analysis *(Sato et al. 1988)*.

4.2.8 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli *(1970)* and modified by Xu et al. *(2015)* using a 7.5% separating gel slab. The crude collagen extracted from muscle (30 g) was added to a 20 ml sample solution (5% SDS, 10 mM EDTA, 20 mM Tris-HCL (pH 8.0), 0.002% bromophenol blue, 8 M urea, and 15% 2-mercaptoethanol). The homogenate mixture was stored at room temperature for one night to dissolve the collagen and analyzed by SDS-PAGE. The gel was stained with 0.1% coomassie brillant blue R-250 in 50% methanol and 10% acetic acid, and then destained with 50% methanol and 7.5% acetic acid.

4.2.9 Measurement of the breaking strength

The dorsal muscle was cut vertically to the orientation of muscle fibers at a thickness of 10 mm. We used a cylindrical plunger (diameter 3 mm) to measure firmness. The plunger was inserted into the sliced block parallel to the orientation of muscle fibers at a speed of 1 mm/s using a Rheoner model RE-3305s rheometer (Yamaden Co., Tokyo, Japan). The maximum force needed to penetrate the muscle was regarded as the breaking strength (N) *(Mora et al. 2007)*.

4.2.10 Protein concentration

Protein concentration was determined and rectified using the biuret method according to Gornall et al. *(1949)*.

4.2.11 Statistical analyses

All data analyses were performed in the R statistical environment (v.1.1.442). The values of the continuous variables were compared using the student t-test. p values that * < 0.05, ** < 0.01, *** < 0.001 were considered statistically significant *(O'Mahony 1985)*.

4.3 Results

4.3.1 Change in color a*

The color a* of SA group was more intense than that of SCD group. The color a* was higher in SA-under (4.06 ± 1.69) than in the SA-upper (1.86 ± 1.16) at 96 h. In our previous study, the main factor responsible for color change in yellowtail was residual blood *(Jiang et al. 2019)*. As a consequence, we considered that this color a* change was resulting from blood deposition.

4.3.2 Light microscopic observations

The upper and under-side muscles of the yellowtail for both the SCD and SA groups were observed under the light microscope as shown in Fig. 4-1. After the compression test, detachment of muscle fibers occurred at 4 h of the SA group (Fig. 4-1 c and d), whereas muscle fibers were mainly intact at 4 h of the SCD group (Fig. 4-1 a and b). These results indicated that the collagen fiber, which integrated muscle fibers, was degraded by the blood as compared with the bleeding groups during the 4 h storage. Meanwhile, the collagen fiber of the under-side (Fig. 4-1 d) had more degradation than

the upper-side (Fig. 4-1 c) in the SA group at 4 h, whereas there was no obvious difference between the under-side (Fig. 4-1 b) and the upper-side (Fig. 4-1 a) in the SCD group. After 96 h storage, both SCD and SA groups could not maintain the muscle structure. The collagen fiber of SA groups (Fig. 4-1 g and h) had larger structural damage than the SCD groups (Fig. 4-1 e and f). Moreover, the SA-under (Fig. 4-1 h) had the largest damage in muscle structure and the collagen fiber among the other groups. Thus, we propose that the blood deposition corresponding to influence the Type-I collagen stability is related to the structural changes in ordinary muscle of yellowtail.

4.3.3 SEM observations

Histological observations revealed a tight and tidy honeycomb-like extracellular matrix (ECM) structure, with smooth cut surfaces and a neat collagen fibril networks in both upper- and under-sides of the SCD groups (Fig. 4-2 a, b, e and f) at 4 h and 96 h. By contrast, the cut surfaces were rough in the SA groups (Fig. 4-2 d, g and h), and the honeycomb structures collapsed especially in the under-sides of SA groups at 96 h (Fig. 4-2 h). The pericellular connective tissue in the yellowtail muscle became thinner at 96 h than 4 h, in the upper- and under-sides of both SCD and SA groups.

At the 4 h and 96 h, there was no obvious difference between upper-sides (Fig. 4-2 a and e) and under-sides (Fig. 4-2 b and f) of the SCD groups. The honeycomb-like ECM structure was more heavily collapsed in under-side (Fig. 4-2 d and h) than in upper-sides (Fig. 4-2 c and g) of the SA groups both at 4 h and 96 h. Furthermore, the

honeycomb-like ECM structure almost could not be observed in SA-under at 96 h (Fig. 4-2 h) after blood deposition took place in the yellowtail ordinary muscle.

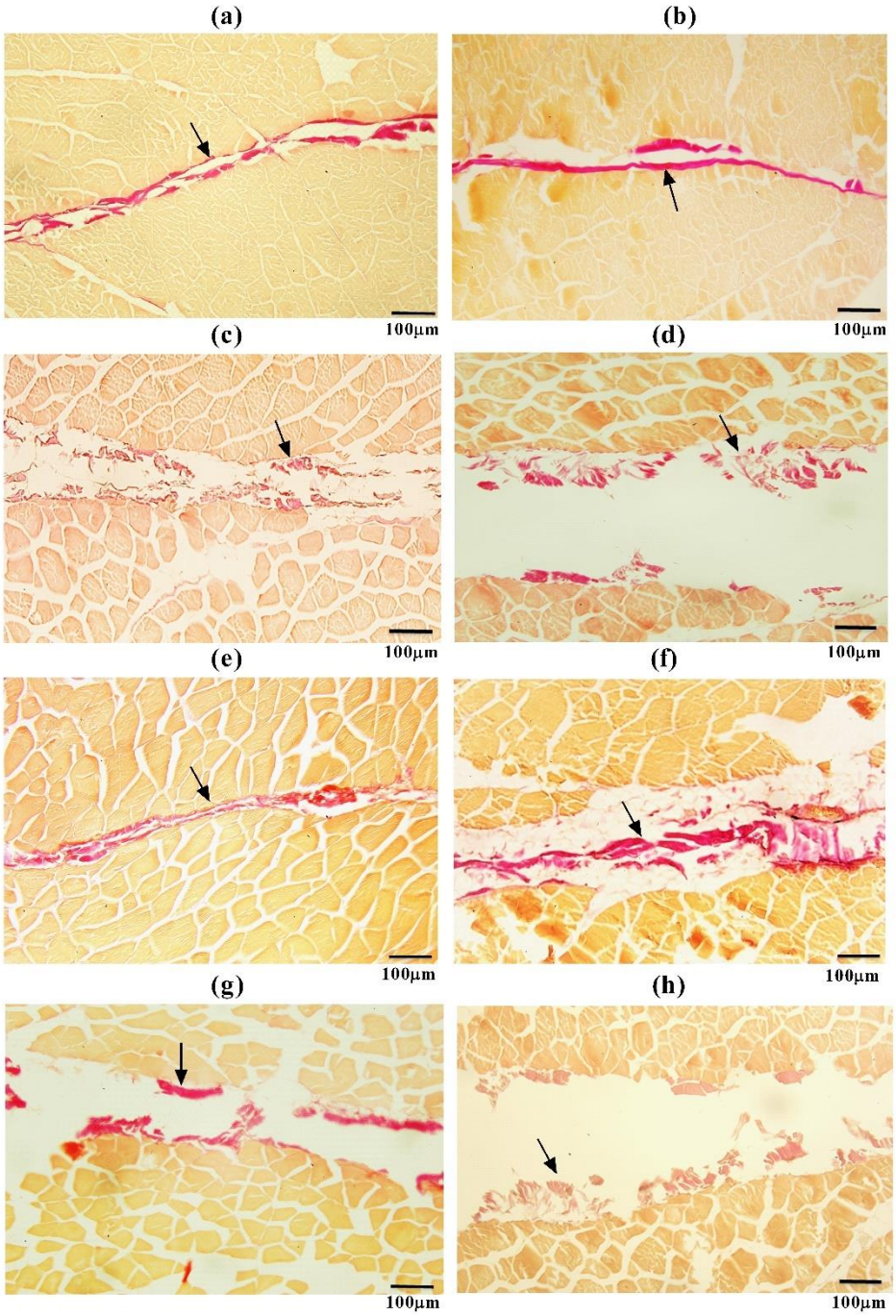

Figure 4-1 Light micrographs of the ordinary muscle in yellowtail from the upper-side and under-side during storage at 10 °C. (a) SCD-upper at 4 h; (b) SCD-under at 4 h; (c) SA-upper at 4 h; (d) SA-under at 4 h; (d) SCD-upper at 96 h; (d) SCD-under at 96 h.

(g) SA-upper at 96 h; (f) SA-under at 96 h. Arrows: myocommata. Scale bars represent 100 μm.

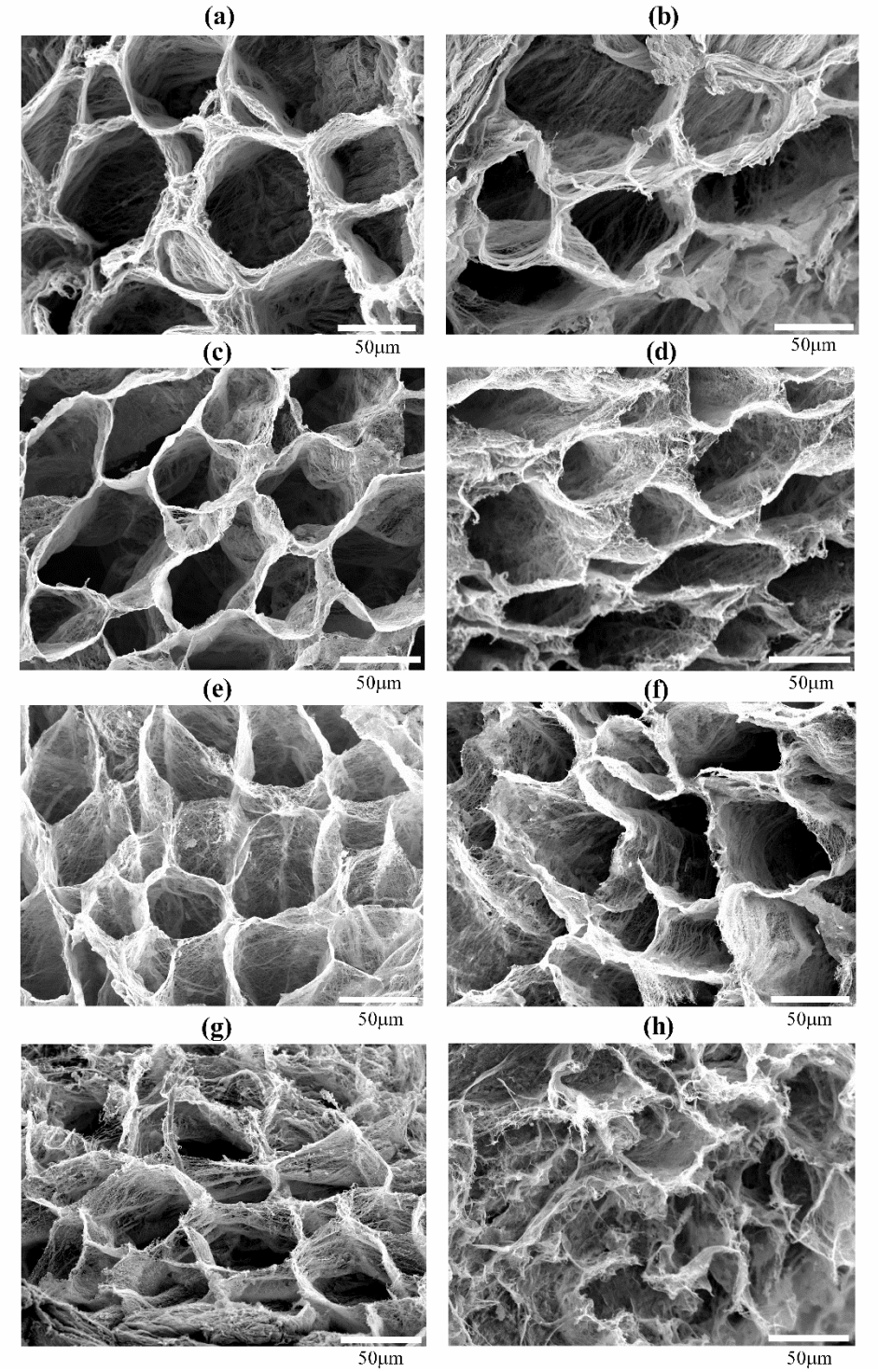

Figure 4-2 Scanning electron micrographs of extracellular matrices of SCD (spinal-cord destruction with blood removal) and SA (suffocation in air without blood removal) groups, from upper-side and under-side muscles in yellowtail during storage at 10 ℃.

(a) SCD-upper at 4 h; (b) SCD-under at 4 h; (c) SA-upper at 4 h; (d) SA-under at 4 h; (e) SCD-upper at 96 h; (f) SCD-under at 96 h. (g) SA-upper at 96 h; (h) SA-under at 96 h. Scale bars represent 50 μm.

4.3.4 Gelatin zymography of muscle and blood

The gelatin zymography of yellowtail blood showed that active bands could be seen in whole blood, white blood cell, red blood cell and plasma in both pH 6.5 and pH 7.4 conditions (Fig. 4-3 a and b). In addition, the following active bands were detected: 110 kDa (band B), 100 kDa (band C), 97 kDa (band D), 70 kDa (band E), 65 kDa (band F), 55 kDa (band G) in white blood cell; 66 kDa (band H) in plasma and strong active bands of 90 kDa (band A) in blood, white blood cell, red blood cell and plasma. The strong active band (band A) had high activity in white blood cells and plasma, but had low activity in red blood cells in both pH 6.5 and 7.4 conditions.

Various active bands between 50 and 97 kDa were observed in the ordinary muscle tissue extract at both pH 6.5 and pH 7.4 conditions (Fig. 4-3 c and d). Under an acidic condition, the gelatinolytic activities in the muscle (Fig. 4-3 c) were induced, but activity at the 90 kDa (band I) was higher in the under- than the upper-side of the SA group during 4 h storage. After 96 h storage, lower gelatinolytic activities were observed in SA group (band I, J and band K). The gelatinolytic activity in the muscle at pH 7.4 (Fig. 4-3 d) showed the same result with pH 6.5, while the 90 kDa (band I) in the under-side of the SA group always maintained a higher activity than the upper-side at both 4 h and 96 h.

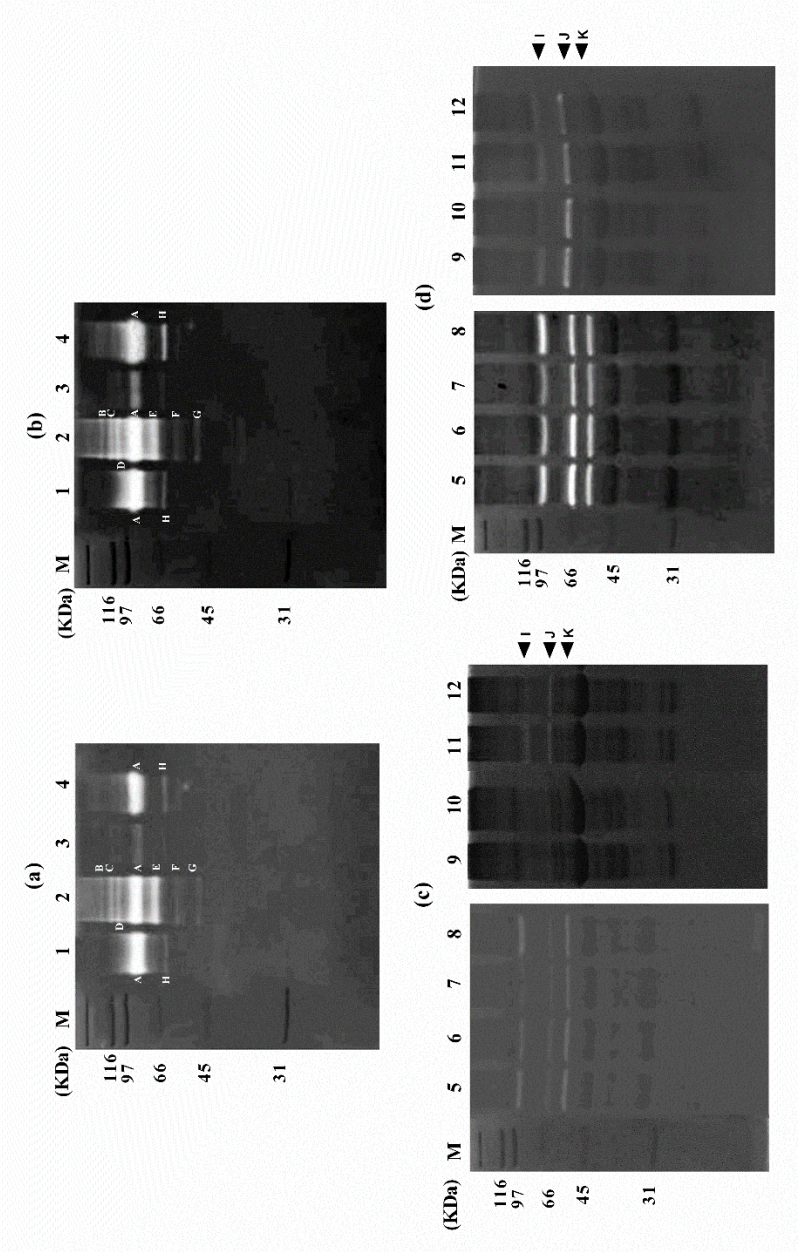

Figure 4-3 Gelatinolytic activities in blood (a and b) and ordinary muscle (c and d) of yellowtail during 96 h storage at 10 ℃. (a) and (c) were pH 6.5. (b) and (d) were pH 7.4. Lane 1: whole blood. Lane 2: white blood cells. Lane 3: red blood cells. Lane 4: plasma. Lane 5: SCD-upper muscle at 4 h. Lane 6: SCD-under muscle at 4 h. Lane 7: SA-upper muscle at 4 h. Lane 8: SA-under muscle at 4 h. Lane 9: SA-upper muscle at 96 h. Lane 10: SA-under muscle at 96 h.

4.3.5 The crude collagen extracts and SDS-PAGE

The SDS-PAGE of the crude collagen extracts at different storage time is shown in Fig. 4-4. At 4 h and 96 h, the bands between 100 kDa and 200 kDa were thinner in the SA group than in the SCD group, both for upper- and under-sides (Fig. 4-4). This indicates that the influence of blood may have accelerated the degradation of collagen protein during storage. Because the crude extract is not digested by pepsin, type V collagen cannot be formed without the telopeptide being cleaved. Therefore, we failed to observe the type V collagen bands in SDS-PAGE. Comparison of the upper-sides and under-sides of SCD groups showed no significant difference in total collagens between the SCD-upper and SCD-under group at 4 h and 96 h. By contrast, the type I (β(I)) collagen in SA-under group was reduced at both 4 h and 96 h compared to SA-upper. Therefore, we consider that the difference in crude collagen content between SA-upper and SA-under is due to blood deposition.

4.3.6 breaking strength

All groups had lower breaking strength values at 96 h compared with 4 h. The SA groups had lower breaking strength values than the SCD groups at both 4 h and 96 h. In addition, the breaking strength value of the SA-under group was slightly lower than that of the SA-upper at both 4 h and 96 h, but there was no significant difference. (Fig. 4-5).

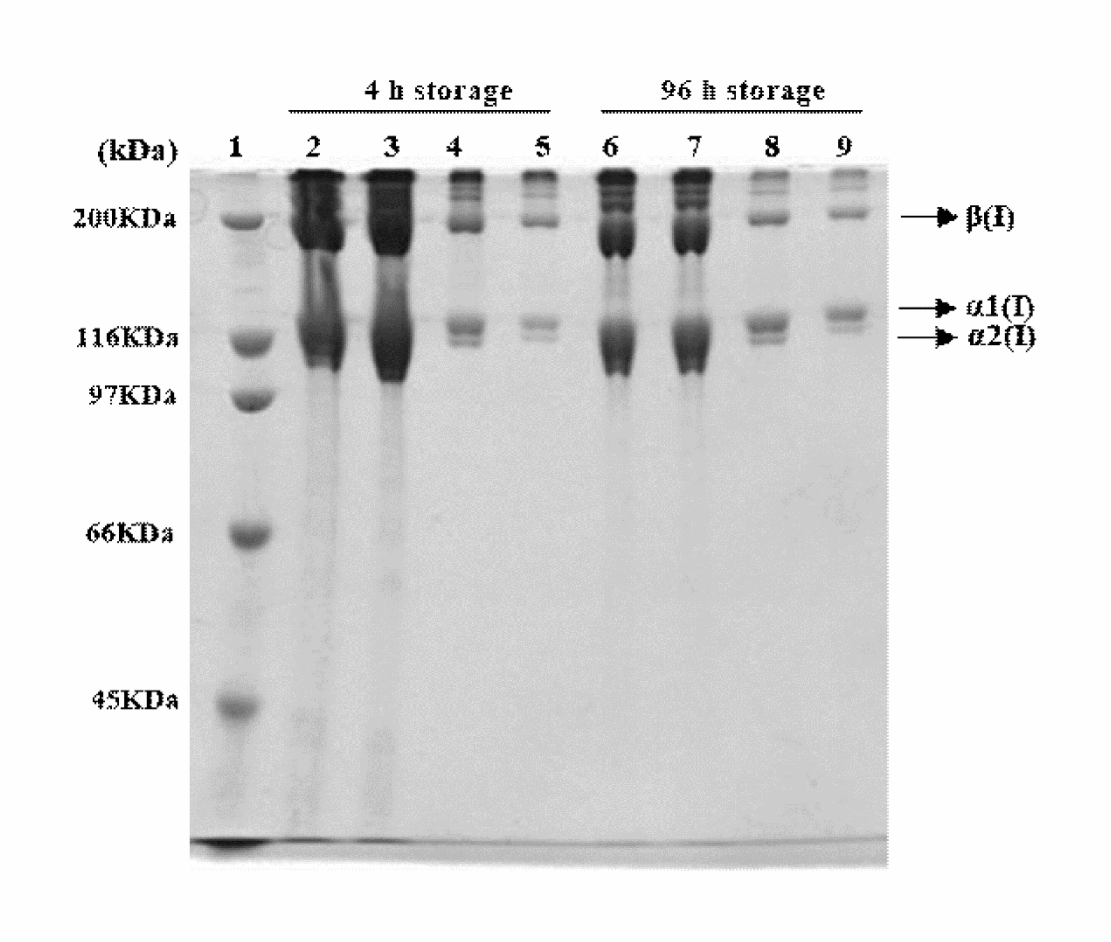

Figure 4-4 Protein profiles on SDS-PAGE of crude collagen extracts from ordinary muscle of yellowtail during storage at 10 ℃. Lane 1: maker. Lane 2: SCD-upper. Lane 3: SCD-under. Lane 4: SA-upper. Lane 5: SA-under. Lane 6: SCD-upper. Lane 7: SCD-under. Lane 8: SA-upper. Lane 9: SA-under.

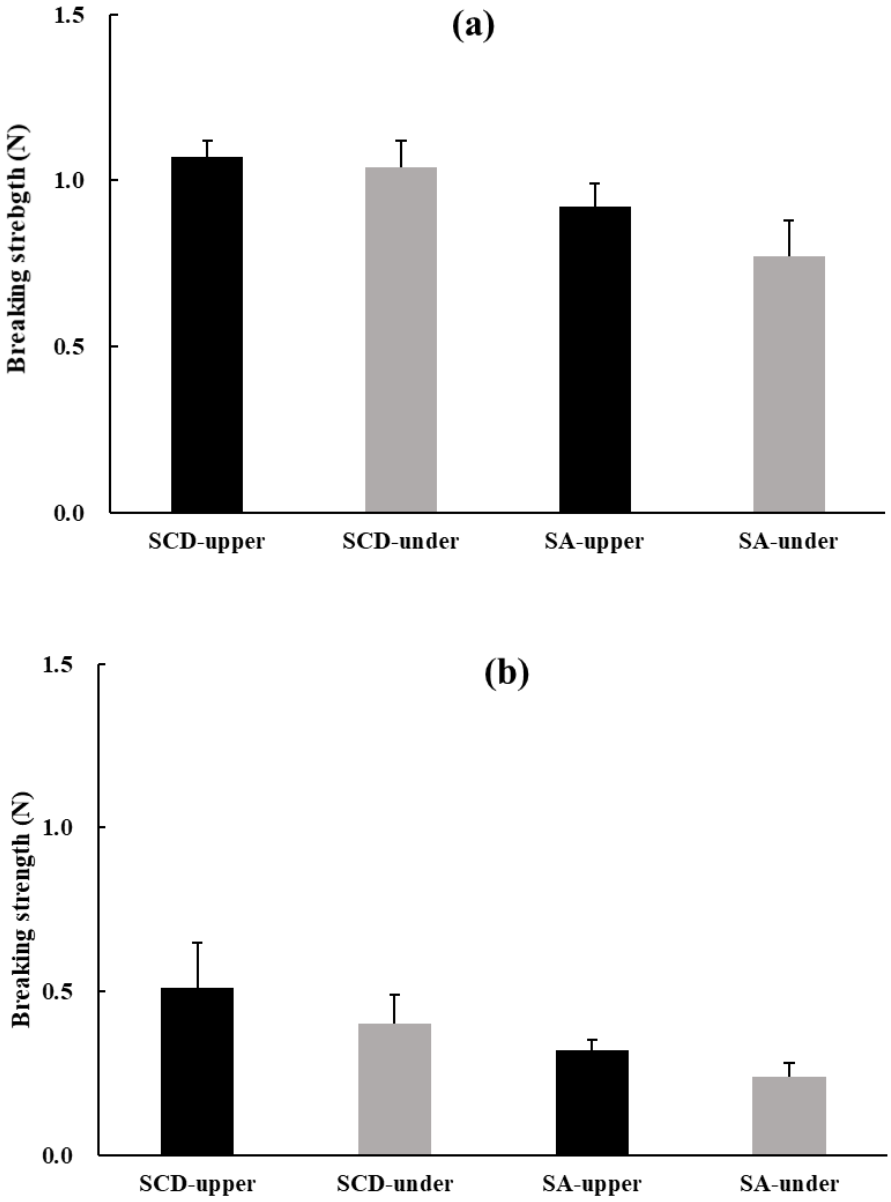

Figure 4-5 Breaking strength of upper- and under-sides of ordinary muscle from blood deposition during storage at 10 ℃. Data are mean ± standard deviation (n = 3).

4.4Disscussion

In our previous study, we found out that deposited blood had an influence on myofibril protein (myosin heavy chain) degradation during storage *(Jiang et al. 2019)*. Blood deposition accompanied by acid proteases downside movement affected the flesh quality of the under-side. The same result can be seen in Fig. 1, where both SCD-upper and SCD-under had an almost intact myofibril structure after the compression test, compared to SA group at 4 h. Meanwhile, the structure of the collagen fibers (type I collagen) was more damaged in no-bleeding group than in bleeding group, further, the deposited blood cause a greater damaged of collagen fiber in SA-under during 96 h storage. A slight decomposition of type I collagen in yellowtail muscle might have occurred corresponding to postmortem softening during chilled storage *(Shigemura et al, 2004)*. Kimiya et al. *(2005)* suggested that both type I and type V collagens might have been involved in the post-mortem softening in Japanese flounder during chilled storage. In order to clarify the degradation in pericellular connective-tissue, we used SEM to observe the structure of collagen fibrils. In SCD groups, there was no obvious difference between the upper-sides and under-sides at both 4 h and 96 h. In the SA groups, however, more damage in the structure of collagen fibrils was observed in the under-side than in the upper-side at both 4 h and 96 h. Bleeding caused the delay of degradation in pericellular collagen fibrils which delay of muscle softening in pelagic fish *(Ando et al., 1999)*. In our result, the pericellular connective tissue (type V collagen fibrils) seemed thinner in no-bleeding group than in bleeding group. This confirmed that blood influenced the structure of pericellular connective-tissue during storage.

Breaking strength was higher in SCD group than in SA group during 96 h storage, and SA-upper showed slightly higher value of breaking strength than SA-under. We confirmed this in histological observations, wherein the blood deposition phenomenon influenced collagen fibrils decomposition, leading to the decrease in breaking strength during storage.

Gelatinolytic activities in the muscle of ayu *plecoglossus altivelis* were due to both metalloproteinase and serine proteinase activities *(Kubota et al. 2000)*. Matrix metalloproteinases (MMPs) had the ability to degrade several of the matrix components, such as collagens and proteoglycans *(Woessner 1991; LØdemel and Olsen 2003)*. In addition to MMPs, matrix serine proteinases (MSPs) might also play an important role in degradation of the ECM *(Koshikawa et al. 1992)*. In our study (Fig. 3), we found that strong gelatinolytic activities came from white blood cells and plasma at 90-97 kDa (Fig. 3 a and b), whereas the gelatinolytic activities was also observed at 90-97 kDa of the muscle extract, with stronger activities observed in the under-side than the upper-side of the SA groups (Fig. 3 c and d). However, gelatinolytic activities at 50-66 kDa of the muscle extract had no obvious difference between the upper-sides and under-sides in both SCD and SA groups at 4 h (Fig. 3 c and d). There were no obvious gelatinolytic activities at 50-66 kDa of the blood extract (Fig. 3 a and b). The main component removed during bleeding was considered to be the blood, while part of the plasma still remained in the muscles. According to the results of the gelatin zymography in the blood (Fig. 3 a and b), plasma also contains a large amount of gelatinolytic enzyme activity. Therefore, these results raise the possibility that the SCD group retains

gelatinolytic enzyme activity at 4 h storage. On the other hand, gelatinolytic enzyme activity is deposited from SA-upper to SA-under at 4 h, suggesting that the enzyme derived from the plasma and blood cells. Due to the autolysis of proteases, the activity of gelatinolytic enzyme was disappeared at pH 6.5 and exhibits a low activity at pH 7.4 after 96 h storage. In this work, we chose gelatin zymography as our method of investigating the presence of tissue-degrading proteases, because gelatin is partly denatured collagen and therefore the enzyme's ability to degrade gelatin implies a possible ability to degrade collagen, the most important structural protein in the connective-tissue *(Felberg et al. 2009)*. Gelatin zymography results indicated that deposited blood influenced the distribution of gelatinolytic activities at 90-97 KDa of the fish muscle and this might have affected the integrity of collagen fibrils structure during the storage. We extracted crude collagen from yellowtail ordinary muscle and stored at different times. At 4 h and 96 h, there was higher degradation of total collagen proteins in the SA group than in the SCD group. Type I collagen degradation was especially higher in SA-under than SA-upper. These show that the blood deposition phenomenon has a greater influence on the degradation of type I collagen. The increase in gelatinolytic activities accompanied by collagen proteins degradation in ordinary muscle during storage indicates that the blood deposition phenomenon changed the distribution of the gelatinolytic activities, and consequently accelerated collagen degradation.

In summary, flesh quality was significantly influenced by blood, which degraded myofibrils and collagen fibrils, and particularly by the difference in degradation which

could be attributed to the blood deposition phenomenon during storage. Blood deposition affected the extracellular matrix and showed strong gelatinolytic activities in blood components which accelerated the degradation of the collagen proteins and fibrils in the under-side of the stored fish. Blood deposition might have been given little attention, but this phenomenon could lead to the decrease in the quality of the under-side fish flesh, consequently reducing the commercial value of the fish.

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Chapter V General Discussion

In the distribution of fresh fish, the handling volume of fish increases year by year, and the handling volume of natural fish outperforms cultured fish. In Japan, the net and fishnet fishery is a fishing method for high-catching migratory fish, and it is regarded as a central fishery in Japan in terms of catch. after fishing, fish are stored in fish storage until landing at fish markets etc. Fish caught with a net, etc., will be killed in the condition of low temperature shock and bitter melon in low temperature seawater and water ice in fish storage. In these processes, fish are generally not treated after blood loss, and they are customary in fish market unique to Japan, with the head on the left and arranged in a box (ie the right side of the fish downwards, the left side) Are distributed upward). After death, the blood pressure of the fish that did not lose blood is lowered, and the blood in the body causes a blood drop phenomenon that gathers under the fish by the action of gravity. Therefore, it is considered that blood is stored in the lower side of the fish, that is, on the right side. This difference in blood distribution was thought to cause a difference in fish meat softening between the upper and lower portions of the fish. By the way, it is generally said that the main cause of fish meat softening is due to the decomposition of myofibrillar proteins and extracellular matrix (EMC) components by tissue proteases and the like. First, in myofibrillar proteins, it is known that degradation of myosin heavy chain and Z-ray in normal muscle is involved in post-mortem flesh softening of fish muscle. In addition, with regard to EMC, it is known that collagen I, which is a main component, is degraded and that V is solubilized during storage. In this study, the influence of the blood deposition phenomenon on the

flesh quality of yellowtail muscle was examined from the point of the flesh quality in the right side where the blood concentrates during storage and the left side not muscle.

In order to examine the effect of blood deposition on physical properties and myosin heavy chains during storage of yellowtail common muscle, the cultured yellowtail is sacrificed to prevent hemorrhage and the blood deposition phenomenon is produced and used as an experimental group. As a control, a fish body subjected to blood removal after spinal cord destruction was used as a sample fish. Each sample fish was stored at 10 ° C, and the sensory chromaticity (redness: a *) of normal muscle during storage, the content of hemoglobin, and the occurrence of blood drop were examined. In addition to measuring the breaking load during storage, the cathepsin-like enzyme activity in muscle and blood was examined, and the degradation of the blood-derived cathepsin-like enzyme and myosin heavy chain was examined.

Sensory color a * and hemoglobin contents during storage at 10 ° C were higher than those from the upper side (SA-upper) of bitter writhing (SA-under) from the 4th hour of storage and both sides of the fish after debulking after spinal cord destruction The blood retention phenomenon was confirmed in the bitter area.

The breaking load was low throughout the storage period under bitter scorching (SA-under).

In immunoblotting of myosin heavy chain, myosin heavy chain was not degraded at 4 hours of storage in the spinal cord destruction and blood removal (both sides), but degradation of myosin heavy chain was observed from 4 hours in the writhing area, and the storage time was The lower side (SA-under) was more degraded than the upper side

(SA-upper) due to the blood deposition phenomenon.

In normal muscle, cathepsin B and cathepsin B + L-like activities were higher in the lower part (SA-under) of bittern than in the upper side (SA-upper), and in the leukocyte fraction both cathepsin-like activities were higher than red blood cells and plasma.

From the above, it was found that blood stasis phenomenon occurs during normal muscle preservation in bitter-killed yellowtail, and softening phenomenon of the lower side (SA-under) progresses rapidly, and degradation of myosin heavy chain is involved in this flesh softening It is considered that the cause is related to the group of enzymes such as cathepsin derived from white blood cells due to blood deposition phenomenon.

We examined changes in myofibrillar proteins during storage of yellowtail muscle and changes in ultrastructure using transmission electron microscopy. That is, using the cultured yellowtail, store the same sample fish as in Chapter 2 at 10 ° C, and change the value of α-actinin constituting the K value and Z line during storage at 10 ° C of ordinary muscle, and use the transmission electron microscope We examined the change of the fine structure. The increase in K value at 10 ° C did not differ between the upper and lower sides of the wriggling area (SA-under, SA-upper) or the spinal cord-disrupting blood removal area. The cathepsin B-like activity was significantly higher in the lower (SA-under) than in the upper (SA-upper) at 4,24 hours after storage. The degradation of α-actinin by immunoblotting was larger in the lower part (SA-under) of the bitter area than in the upper part (SA-upper), and Z-ray rupture and sarcoplasmic reticulum collapse were also quick by transmission electron microscopy. From the

above, the increase in blood distribution to the lower side of the fish body due to the phenomenon of blood deposition affects the distribution of enzymes such as cathepsin derived from leukocytes, which in turn results in the structural collapse of α-actinin and sarcoplasmic reticulum that constitute the Z-ray. It was believed to accelerate and cause loss of normal muscle mass during storage.

In order to clarify the texture softening mechanism of blood deposition, we focused on the intimal structure of the myocardium and examined the effect on extracellular matrix (EMC) degradation during storage. That is, using a cultured yellowtail, the sample fish was made to writhe to make a blood-bearing phenomenon model fish, and a spinal cord-disrupted blood sample was used as a control. Each sample fish was stored at 10 ° C, and EMC of normal muscle was observed during storage using light microscope and scanning electron microscope. We also measured the breaking load during storage and examined the degradation of muscle and blood derived gelatinolytic enzymes and collagen. In the writhing area at 96 hours of storage, the a * value was higher in the lower side (SA-under) than in the upper side (SA-upper), and a blood drop phenomenon was observed. In addition, the breaking strength at the lower side (SA-under) of the bitter area was the lowest, and the morphological dissection of EMC was large. On the other hand, the gelatinolytic enzyme activity of the bitter-sweet area (SA-under) at the same storage time was the highest, and the collagen content was low. From the above, it is thought that the increase in blood distribution to the lower side of the fish body due to the blood deposition phenomenon accelerates the collagen degradation of normal muscle during storage.

The results of this study were comprehensively summarized and comprehensively considered. Among them, it was considered that the increase in blood distribution to the lower side of the fish body at the time of occurrence of the blood deposition phenomenon of fish did not greatly affect the change in chemical freshness as seen from the K value. However, this phenomenon is thought to accelerate meat softening during storage, which includes the degradation of myofibrils and the disintegration of myocyte subcellular organelles by white blood cell-derived cathepsins and gelatinolytic enzymes in blood, It was considered that this was due to the disintegration of the extracellular matrix, in particular, the degradation of collagen fibrils, which is a major component of the intima of the myocardium.

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