The Influence of Characteristics of Actomyosin on the Difference of Rigor Mortis Progress between Cultured Red Sea Bream and Japanese Flounder

Kyung Hee Lee, Mutsuyosi Tsuchimoto, Osamu Yada,
Tosio Misima, Abdul Jabarsyah, Qin Wang,
Paula Andrea Gómez Apablaza, and Katsuyasu Tachibana

Laboratory of Fishery Nutritional Sciences, Graduate School of Marine Science and Engineering,
Nagasaki University, Nagasaki 852-8131, Japan

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As part of a study program to clarify the cause of differences in rigor mortis progress between cultured red sea bream and Japanese flounder, the superprecipitation reaction, the Mg^{2+} -ATPase activity, and the Ca^{2+} -sensitivity of actomyosin were measured at several Ca^{2+} and ATP concentrations, and these discrepant points were examined between both fishes. The superprecipitation reaction became higher with the increase of Ca^{2+} concentration from pCa 7.0 to pCa 5.0 and with the decrease of ATP concentration from 3.0 mM to 0.5 mM in both fishes, and the level was remarkably lower in cultured Japanese flounder than in cultured red sea bream. In both the Mg^{2+} -ATPase activity and the Ca^{2+} -sensitivity, similarly changing aspects were observed and these levels were also remarkably lower in cultured Japanese flounder than in cultured red sea bream. Although the significantly positive correlation was found between the superprecipitation reaction and the Mg^{2+} -ATPase activity in both fishes, the distributive position of both values was clearly different between both fishes, and the superprecipitation reaction was higher in cultured Japanese flounder than in cultured red sea bream when the Mg^{2+} -ATPase activity was the same level. This results suggest that the characteristics of actomyosin corresponding to muscular contraction might be remarkably different between both fishes and provide support to the difference of rigor mortis progress between both fishes.

Key words: cultured red sea bream, cultured Japanese flounder, actomyosin, superprecipitation reaction, Mg^{2+} -ATPase activity, Ca^{2+} -sensitivity, rigor mortis progress

It has been known that the rigor mortis progress in fish differs remarkably among fish species,^{1,2)} habitat temperatures,^{3,4)} storage temperatures,²⁻¹¹⁾ and killing procedures,^{9,11-13)} then again between cultured and wild fish of the same fish species.^{1,9,10)} In regard to the cause of these differences, they are attributed to several causes as the resolution speed of total ATP and the accumulation of lactic acid,^{2-4,6,7,9-14)} myofibrillar Mg^{2+} -ATPase activity,^{1,3,8,14,15)} and Ca^{2+} uptake by sarcoplasmic reticulum.^{3,8)}

Regarding the difference between cultured red sea bream and Japanese flounder, it is already reported that the rigor mortis progress of cultured Japanese flounder is very late in reaching the maximum level and is remarkably lower in the maximum level than that of cultured red sea bream.⁵⁾ Watabe *et al.*⁸⁾ examined the differences of the myofibrillar Mg^{2+} -ATPase activity and Ca^{2+} uptake by sarcoplasmic reticulum between wild red sea bream and Japanese flounder. Tsuchimoto *et al.*¹⁾ considered that the difference of rigor mortis progress among several fishes might be due to the Ca^{2+} concentration around the myofibrils. However, there have been few studies on the difference in the rigor mortis progress between both fishes. Therefore, the cause of the difference in rigor mortis progress between both fishes has not been examined thoroughly yet. On the other hand, Wang *et al.*¹⁶⁾ measured the superprecipitation reaction and the Mg^{2+} -ATPase activity of actomyosin to clarify

the cause of the difference in rigor mortis progress between cultured and wild red sea bream, and considered that the difference might be due to the actomyosin characteristics.

Therefore, we attempted to clarify the cause of the difference in rigor mortis progress between cultured red sea bream and Japanese flounder. As a part of this study program, we compared and examined the characteristics of actomyosin between cultured red sea bream and Japanese flounder.

Material and Methods

Sample Fish

The cultured (omitted hereafter) red sea bream *Pagrus major* and the cultured (omitted hereafter) Japanese flounder *Paralichthys olivaceus*, which are already known to be remarkably different in the speed, pattern, and time and maximum level reached of rigor mortis progress,¹⁶⁾ were used as sample fish species. The two years-old fish had been cultivated in Toisu Bay, Nagasaki Prefecture, and were purchased from a fish farmer in the summer at a habitat temperature near 25°C. These sample fish were carried back to the laboratory alive and held in FRP tanks with water at 25°C. To adapt these fish to 25°C because of the slightly different collecting time of purchase, they were

Table 1. The standard body length and body weight of sample fish used for experiments of superprecipitation reaction, Mg^{2+} -ATPase activity, and Ca^{2+} -sensitivity of actomyosin

Species	Code no.	Standard body length (cm)	Body weight (g)
Cultured red sea bream	CR-35	29.2	762.5
	CR-36	28.2	726.7
	CR-37	29.9	837.1
n	3	3	3
Mean $\pm$ S.D.		29.1 $\pm$ 0.9	775.4 $\pm$ 56.3
Cultured Japanese flounder	CJ-30	31.8	557.6
	CJ-31	31.2	511.2
	CJ-32	32.2	569.0
n	3	3	3
Mean $\pm$ S.D.		31.7 $\pm$ 0.5	545.9 $\pm$ 30.6

held continuously in tanks with water at a habitat temperature of 25°C for two months or more and fed on moist pellets daily. After this, the fish were used as experimental sample fish.

Furthermore, the body size of sample fish is shown in Table 1. The mean values of standard body length and body weight were 29.1 $\pm$ 0.9 cm and 775.4 $\pm$ 56.3 g in red sea bream, and 31.7 $\pm$ 0.5 cm and 545.9 $\pm$ 30.6 g in Japanese flounder, respectively (Table 1). The deviations of body size among fish specimens were very small for both fishes.

Preparation of Actomyosin

We followed the methods of Takashi *et al.*¹⁷⁾ and Konno *et al.*¹⁸⁾ in the preparation of actomyosin. The dorsal ordinary muscle of sample fish was minced. This mince of 10 g was homogenized in three times as much volume of low ion strength phosphate buffer ($I=0.05$, pH 7.5). The homogenate was centrifuged for 10 min at 3,000 $\times g$. Redispersion and centrifugation were repeated 4 times with stirring. Three times as much volume of 0.6 M KCl phosphate buffer (pH 7.5) was added to the residue to dissolve actomyosin, which was centrifuged for 20 minutes at 20,000 $\times g$ after 2 h with occasional stirring. The supernatant was dropped little by little into 10 times as much volume of cold distilled water, which was then adjusted to pH 6.6 with 5% acetic acid and centrifuged for 10 minutes at 6,000 $\times g$. The residue was dissolved in 3 M KCl to bring the final concentration of KCl to 0.6 M, which was dialyzed overnight against 0.6 M KCl, 20 mM Tris-maleate buffer (pH 7.5), then centrifuged for 1 h at 20,000 $\times g$. Well-washed actomyosin was acquired from the supernatant. These procedures were carried out at low temperature, less than 4°C.

Superprecipitation Reaction of Actomyosin

We followed the method of Ebashi¹⁹⁾ in the measurement of the superprecipitation reaction. Before measurement, the prepared actomyosin was diluted in 20 times as much volume of cold 0.3 mM $NaHCO_3$, which was centrifuged for 10 min at 6,000 $\times g$. The actomyosin from the residue was suspended in 0.3 mM $NaHCO_3$, which then was used for measurement. Before that, the concentration of actomyosin in suspension was colorimetrically moni-

tored using the biuret method²⁰⁾ and adjusted to 0.6 mg protein/ml by diluting with this medium. KCl- Mg^{2+} buffer solution of 0.9 ml containing 0.1 M KCl, 1 mM $MgCl_2$, and 20 mM Tris-maleate as final concentration, 1 ml of Ca-EGTA[Ethylene Glycol-O,O'-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid] Buffer^{21,22)} amounting to the requisite concentration, and 1 ml of actomyosin suspension were put into a quartz cell of 4 ml in total volume and 1 cm in light path, then stirred. After actomyosin in the reaction media was preincubated until it reached the reaction temperature of 25°C, the superprecipitation reaction was started by adding 0.1 ml of ATP solution amounting to the requisite concentration and was measured at 660 nm with the lapse of time under such conditions by stirring with a magnetic stirrer. The measurement time for each was 0 s, 15 s, 30 s, 1 min, 1.5 min, 2 min, 3 min, and 4 min. Further, the combinations of Ca^{2+} concentration of Ca-EGTA buffer and ATP concentration of substrate solution in final concentration were carried out in two cases each of which were pCa 7.0, 6.5, 6.0, 5.0, 4.0, and 3.0 at constant 1.0 mM ATP and 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mM ATP each at constant pCa 5.0, respectively.

Mg^{2+} -ATPase Activity of Actomyosin

The Mg^{2+} -ATPase activity of actomyosin was measured in the same reaction media as the two cases of superprecipitation reaction, and furthermore each was also measured at 0.040, 0.045, 0.060, 0.090, and 0.190 mM of low ATP concentration and a half concentration (0.3 mg protein/ml) of actomyosin suspension at constant pCa 5.0. The reaction was carried out at the same temperature, 25°C, as the cases of superprecipitation reaction for 0, 1, 2, 3, 4, and 5 min and was terminated by adding 1 ml of 40% PCA. The activity was measured by analyzing the liberated inorganic phosphate²³⁾ in the cases of high ATP concentrations, and by analyzing the degraded ATP and produced ADP in another case of low ATP concentrations using the method of Tsuchimoto *et al.*²⁴⁾ after extraction by the method of Ehira *et al.*²⁵⁾ The reaction velocity was calculated by the following formula because the activity showed a first-order reaction. $v=2.303 \times So \times \phi$ (So : initial substrate concentration; ϕ : slope of regression line between time and log ATP concentration).

Ca^{2+} -sensitivity of Actomyosin Mg^{2+} -ATPase

Ca^{2+} -sensitivity was calculated from actomyosin Mg^{2+} -ATPase activity in the presence of either Ca^{2+} (pCa 7.0, 6.5, 6.0, 5.0, 4.0, and 3.0) or EGTA under constant 1.0 mM ATP as follows.^{26,27)}

Ca^{2+} -sensitivity(%)

$$= \left(1 - \frac{\text{ATPase activity in the absence of } Ca^{2+}}{\text{ATPase activity in the presence of } Ca^{2+}} \right) \times 100$$

Results

Superprecipitation Reaction of Actomyosin

The change of superprecipitation reaction of actomyosin with the increase of Ca^{2+} concentration is shown in Fig. 1. The superprecipitation reaction became higher with the increase of Ca^{2+} concentration from pCa 7.0 to pCa 5.0 for both fishes. Within the range from pCa 5.0 to pCa

Fig. 1. The change of superprecipitation reaction of actomyosin with the increase of Ca^{2+} concentration within the range from pCa 7.0 to pCa 3.0 at constant 1.0 mM ATP.

3.0, however, it showed almost no change although that in Japanese flounder decreased slightly from pCa 5.0 toward pCa 4.0. Therefore, the superprecipitation reaction reached the maximum level at near pCa 5.0 for both fishes. Regarding the level between both fishes, the level was remarkably lower in Japanese flounder than in red sea bream at all Ca^{2+} concentrations with the exception of near pCa 7.0 and the difference was magnified with the increase of Ca^{2+} concentration.

On the other hand, the change with the decrease of ATP concentration from 3.0 mM to 0.5 mM is shown in Fig. 2. The superprecipitation reaction became higher with the decrease of ATP concentration for both fishes. In comparison of the level between both fishes, the level was also remarkably lower in Japanese flounder than in red sea bream at all ATP concentrations. This difference was very similar to the result (Fig. 1) with the increase of Ca^{2+} concentration. The difference, however, exhibited a tendency to become remarkably smaller with the decrease of ATP concentration.

Mg^{2+} -ATPase Activity of Actomyosin

The change of actomyosin Mg^{2+} -ATPase activity with the increase of Ca^{2+} concentration is shown in Fig. 3. The activity became higher in response to the increase of Ca^{2+} concentration from pCa 7.0 to pCa 5.0 for both fishes. Within the range from pCa 5.0 to pCa 3.0, however, it showed almost no change although that in Japanese flounder decreased slightly from pCa 5.0 toward pCa 4.0. These aspects were very similar to the result (Fig. 1) of superprecipitation reaction in the same reaction medium. The level was remarkably lower in Japanese flounder than in red sea bream at all Ca^{2+} concentrations, then the difference between both fishes became remarkably larger with the increase of Ca^{2+} concentration although it was very small at low Ca^{2+} concentrations of near pCa 7.0.

Fig. 2. The change of superprecipitation reaction of actomyosin with the decrease of ATP concentration within the range from 3.0 mM to 0.5 mM at constant pCa 5.0.

Fig. 3. The change of actomyosin Mg^{2+} -ATPase activity with the increase of Ca^{2+} concentration within the range from pCa 7.0 to pCa 3.0 at constant 1.0 mM ATP.

On the other hand, the change of the activity with the decrease of ATP concentration from 3.0 mM to 0.5 mM and from 0.19 mM to 0.04 mM is shown in Fig. 4. The activity became higher with the decrease of ATP concentration

Fig. 4. The change of actomyosin Mg^{2+} -ATPase activity with the decrease of ATP concentration within the range from 3.0 mM to 0.04 mM at constant pCa 5.0.

from 3.0 mM to 0.5 mM for both fishes. This aspect was also very similar to the result (Fig. 2) of superprecipitation reaction in the same reaction medium. Furthermore, this result suggested that the actomyosin Mg^{2+} -ATPase activity could be inhibited by the substrate ATP within the range from 3.0 mM to 0.5 mM for both fishes. Regarding the comparison of level between both fishes, the level was also remarkably lower in Japanese flounder than in red sea bream at all ATP concentrations. This difference was also very similar to the foregoing result (Fig. 2) of the superprecipitation reaction in the same reaction medium, although the difference became larger with the decrease of ATP concentration by contrast to the foregoing result.

Furthermore, within the lower range from 0.190 mM ATP to 0.040 mM ATP, the activity in red sea bream increased remarkably with the decrease of ATP concentration in succession from the foregoing result. On the contrary, the activity in Japanese flounder exhibited no change or decreased slightly with the decrease of ATP concentration. Namely, the change within the lower range of ATP concentration differed remarkably between both fishes. Therefore, the level was remarkably lower in Japanese flounder than in red sea bream at all low ATP concentrations, then the difference was magnified remarkably with the decrease of ATP concentration.

Ca^{2+} -sensitivity of Actomyosin Mg^{2+} -ATPase

The change of Ca^{2+} -sensitivity of actomyosin Mg^{2+} -ATPase with the increase of Ca^{2+} concentration is shown in Fig. 5. The Ca^{2+} -sensitivity became higher in response to the increase of Ca^{2+} concentration from pCa 7.0 to pCa 5.0 for both fishes. Within the range from pCa 5.0 to pCa 3.0, however, it showed no change although that in Japanese flounder decreased slightly from pCa 5.0 toward pCa 4.0. Therefore, the Ca^{2+} -sensitivity reached the maximum level at near pCa 5.0 for both fishes. In comparison of the level between both fishes, the level exhibited a ten-

Fig. 5. The change of Ca^{2+} -sensitivity of actomyosin Mg^{2+} -ATPase with the increase of Ca^{2+} concentration within the range from pCa 7.0 to pCa 3.0 at constant 1.0 mM ATP.

dency to be lower in Japanese flounder than in red sea bream at all Ca^{2+} concentrations, and the difference between both fishes was almost constant at all Ca^{2+} concentrations.

Relationship between Mg^{2+} -ATPase Activity and Superprecipitation Reaction of Actomyosin

The relationship between Mg^{2+} -ATPase activity and superprecipitation reaction of actomyosin at several Ca^{2+} and ATP concentrations is shown in Fig. 6. The relation between both values exhibited the significantly positive correlation that the superprecipitation reaction of actomyosin became higher in response to the increase of actomyosin Mg^{2+} -ATPase activity in the total number of fish specimens and in both fishes. The distributive positions, however, were remarkably different between both fishes, and exhibited a tendency to be distributed more upward in Japanese flounder than in red sea bream. The regression line and coefficient of correlation between the superprecipitation reaction (Y) and Mg^{2+} -ATPase activity (X) of actomyosin were calculated as $Y=0.728X-0.017$, $n=35$, $r=0.880$ ($p<0.001$) in red sea bream and $Y=1.471X-0.032$, $n=36$, $r=0.817$ ($p<0.001$) in Japanese flounder. The slope value of regression line was remarkably larger, about two times in Japanese flounder than in red sea bream. This result suggested that the increasing rate of superprecipitation reaction in relation to the increase of Mg^{2+} -ATPase activity was different between both fishes and was remarkably larger in Japanese flounder than in red sea bream. Furthermore, these results suggested that the superprecipitation reaction was higher in Japanese flounder than in red sea bream when the actomyosin Mg^{2+} -ATPase activity was at the same level, and

Fig. 6. The relationship between actomyosin Mg^{2+} -ATPase activity and superprecipitation reaction at several Ca^{2+} and ATP concentrations.

the difference was magnified with the increase of Mg^{2+} -ATPase activity.

Discussion

It is generally known that the Ca^{2+} concentration around the myofibrils in live muscle is controlled within the range from pCa 7.0 in relaxation to pCa 5.0 in strain by the sarcoplasmic reticulum.²⁸⁾ We, however, carried out the experiment not only at these physiological Ca^{2+} concentrations but also at higher Ca^{2+} levels beyond pCa 5.0, because these phenomena were thought of as cases in fish muscle after death. The results of no change in both the superprecipitation reaction and the Mg^{2+} -ATPase activity beyond pCa 5.0 in both fishes differed from the finding by Yamada *et al.*²⁹⁾ which decreased remarkably at high Ca^{2+} levels beyond pCa 5.0 in both the parameters of myosin B in carp. Also, they considered that these phenomena were caused by the decrease of Ca^{2+} sensitivity in Mg^{2+} -ATPase of myosin B at high Ca^{2+} levels beyond pCa 5.0. Our result in red sea bream, however, exhibited that the Ca^{2+} sensitivity of actomyosin Mg^{2+} -ATPase also did not change at Ca^{2+} levels beyond pCa 5.0. From our results, it was suggested that the rigor mortis progress was the fastest and highest at pCa 5.0 for both fishes, and it did not exhibit a remarkable change at Ca^{2+} levels beyond pCa 5.0.

In the changes of superprecipitation reaction and Mg^{2+} -

ATPase activity of actomyosin with the decrease of ATP concentration (Figs. 2 and 4), the former result of superprecipitation reaction agreed with the finding by Suzuki *et al.*^{30,31)} that it was higher at 0.5 mM ATP than at 1.0 mM ATP, and the latter result of actomyosin Mg^{2+} -ATPase activity also agreed with the finding by Perry *et al.*³²⁾ Regarding the cause of these phenomena, Perry *et al.*³²⁾ considered that the activity was inhibited by substrate ATP because free ATP was not split but was bound at the enzymatically active sites of the actomyosin system. The aspect of substrate inhibition, however, disappeared at lower ATP concentrations less than 0.2 mM in Japanese flounder (Fig. 4). Therefore, from these results it was considered that the rigor mortis progress was smaller at high ATP concentration than at low ATP concentration.

Comparing the levels of superprecipitation reaction and Mg^{2+} -ATPase activity of actomyosin between both fishes, the distributive position of both values differed clearly between both fishes and the superprecipitation reaction was higher in Japanese flounder than in red sea bream when the Mg^{2+} -ATPase activity was at the same level (Fig. 6). This phenomenon suggested that the contractile characteristic of actomyosin in ordinary muscle might be remarkably different between both fishes. Furthermore, it was considered that the difference of contractile characteristic in actomyosin might be due to the difference of muscle fiber type in ordinary muscle between both fishes, because the ordinary muscle of most red sea bream was organized from two muscle fibers as two white muscle fibers of IIa (comparatively aerobic fast muscle fiber) and IIb (anaerobic fast muscle fiber) subtypes or one white muscle fiber of IIa or IIb subtype and pink muscle fiber,³³⁾ while that of Japanese flounder was organized from only one white muscle fiber of IIa subtype. Regarding this consideration, it is generally known that the organization of dorsal skeletal muscle in fish is arranged in three distinct layers of dark muscle, intermediate muscle, and ordinary muscle, and these muscles are composed of red muscle fibers (type I), pink muscle fibers, and white muscle fibers (type II), respectively. Buttkus³⁴⁾ reported that the rigor contraction and rigor tension of muscle in lingcod *Ophiodon elongatus* were about three times greater in red muscle than in white muscle. Furthermore, we reported that the organization of muscle fiber types in ordinary muscle was remarkably different among fishes, and it was found that the maximum level reached in rigor mortis progress exhibited a consistent phenomenon to be higher in fishes with the presence of pink muscle fiber in ordinary muscle than in fishes with the absence of pink muscle.³⁵⁾ Thus, our results of superprecipitation reaction and Mg^{2+} -ATPase activity of actomyosin provided support to the difference of rigor mortis progress between red sea bream and Japanese flounder.

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