Changes in type I collagen of cultured yellowtail *Seriola quinqueradiata* burnt meat

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Yellowtails (*Seriola quinqueradiata*), which were cultured in summer at the water temperature of around 30-31℃, were used to make the model of burnt meat for the purpose of investigating the changes in type I collagen during the occurrence of burnt meat. "Burnt meat" (with lightness parameter, $L^* \geq 55$) was observed just after slaughter in suffocate in air (SA) group and after 2 h storage in spinal cord destruction (SCD) group. Type I collagen decreased (33-37%) during the occurrence of burnt meat in SCD group. In addition, collagenase like protease activity was detected in muscle meat of yellowtail by using synthetic substrate, and was inhibited by EDTA. EDTA and pepstatin A suppressed the decrease of type I collagen, indicating that collagenase like protease and aspartic proteases may cause the changes in type I collagen.

Key Words: burnt meat, yellowtail, type I collagen, aspartic protease, collagenase

Yellowtail *Seriola quinqueradiata* is one of the most important cultured fish among cultured species in Japan. The meat of yellowtail is delicacy and popularly eaten raw as sashimi. Meat texture is a major influencing for consumer acceptance and product value. However, a variation of the raw meat of cultured yellowtail has been occurred during the summer time, and concerned with quality of the meat, especially flavor, color and texture. This is referred to as "burnt meat".[1)]

"Burnt meat" has a pale, grainy and exudative, and is considered unsuitable for consumption as raw meat. The similar phenomenon was also observed in the other fish species, such as tuna and frigate mackerles,[2-5)] and pork.[6)] Previous researches suggested that the deterioration in the quality of burnt meat may be caused by high temperature in the environmental, low ultimate muscle pH and slaughter methods.[2-5)] Recently, several researches have suggested that the denaturation of muscle proteins is also a possible cause of the quality loss in the burnt meat.[1, 7-9)]

The muscle proteins are consisted of sarcoplasmic protein, myofibrillar protein and myostroma protein. Previous researches have pointed out that myofibrillar proteins and sarcoplasmic proteins were changed during the occurrence of burnt meat. Concerning myofibrillar protein, myosin heavy chain (MHC) and α-actinin were degraded during appearance of burnt meat of yellowtail.[1, 8)] In our previous studies, the changes in myofibrillar proteins of burnt meat of cultured yellowtail were also investigated, and suggested that MHC was degraded during the occurrence of burnt meat, and it may be caused by a myofibril-bound EDTA-sensitive protease (MBESP).[7)] Concerning sarcoplasmic protein, Ochiai[9)] reported that some scarcoplasmic proteins, such as creatine kinase (40 kDa) in the burnt meat of wild or cultured bluefin tuna disappeared and it may be hydrolysed by cathepsins B and L. In our previous studies, some sarcoplasmic proteins, such as glycogen phosphorylase (PYGM), glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and aldolase were insolubilized and shifted to myofibrillar fraction, when the burnt meat occurred in cultured yellowtail.[10)]

While, there are few report on the changes in myostroma protein of the burnt meat. Myostroma proteins are consisted of collagens and elastin. Collagens are a family of fibrous proteins found in all multicellular animals, and accounting for 30% of all proteins in mammals.[11)] In fish muscle, type I and V collagens are reported to exist, both of which are fibrous collagens.[12)] The quantity of type I collagen is over 95% of whole collagen, whereas the amount of type V collagen is only 5% or less of the remainder.[12)] Muscle collagens have been found to influence fish meat texture.[13)] Thakur *et al.* reported that collagen is a primary muscle biochemical constituent which affects the raw meat

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texture of cultured yellowtail.[13]

In the present study, the authors investigated the changes in one of the myostroma protein, type I collagen, of burnt meat of cultured yellowtail. Furthermore, the protease activities, which lead to the changes of type I collagen was also investigated to clarify the reason of changes in the collagen.

Materials and methods

Materials

Yellowtail *Seriola quinqueradiata* (average fork length 67.0 ± 6.9 cm, average weight 3.8 ± 0.9 kg) were obtained from a culture farm in Nagasaki Prefecture, Japan, during the summer periods. Water temperature at the sampling site was around 30-31℃ . The fish were transported by a car within 30 min to Marine Biochemistry Laboratory of Nagasaki University, Nagasaki, Japan. After carried back to the laboratory, the fish were dead and considered as just after slaughter.

Slaughter methods and sampling

Two slaughter methods including spinal cord destruction (SCD) and suffocation in air (SA) were conducted following the procedure described by Mora *et al.*.[1] After slaughtering, the viscera of the fish were removed, and the heads and tails were cut off. Fish blocks were wrapped up in the paper towels (Kimtowel, Crecia, Tokyo, Japan), containing with 10 mM sodium azide solution to avoid the pollution of microorganism, and then placed in plastic bags and stored at 30℃ . Sampling was made at 0 (immediately after slaughtering), 2 and 4 h of storage from the blocks. The samples of dorsal ordinary muscle from the fish were frozen at -30℃ until use.

Evaluation of meat quality

One centimeter thick slices of dorsal ordinary muscles (per sampling time) were used for color measurement for each fish used. The color of sliced muscles was measured with a Minolta CR-200b color chromameter (Konica-Minolta, Tokyo, Japan). Color variations were obtained as L^* (lightness).[7] The measurement was carried out four times for each sliced muscle and the values were averaged. The muscle was judged to be burnt meat by the lightness values of $L^* \geq 55$.[1] In addition, 1 g samples of the ordinary muscle (per sampling time) were homogenized in 9 ml of distilled water. The pH value of the homogenate was immediately measured by SevenEasy S20 pH meter (Mettler-Toledo International Inc., Tokyo, Japan). Expressible water measurement was used following the method of Mora *et al.*:[1] 1 g samples of ordinary muscle were measured by using an expressible water meter (Chuo Riken Co., Tokyo, Japan), with a force of 10 kg for 2 min. The mean of four measurements was presented as a percent value. Throughout all experiments, three fishes were used.

Preparation of type I collagen

Type I collagen from muscle was extracted according to the method of Sato *et al.*.[14] All procedures to prepare the type I collagen were carried out at 4℃ . The ordinary muscle (per sampling time) of cultured yellowtail was homogenized with 10 volumes (v/w) of 0.1 N NaOH and the suspension was centrifuged at 10,000×g for 20 min. Then ten volumes of 0.1 N NaOH was added to the precipitate. The suspension was stirred overnight and centrifuged at 10,000×g for 20 min. This alkali-extraction was repeated 4 times. The final precipitate was washed with distilled water for 2.5 h, and then mixed with 10 volumes of 0.5 M acetic acid. After stirring overnight, the suspension was centrifuged at 10,000×g for 20 min. This acetic acid-extraction was repeated 3 times. All the suspensions were pooled and used as the type I collagen fraction.

Effects of protease inhibitors on the changes of type I collagen

The inhibition effects of different kinds of protease inhibitors on the degradation of type I collagen were investigated. The experiment was performed using 1 g muscles suspended in four volumes of 50 mM acetic acid-sodium acetate buffer (pH 5.8, the pH values of yellowtail burnt meat) containing different types of protease inhibitor (Pefabloc SC, E-64, pepstatin A or EDTA). The mixture was incubated at 30℃ for 24 h and centrifuged at 10,000×g at 4℃ for 10 min. The pellets were used to extract the type I collagen, and analyzed the effects of the inhibitors on the degradation of type I collagen by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE analysis

SDS-PAGE was performed under reducing conditions with 7% gel according to the method of Laemmli.[15] After electrophoresis, the protein bands were detected by staining with Coomassie Brilliant Blue R-250. Gels were also scanned and analysed with CS Analyzer Ver. 3.0 (ATTO corporation, Tokyo, Japan).

Enzyme extraction

Five grams of muscle were minced and homogenized with 2 volumes of ice-cold distilled water using a Physcotron NS-50 homogenizer (Microtec co., LTD, Funabashi, Japan). After centrifugation at 4,000×g at 4℃ for 20 min, the supernatant containing the water-soluble proteins were considered as the enzyme fractions.

The protein concentration of the enzyme fractions was determined by the method of Lowry *et al.*[16] after proper dilution. Bovine serum albumin was used as a standard.

Assay of collagenase activity

Collagenase like activity was measured according to Kojima *et al.*.[17] The principle of the assay for collagenase activity using Succinyl-Glycyl-L-Prolyl-L-Leucyl-Glycyl-L-Proline 4-Methyl-Coumaryl-7-Amide (Suc-Gly-Pro-Leu-Gly-Pro-MCA) (Peptide institute. Inc., Osaka, Japan) as a substrate is based on the fluorometric measurement of aminomethylcoumarin (AMC) liberated from reaction product -Pro-Leu-Gly-Pro-MCA by second enzyme reaction with dipeptidyl aminopeptidase (DPP) from pork. The standard incubation mixture contained 50 μl of 50 mM acetic acid buffer (pH 5.8-6.6, the pH values of each sample muscles) with 2.4 mM Suc-Gly-Pro-Leu-Gly-Pro-MCA, and 100 μl of crude enzyme were incubated at 30℃ for 30 min. 70μl of 1 M Tris-HCl buffer (pH 7.5) was added to the mixture, and boiled at 100℃ for 10 min. After boiling the mixture was added to 10μl DPP and incubated at 37℃ for 1 h, the reaction was stopped by adding 1.5 ml of 1 M sodium acetate buffer (pH 4.3). The fluorescence intensity of AMC liberated by hydrolysis was measured in a spectrofluorophotometer (RF-1500, SHIMADZU, Kyoto, Japan) at an excitation wavelength of 380 nm and an emission wavelength of 470 nm. One unite of enzyme activity was defined as the amount of activity that released 1 μmol AMC per minute at 37℃ .

Results

Changes in muscle color

By the megascopic, the normal muscle color of the yellowtail in summer was brilliant transparent pink after slaughtering, while the burnt meat muscle showed turbid and white color (Fig. 1). Changes in lightness (L^*) on yellowtail ordinary muscle from two slaughter methods during storage at 30℃ are as shown in Fig. 2. Muscles with lightness values of $L^* \geq 55$ were evaluated as burnt meat.[1] In SCD group, the burnt meat was observed within 2 h and the L^* value was 60. The L^* value of SA group was 65 just after slaughter, indicating that the burnt meat phenomenon had progressed.

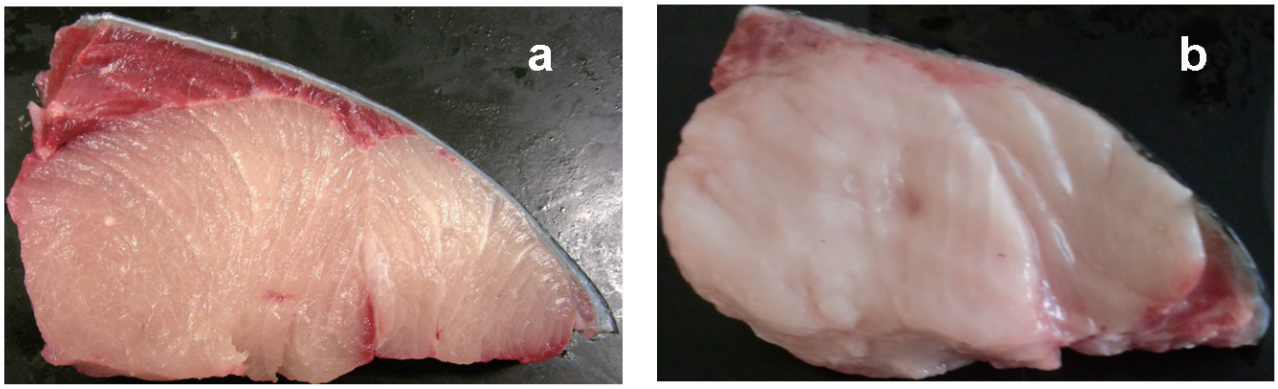

Fig.1 Appearance of (a): normal meat (SCD 0 h storage, L^*=46) and (b): burnt meat (SA 0 h storage, L^*=65) from cultured yellowtail in summer.

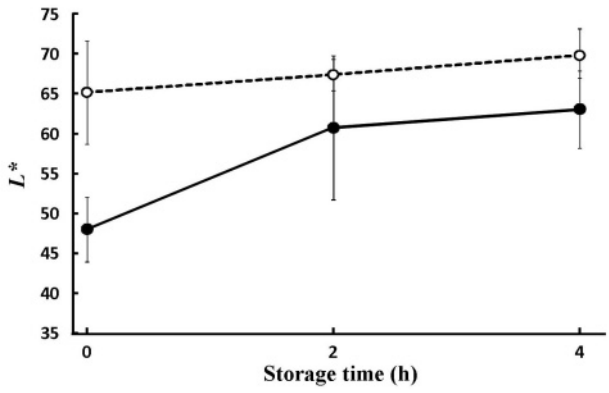

Fig.2 Changes in lightness L^* of the ordinary muscle of cultured yellowtail from two slaughter methods, during storage at 30℃. *Filled circles* SCD; *open circles* SA. Data are means (±SD) of 3 replicates.

Changes in expressible water

Changes in expressible water of yellowtail ordinary muscle from two slaughter methods during storage at 30℃ are as shown in Fig. 3. In SCD group, the value of expressible water at 0 h storage was 25%, which was lower than that of SA group. The value of that increased during the 4 h storage and the value was highest at 42%. In SA group, the value of expressible water was 38% at 0 h storage and showed no significant changes during 4 h storage at 30℃ .

Changes in ultimate muscle pH

Changes in ultimate muscle pH of yellowtail ordinary muscle from two slaughter methods during storage at 30℃ are as shown in Fig. 4. In SCD group, the pH value at 0 h

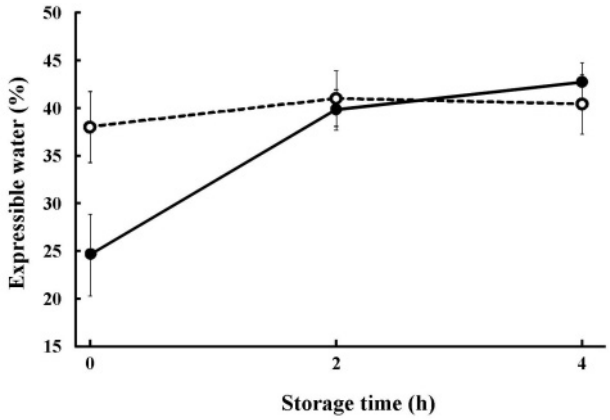

Fig.3 Changes in expressible water of the ordinary muscle of cultured yellowtail from two slaughter methods, during storage at 30℃. *Filled circles* SCD; *open circles* SA. Data are means (±SD) of 3 replicates.

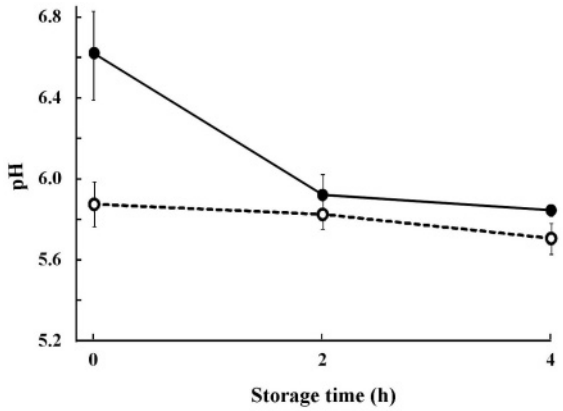

Fig.4 Changes in ultimate muscle pH of the ordinary muscle of cultured yellowtail from two slaughter methods, during storage at 30℃. *Filled circles* SCD; *open circles* SA. Data are means (±SD) of 3 replicates.

storage was 6.6, which was higher than that of SA group. The value declined rapidly to pH 5.9 during the 4 h storage. In SA group, pH value was 5.9 at 0 h storage and showed no significant changes during 4 h storage at 30℃.

Changes in type I collagen

The changes in SDS-PAGE profile of type I collagen from two slaughter methods with storage time is shown in Fig. 5. For both groups, SDS-PAGE gel showed that β-chain, α1-chain and α2-chain of type I collagen decreased with time during the 4 h storage. The gel was scanned, and band intensities were quantified by densitometry. Compared with the collagen bands of 0 h storage in SCD group, the band intensities of β-chain, α1-chain and α2-chain in SCD group were reduced by 35%, 37% and 33%, respectively, during the 4 h storage. Concerning the results of SA group, the collagen bands of 0 h storage were already thinner than that of SCD 0 h storage, and β-chain, α1-chain and α2-chain were reduced by 20%, 18% and 2%, respectively. After 4 h storage, the band intensities of the three chains were reduced by 70%, 76% and 70%, respectively, which were compared with SCD 0 h storage. In SCD group, increases of the band around 60 kDa were observed during the storage. In SA group, degradation of type I collagen during storage was faster than that of SCD group. The band around 60 kDa were found in SA 0 h storage, and then disappeared during the 4 h storage.

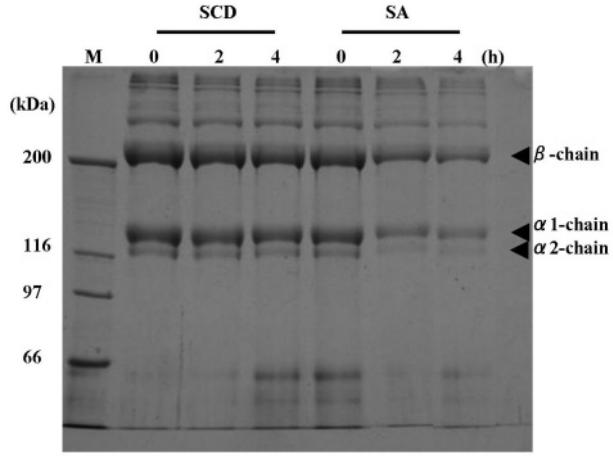

Fig.5 SDS-polyacrylamide gel electrophoresis (7% gel) of type I collagen degradation on cultured yellowtail ordinary muscle from two slaughter methods during storage at 30℃. Lanes: M, Molecular-weight marker; 0-4 is storage times. Arrowheads indicate the positions of β-chain, α1-chain and α2-chain.

Effect of protease inhibitors on the changes in type I collagen

Suppressive effects of different protease inhibitors on proteolytic activity in type I collagen are shown in Fig. 6. The gel was scanned, and band intensities were quantified by densitometry. Compared to the 0 h, the band intensities of β-chain, α1-chain and α2-chain in control were reduced to 20%, 29% and 27%, respectively, during the 24 h storage at 30℃. When metalloprotease inhibitor EDTA was added, the band intensities of β-chain, α1-chain and α2-chain increased to 123%, 116% and 105%, respectively, during the storage. Aspartic protease inhibitor pepstatin A showed the similar effects like EDTA, and the bands of β-chain, α1-chain and α2-chain increased to 157%, 175% and 155%, respectively, during the storage. Metalloproteases inhibitor EDTA and aspartic protease inhibitor pepstatin A showed significant effects on the suppression of the decrease of type I collagen.

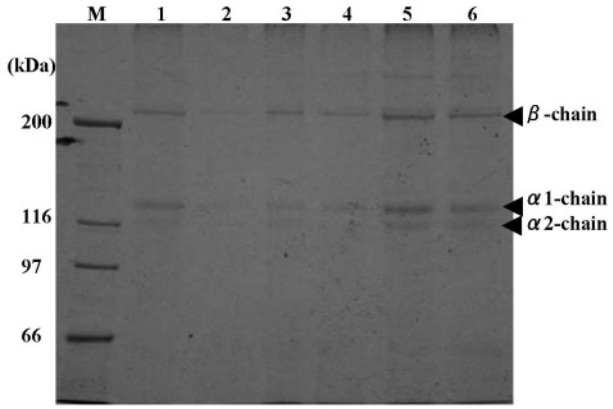

Fig.6 SDS-polyacrylamide gel electrophoresis (7% gel) analysis of the inhibitory effect of protease inhibitors on decrease of type I collagen from cultured yellowtail. Type I collagen were isolated from muscles after incubated in 50 mM acetic acid-sodium acetate buffer (pH 5.8) and different type inhibitors. Lanes: M, Molecular-weight marker; (1), 0 h; (2) no inhibitor 24 h (control); (3) Pefabloc SC to the final concentration of 5 mM; (4) E-64 to 0.01 mM; (5) pepstatin A to 0.01 mM; (6) EDTA to 5 mM, respectively, incubated at 30℃ for 24 h. Arrowheads indicate the positions of β-chain, α1-chain and α2-chain.

Activity of collagenase like protease

The activities of collagenase like protease in the normal and burnt meat of cultured yellowtail are as shown in Fig. 7. The protease had the activities in all the groups during 4 h storage at 30℃ . The authors had confirmed that the activities of collagenase were inhibited by EDTA, an inhibitor of metalloprotease (Data not shown).

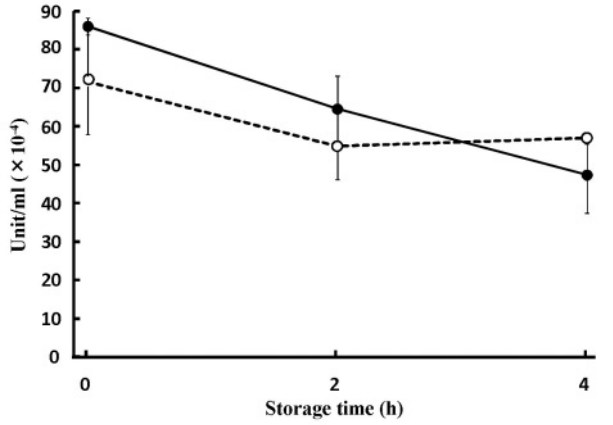

Fig.7 Changes in collagenase like proteases activities of the enzyme fractions of yellowtail from two slaughter methods, during storage at 30℃. *Filled circles* SCD; *open circles* SA. Data are means (±SD) of 3 replicates.

Discussion

In previous studies on the changes of muscle proteins of the burnt meat of cultured yellowtail, the authors investigated the degradation of myofibrillar proteins and suggested that the degradation of MHC may be caused by MBESP.[7] Furthermore, some sarcoplasmic proteins, such as PYGM, GAPDH and aldolase were insolubilized and shifted to myofibrillar fraction, when the burnt meat occurred in cultured yellowtail.[10] In this study, the authors focused on the changes in myostroma proteins, type I collagen, of the burnt meat of cultured yellowtail. In order to know the changes of type I collagen in burnt meat of cultured yellowtail, the authors made the model samples of normal and burnt meat, and extracted type I collagen from them respectively. Moreover, the activities of collagenase like protease and the effects of protease inhibitors on the changes in type I collagen were investigated to know the reason of the changes.

Muscle color (L^*) and ultimate muscle pH are useful as indicators for assessment in the making process of burnt meat.[6] When the fish was slaughtered and stored at 30℃, burnt meat (with muscle color parameter, $L^* \geq 55$) was observed after 2 h storage in the SCD group ($L^*=60$), and at 0 h in SA group ($L^*=65$) (Fig. 2). These results imply that slaughter method play an important role in the occurrence of burnt meat in yellowtail. The finding is consistent with the report by our previous studies. In the present study, the muscle pH value ranged from 5.8 to 6.0 when the burnt meat appeared (Fig. 4). The pH values of the SA group were lower than those of the SCD group. In SCD group, pH values dramatically decreased during the initial stage of storage. The lower muscle pH can lead to negative effects on the quality of fish, such as poor liquid-holding capacity and a rapid degradation of the muscle proteins.[18-20] In *post-mortem*, high muscle temperature caused rapid glycolysis which increased lactic acid content and consequently caused the drop in pH.[1, 9]

In 2007, Mora *et al.*[1] investigated the characteristics of the burnt meat of cultured yellowtail and found that a larger pericellular area in the burnt meat samples than in the normal samples on the microscopic data, suspected the possible degradation of collagen from connective tissue of muscle. In the present study, the changes in SDS-PAGE profile of type I collagen from two slaughter methods with storage time were shown in Fig. 5. The bands of β-chain, α1-chain and α2-chain were decreased with time during the 4 h storage in both groups. These results are consistent with the theory by Mora *et al.*.[1] In addition, the band around 60 kDa was appeared with the occurrence of burnt

meat, and then disappeared by subsequent degradation during the storage in SA group. This protein band may be a proteolytic product of type I collagen in yellowtail burnt meat.

Several researches reported that, the different types of enzymes, such as the matrix metalloproteinase (MMP) family,[21, 22] lysosomal cysteine proteinases (cathepsins L)[23, 24] or collagonolytic serine protease[25] lead to the degradation of collagens. In order to identify which protease caused the decrease of type I collagen in burnt meat of yellowtail, specific protease inhibitors including, EDTA (metalloprotease inhibitor) E-64 (cysteine proteinase inhibitor), Pefabloc SC (serine protease inhibitor) and pepstatin A (aspartic protease inhibitor) were added to the muscles and their inhibiting effects on hydrolytic activity were investigated (Fig. 6). Compared to the control, EDTA and pepstatin A had significant inhibiting effects on the decrease of type I collagen. This result suggested that the proteases including MMP and aspartic protease are possibly involved in type I collagen decreasing of burnt meat.

Collagenases is a subfamily of the MMP family, are capable of cleaving collagen.[22] In the present study, a substrate (Suc-Gly-Pro-Leu-Gly-Pro-MCA) was used to measure the activity of collagenase like protease during the storage in both groups (Fig. 7). The activity of collagenase like protease was observed during the occurrence of burnt meat, suggested that collagenase like proteases might cause the decrease of type I collagen during the occurrence of burnt meat of yellowtail. On the other hand, there are few reports on the collagonolytic aspartic protease. Bae *et al.*[26] reported that, the denaturation temperatures of acid-soluble collagen in some fish species, such as tiger puffer, dusky spinefoot and sea chub were around 30℃, which lower than that of mammal. Thus, the authors suspected that, the collagens of cultured yellowtail were already denatured during the storage at 30℃, and gelatin which produced from collagen might be degraded by the aspartic protease.

In future, the warrants of the changes in type I collagen should be investigated by using histological analysis and immunological analysis, to clarify the mechanism of quality loss in cultured yellowtail burnt meat.

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養殖ブリやけ肉におけるⅠ型コラーゲンの変化

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海水温30－31℃で飼育されたブリを用いて，やけ肉モデル魚を作成し，筋肉Ⅰ型コラーゲンの変化を調べた。苦悶死ブリは致死直後に，即殺死ブリは保存２時間後にやけ肉 ($L^* \geq 55$) が発生した。即殺死ブリでは，やけ肉発生に伴いⅠ型コラーゲンの減少(33－37%)が見られた。また，ブリ筋肉中に，合成基質を分解するコラゲナーゼ様酵素活性が認められ，その活性はEDTAにより阻害された。Ⅰ型コラーゲンの減少はEDTA及びペプスタチンAにより抑制されたことから，その減少にはコラゲナーゼ様酵素及びアスパルティックプロテアーゼが関与する可能性が示唆された。

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