INHIBITON OF POST-MORTEM MUSCLE SOFTENING FOLLOWING *IN SITU* PERFUSION OF PROTEASE INHIBITORS IN FISH

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Background

Fish and shellfish are kept in cold storage after capture and are generally consumed within several days, but the muscle freshness deteriorates even under cold storage. Because Japanese people like fresh slices of raw fish, consumers are interested in the freshness and the muscle texture is an especially important factor for freshness. The muscle structure is known to deteriorate during the 1st day of cold storage, affecting the muscle texture before rigor mortis occurs (Toyohara and Shimizu 1988, Ando *et al.*1991). Such a muscle-softening phenomenon in post-mortem is thought to be caused about by proteolysis of the muscle structure. Several proteases have been studied as being causative factor for fish muscle softening such as cathepsins B, D, H and L (Yamashita 1994), calpain (Tsuchiya *et al.* 1992) and matrix metalloproteinase (Kubota *et al.* 2000). Because of the low level of protease activities in ordinary fish muscle, the material in most of these studies was drawn from particular meat that has an unusually soft texture and extremely high protease activity such as jellied meat from Japanese flounder and matured semelparity fish. In addition, anadromous and amphidromous fish die certainly after spawning and the death is thought to be programmed as apoptosis. If the soft muscle texture at the spawning stage is also programmed as part of apoptosis, there are some possibilities that caspase, a key protease of apoptosis (Yamashita 2003), relates to post-mortem muscle softening.

Objectives

In order to demonstrate involvement of protease in the post-mortem softening in ordinary fish muscle, we have developed an *in situ* perfusion technique, which introduced other chemicals into blood vessels in fish muscle. In the present study, using the *in situ* perfusion technique, protease inhibitors were perfused to identify the type of proteases that led to post-mortem muscle softening in fish, tilapia and yellowtail.

Materials and methods

***In situ* perfusion:** Tilapia *Oreochromis niloticus* and Yellowtail *Seriola quinqueradiata* were obtained from a private fish farm and reared for several months before use. Fish were anesthetized with 200 ppm MS222 (Tricaine methanesulfonate, Acros Organics, NJ, USA) and a cannula connected to a hypodermic syringe that was filled with physiological saline (0.7489% NaCl, 0.1294% KCl, 0.199% $CaCl_2$) was inserted into the bulbus arteriosus. A polyethylene tube (1.5 mm in diameter, 10 cm long) was inserted into the ventricle to bring the venous blood out of the fish body, so that it prevented the circulation of the venous blood and helped the physiological saline perfusion. Soon after the operation, the color of the fish gills changed to white from red and the color of the fluid from the polyethylene tube became clear about 10 min later. Finally after approximately 30 min, the liver color was observed to become whitish (Fig. 2).

Eosin perfusion: Eosin (Wako Pure Chemical Industries, Osaka, Japan) was dissolved in physiological saline to obtain a 10% solution and this solution was perfused into tilapia (359 - 404 g, n = 2) using the *in situ* perfusion technique. After the perfusion of 40 mL of eosin solution (about

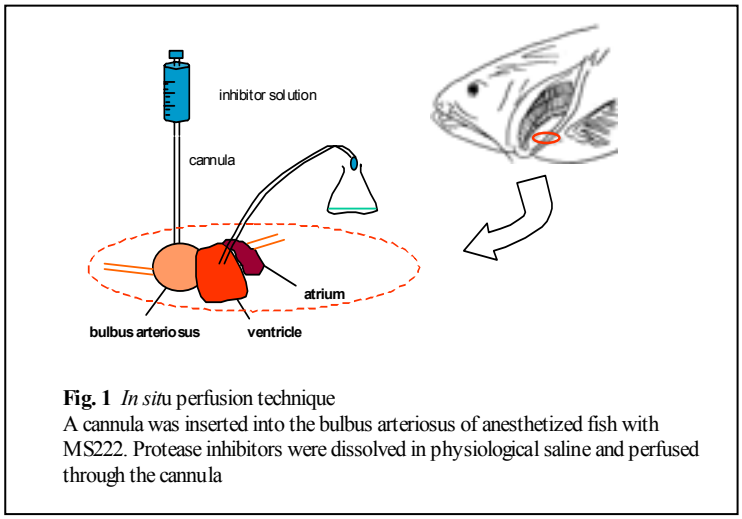

Fig. 1 *In situ* perfusion technique
A cannula was inserted into the bulbus arteriosus of anesthetized fish with MS222. Protease inhibitors were dissolved in physiological saline and perfused through the cannula

40 min), the cannula was removed and the upper dorsal muscle was dissected. The muscle section was observed under a fluorescence stereoscopic microscope (R-400; Edge Scientific Instruments, CA, USA) using ultraviolet light to confirm the occurrence of fluorescence from the eosin.

[^{35}S]-methionine perfusion: [^{35}S]-methionine (Amersham Biosciences, NJ, USA) was dissolved in physiological saline to obtain a 1,000 Bq/mL solution. After the perfusion of 25 mL [^{35}S]-methionine solution into the tilapia (448±71 g, n = 3), fish were individually dissected to obtain four samples of dorsal muscle and one liver sample. Each 1 g-tissue sample was dissolved in 0.1 M NaOH and mixed with scintillation cocktail (PICO-FLOUR 40, Packard Instrument, CT, USA), and the radioactivity was measured using a liquid scintillation analyzer (TRY-CARB 200CA, Packard Instrument).

Protease inhibitor perfusion: Each protease inhibitor, leupeptin (Peptide Institute, Osaka, Japan; 1 mg/mL, a serine and cysteine protease inhibitor), chymostatin (Peptide Institute, 1 mg/mL, a serine protease inhibitor), benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk, Peptide Institute, 1 mg/mL, a caspase inhibitor), and E-64 (Peptide Institute; 1 mg/mL, a cysteine protease inhibitor) was dissolved in physiological saline. *o*-phenanthroline (Aldrich Chemical, WI, USA, 1 mM, a metalloprotease inhibitor) was dissolved in a small volume of methanol and diluted with physiological saline. These inhibitor solutions and physiological saline as the control sample were perfused into fish for 60 min. Every fish was perfused with at least 30 mL perfusion solution. Each experimental group consisted of ten tilapias (867±195 g) or six yellowtails (608±84 g). The cannula was removed and the upper dorsal muscle was sliced to give 1-cm-width strips. The sample slices were wrapped in plastic film wrap individually and kept in a 4 °C refrigerator until each measurement time. The breaking strength of the slices as a parameter of muscle toughness was measured using a rheometer (NRM-20002J, Hudoh, Tokyo, Japan) equipped with a cylindrical plunger of 3 mm diameter. The plunger was applied at the interspace between the myocomma membranes at the cut surface of the dorsal muscle. Measurements were taken at five points for each strip and the average value was calculated. Because in our unpublished experiment of tilapia, E-64 was found not to be effective to prevent muscle softening, E-64 was not used for tilapia perfusion.

Results and discussion

***In situ* perfusion**: Approximately 30 min after the perfusion of 10 mL physiological saline, the color of the fluid from the polyethylene tube became clear in the case of a 200-g tilapia (Fig. 2). This technique excludes the effect of blood fluid in fish muscle, in which factors inducing muscle softening may exist (Ando *et al.* 1999), and is able to examine the effectiveness in muscle components than in blood fluid.

Fig. 2 Color change of the fluid from the ventricle. The tilapia was anesthetized with MS222 and physiological saline was perfused by a cannula inserted into the bulbus arteriosus (A). Finally after approximately 30 min (B), the liver color was observed to become whitish.

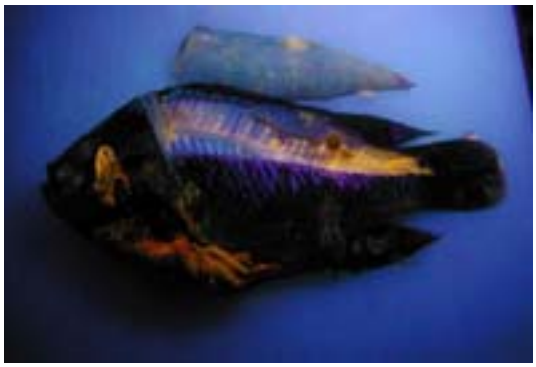

Fig. 3 Fluorescence in the whole body of the eosin-perfused tilapia. Tilapia was anesthetized with MS222 and eosin was perfused by a cannula inserted into the bulbus arteriosus. Orange fluorescence of eosin was observed in the muscle, gill, liver and intestine under ultraviolet light.

Eosin perfusion: Eosin was used for visible confirmation of the *in situ* perfusion technique. Visible fluorescence was observed in the solution from the polyethylene tube, which confirmed the occurrence of eosin, approximately 3 min after the start of the eosin perfusion. Eosin perfusion into the interior organs was confirmed under ultraviolet rays after the upper lateral muscle was removed. Visible fluorescence was observed in the gill, liver, intestine, and dorsal muscle of the eosin treated tilapia (Fig. 3). Eosin perfusion in the blood vessel of the dorsal muscle was confirmed under a fluorescence stereoscopic microscope with ultraviolet rays, and was not observed in the control muscle sample (Ishida *et al*.2003).

[^{35}S]- methionine perfusion: ^{35}S-methionine was used as a marker substance for quantitative analysis. The radioactivity measured in the ^{35}S-methionine perfused tilapia showed that this solution was taken into the dorsal muscle and liver at a rate of 7.8 μL/g and 70.2 μL/g, respectively. As the liver received the radioactive substance much more than the dorsal muscle did, the difference between the two organs possibly depended on the blood circulating volume. From these results of the eosin and [^{35}S]-methionine perfusion experiments, this perfusion technique was concluded to be suitable to introduce chemical substances into fish muscle and was applied to protease inhibitor perfusion in the further experiments.

Protease inhibitor perfusion into tilapia: Figure 4 shows the breaking strength of the tilapia in each inhibitor group. At the beginning of storage (0 h), the breaking strength of all the inhibitor-perfused tilapia was 144 - 197 gw with a large variance. At 23 h storage, significant difference was found in the leupeptin (140±36 gw, P = 0.0140) and Z-VAD-fmk (139±36 gw, P = 0.0162) - perfused tilapia, whereas no remarkable effect was seen in the chymostatin-perfused fish (104±25 gw). At 50.5 h storage, the breaking strength was 86 - 98 gw and statistic difference was not seen between the inhibitor-perfused tilapia. On the other hand, those of *o*-phenanthroline-perfused fish at even 0 h was significantly different from other inhibitors by analysis of variance. Then all the *o*-phenanthroline-perfused experiments were not analyzed statistically (23 h, 106±20 gw, 50.5 h, 86±11 gw) and were not able to be compared with the Kubota *et al*. (2001) that showed involvement of metalloprotease in muscle softening.

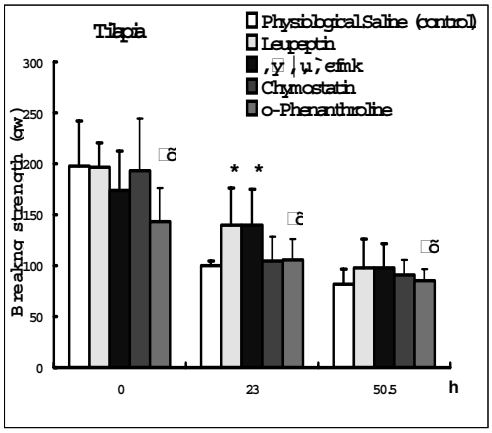

Fig.4 The breaking strength of the tilapia muscle stored under 4°C after the perfusion of the four types of protease inhibitors (leupeptin, Z-VAD-fmk, chymostatin and o-phenanthroline) into the replicate samples (n = 10). Vertical bars represent standard deviation.

* The breaking strength at 23 h storage of leupeptin and Z-VAD-fmk were significantly different by analysis of variance.
† The effect of o-phenanthroline at 23 and 50.5 h were not analyzed statistically, because the breaking strength of o-phenanthroline at 0 h was significantly different from other inhibitor groups by analysis of variance.

Protease inhibitor perfusion into yellowtail: Figure 5 shows the breaking strength of the yellowtail in each inhibitor group. At the beginning of storage (0 h), the breaking strength of all the inhibitor-perfused yellowtail was 191 - 235 gw with a large variance. The E-64 perfused yellowtail was slightly different at 9 h (199±31 gw, P = 0.1102) and significantly different at 46 h (143±13 gw, P = 0.0412) from other perfused fish.

Conclusions

In tilapia, leupeptin (serine and cysteine protease inhibitor) was effective to prevent the muscle softening and it is suggested that trypsin-like protease is the most probable candidate for causative protease of muscle softening. In addition, caspase-3 (Yabu *et al*. 2001) activity was induced in tilapia muscle after death and apoptosis might occur in muscle cells. In yellowtail, E-64 (cysteine protease inhibitor) was effective and cysteine protease was thought to be causative protease in muscle softening. These findings also showed that proteolysis was the one of causative factors for post-mortem muscle softening.

Acknowledgment

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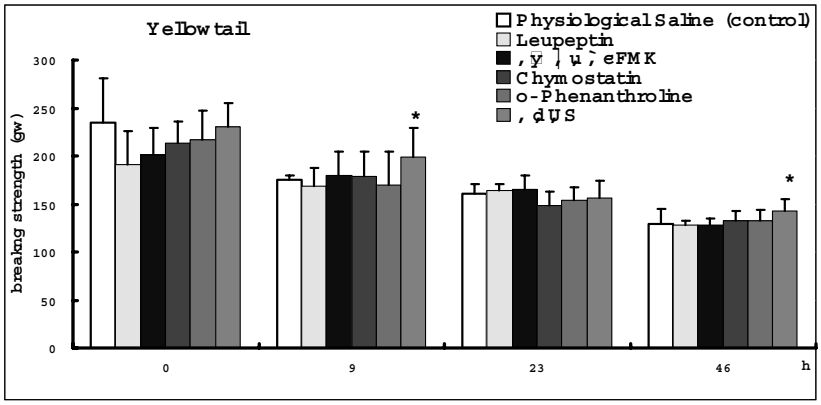

Fig.5 The breaking strength of the yellowtail muscle stored under 4° C after the perfusion of the five types of protease inhibitors (leupeptin, Z-VAD-fmk, chymostatin, o-phenanthroline and E-64) into the replicate samples (n = 6). Vertical bars represent standard deviation.

* The breaking strength of E-64 perfused fish at 9 h and 23 h were different by analysis of variance.

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