Comparison of Interposition Ratios of Pink Muscle Fiber in Dorsal Ordinary Muscle among Various Marine Fishes

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The difference in interposition ratio of pink muscle fiber among various fish species was studied to learn its influence in dorsal ordinary muscle on the post-mortem change of *K*-value. A remarkable mosaic pattern was exhibited in the interpositioned white and pink muscle fibers in many of the species. The relative number and area percentages of these fibers to all muscle fiber types differed greatly among fish species, and distributed from 12.9% to 56.53% in a number, and from 2.22% to 53.68% in an area. The relationship between the area and numerical percentages had significantly high positive correlation. Size of the pink muscle fiber was somewhat smaller than that of white muscle fiber.

Keywords: dorsal ordinary muscle, interposition ratio of pink muscle fiber, size of pink muscle fiber, fish species

Organization of the dorsal skeletal muscle in fish is arranged in three distinct layers of dark muscle, intermediate muscle, and ordinary muscle. Each of these is organized in a single muscle fiber type as follows: red muscle fibers (Type I), pink muscle fibers, and the white muscle fibers (Type II), respectively (Johnston *et al.*, 1974; Gill *et al.*, 1982; Carpena *et al.*, 1982; Hoyle *et al.*, 1986). The red muscle fiber is small in diameter and relies on an aerobic form of metabolism, while the white muscle fiber has a large diameter and operates principally by anaerobic glycolysis. Functionally, the former is implicated in sustained slow swimming and the latter is in bursts of violent activity (Matsuoka & Iwai, 1984). The white muscle fibers are subdivided into Types IIa and IIb, the IIa subtype being used not only for anaerobic metabolism but also for aerobic metabolism, and the IIb subtype especially for anaerobic metabolism in response to typical escape and attack movement; the pink muscle fiber is more particularly for aerobic metabolism than the IIa subtype (Jabarsyah *et al.*, 1999a). The pink muscle fiber is characterized as fast contracting with intermediate resistance to fatigue and intermediate speed of shortening between red and white muscle fibers (Johnston *et al.*, 1977). Most histochemical studies in fish muscle have involved these distinct layers, and only a few studies have dealt with the differences in fiber types in ordinary muscle among various fishes. Korneliussen *et al.* (1978) and Walesby and Johnston (1980) reported that a boundary region between the layers of intermediate muscle and ordinary muscle was not remarkable in the cod *Gadus morhua*, and that the Antarctic teleost *Notothenia rosii* had no pink muscle fiber in its locomotory muscles. In our previous study, we reported that there were not only white muscle fibers but also pink muscle fibers in the dorsal ordinary muscle of many fish species, and that the fiber type in

this muscle was differed remarkably among species, between wild and cultured fish of the same species, and among regions along the length of the body and toward the neural spine (Jabarsyah *et al.*, 1999a).

We also reported that the presence of pink muscle fiber in the ordinary muscle raised the maximum level in rigor mortis in various species (Jabarsyah *et al.*, 1999b), and accelerated the temporal change of *K*-value (one of the freshness indices) in the carp (Yada *et al.*, 2000). However, the influence of muscle fiber type on the temporal change of *K*-value, especially by the presence or absence of pink muscle fiber in the ordinary muscle has not yet been examined thoroughly. In the present study, we attempted to identify the difference in the interposition ratio of this fiber in the dorsal ordinary muscle.

Materials and Methods

Sample fish The twenty-seven fish species used as samples in this study are shown in Table 1. They were collected by fishing from the sea adjacent to the Sakishima Islands in Okinawa prefecture at a habitat temperature of 28°C, and from the sea adjacent to Nagasaki prefecture at a habitat temperature of 17°C during the summer season. The only exception was one cultured species, red sea bream, which was purchased from a local fish farmer in Nagasaki prefecture during the same season. All sample fishes were transferred to the laboratory alive and held in a FRP tank with water similar to their habitat temperatures.

Histological preparation The fish were killed by cutting the hindbrain after anesthetizing them in seawater containing 100 ppm MS-222. Immediately after killing, several muscle blocks (5 mm×5 mm×5 mm) were cut off from the dorsal ordinary muscle underneath the lateral line near the mid-section of the fish's body length. The tissues were mounted on a cryostat chunk and embedded in a commercial mounting medium. They were frozen immediately for 10–15 s by immersion in isopentane, which was

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Table 1. Sample fish species used in the present study.

Waters	Code number	Standard Japanese name	English name	Scientific name
Sea adjacent to Sakishima Islands at 28°C	101	Nanyokaiwari	Kingfish	<i>Caranx ferdau</i>
	102	Shimamochinouo	Barred wrasse	<i>Cheilinus fasciatus</i>
	103	Nokogiridai	Gold-lined sea bream	<i>Gnathodentex aurolineatus</i>
	104	Shirodai	Drab large-eye bream	<i>Gymnocranius japonicus</i>
	105	Amifuefuki	Half-belted emperor	<i>Lethrinus semicinctus</i>
	106	Yosujifuedai	Blue and Yellow snapper	<i>Lutjanus kasmira</i>
	107	Ojisan	Five-barred goatfish	<i>Parupeneus trifasciatus</i>
	108	Kitsuneuo	Yellowstriped whiptail	<i>Pentapodus macurus</i>
	109	Nijihata	Flagtail rock-cod	<i>Cephalopholis urodelus</i>
	110	Akahata	Black-tipped grouper	<i>Epinephelus fasciatus</i>
	111	Ojirobarahata	A kind of rock cod	<i>Variola albimarginata</i>
	112	Kotohiki	Threestriped tigerfish	<i>Terapon jarbua</i>
Total		12		
Sea adjacent to Nagasaki at 17°C	201	Bora	Striped mullet	<i>Mugil cephalus</i>
	202	Maruaji	Round scad	<i>Decapterus maruadsi</i>
	203	Maaji	Horse mackerel	<i>Trachurus japonicus</i>
	204	Sasanohabera	Bambooleaf wrasse	<i>Pseudolabrus japonicus</i>
	205	Ishidai	Japanese parrot fish	<i>Oplegnathus fasciatus</i>
	206	Isaki	Threeline grunt	<i>Parapristipoma trilineatum</i>
	207	Kagokakidai	Footballer	<i>Microcanthus strigatus</i>
	208	Madai	Red sea bream (cultured)	<i>Pagrus major</i>
	209	Masaba	Pacific mackerel	<i>Scomber japonicus</i>
	210	Aigo	Dusky spinefoot	<i>Siganus fuscescens</i>
	211	Izukasago	Izu scorpion fish	<i>Scorpaena izensis</i>
	212	Usubahagi	Unicorn filefish	<i>Aluterus monoceros</i>
	213	Umazurahagi	Black scraper	<i>Navodon modestus</i>
	214	Kawahagi	Filefish	<i>Stephanolepis cirrhifer</i>
	215	Kitamakura	Scribbled tody	<i>Canthigaster rivulata</i>
Total		15		

cooled to its melting point (-165°C) with liquid nitrogen, then placed in a refrigerated cryostat cabinet at -25°C for 1 h. Serial cross sections were then cut at a thickness of about $10\text{ }\mu\text{m}$, mounted on glass cover slips and dried at room temperature.

Preincubation procedure The preincubation procedure of cross sections was carried out according to the method modified by Guth and Samaha (1969), which is commonly used in mammalian studies involving the discrimination of muscle fiber types in skeletal muscle. The method used most frequently to distinguish among muscle fiber types in mammals is dependent on the histochemical actomyosin ATPase activity. By exposing frozen sections to acid or alkaline preincubation before staining for ATPase, it can be shown that the high-ATPase fibers (Type II) are alkali-stable and acid-labile, whereas low-ATPase fibers (Type I) are acid-stable and alkali-labile (Guth & Samaha, 1969). In addition, the fibers having high-ATPase activity (Type II) could be further subdivided into Types IIa and IIb. Brooke and Kaiser (1970) reported that two subtypes of Type II muscle fibers in adult humans could be distinguished successfully on the basis of the acid stability of actomyosin ATPase activity.

A series of seven cross sections was preincubated at each of various pH within the range of pH 4.20–pH 4.80 and 15°C for 0, 0.5, 1.0, 1.5, 2.0, 3.0, and 4.0 min in an acid preincubation solution of 0.1 M potassium acetate buffer containing 18 mM CaCl_2 . Another series of seven cross sections was preincubated at each of various pH within the range of pH 10.35–pH 10.80 and 20°C for 0, 5, 10, 15, 20, 25, and 30 min in an alkaline preincubation solution of 0.1 M 2-amino-2-methyl-1-propanol (AMPro) buffer containing 18 mM CaCl_2 . This procedure involves selective inhibition of muscle fiber types by preincubation under either an acid or alkaline condition. If the pH of acid preincubation was lower or higher than the pH suitable for the same preincubation times,

the actomyosin ATPase activities of muscle fiber types were completely activated or inactivated, and could not be distinguished. The pH inactivation of the ATPase activity was critically dependent on the pH of solution and also on the length of time for which the preincubation was carried out. Small variations in either pH or period made a great difference in the staining pattern of actomyosin ATPase. These two variations (preincubation pH and times) were interdependent. Therefore, we examined the combination of pH and time in all samples, and determined the most suitable condition.

After preincubation under acid and alkaline conditions, the sections were rinsed with 0.1 M AMPro buffer (pH 9.4) containing 18 mM CaCl_2 and 50 mM KCl.

Staining procedure The two previous series of rinsed cross sections were reacted at 20°C for 20 min in a medium of 0.1 M AMPro buffer (pH 9.40) containing 18 mM CaCl_2 , 50 mM KCl, and 3.1 mM ATP, according to the method described by Guth and Samaha (1969). The reacted sections were rinsed with distilled water and immersed in 1% CaCl_2 for 3 min, then immersed in 2% CoCl_2 for 3 min. Finally, the production of phosphate from ATP under the remaining activity of actomyosin ATPase was displaced as cobaltous sulfide using 1% $(\text{NH}_4)_2\text{S}_x$.

Estimation of number and area percentage of pink muscle fibers in dorsal ordinary muscle The relative values (%) of number and area of pink muscle fibers to all muscle fiber types in the dorsal ordinary muscle were estimated using enlarged photographs of the alkaline preincubated section. The area percentage was measured by a tracing device linked to a digital computer (FLOVEL, measure unit MC 300, FLOVEL Co., Ltd., Tokyo).

Results

Muscle fiber types in dorsal ordinary muscle The opti-

mum combination of pH and time for the discrimination of muscle fiber types was examined under both acid and alkaline preincubations for all sample fish species. Photographs of the staining results after both preincubations under optimum conditions are shown in Fig. 1. The results in all sample fish species are classified according to the composition of muscle fiber type in Table 2.

In the drab large-eye bream (code no. 104), all muscle fibers

were stained very weakly at the same intensity both after acid preincubation at pH 4.50 and 1.5 min and after alkaline preincubation at pH 10.40 and 15 min. Therefore, the ordinary muscle of this fish species was composed of only the IIa or IIb-like subtype of white muscle. This same composition of muscle fiber types was not observed in other sample species.

In the threestriped tigerfish (code no. 112), the muscle fibers exhibited a remarkable mosaic pattern after acid preincubation at

Fig. 1. Discrimination of muscle fiber types in dorsal ordinary muscle by stability of actomyosin ATPase activity after acid and alkaline preincubations for four fish species. The W-IIa, W-IIb, and P in photographs denote IIa-like subtype of white muscle, IIb-like subtype of white muscle, and pink muscle, respectively.

pH 4.50 and 1.5 min. Some fibers of similar size were stained strongly and observed among the unstained fibers. However, these were unstained in the same way as other muscle fibers after alkaline preincubation. They were therefore determined to belong to the IIb-like subtype of white muscle, and the ordinary muscle in this fish species was composed of IIa and IIb-like subtypes of white muscle. The mosaic appearance after only acid preincubation was also seen in two fish species (code nos. 208 and 210) from Nagasaki as shown in Table 2.

In the blue and yellow snapper (code no. 106), the actomyosin ATPase activity remained on some muscle fibers not only after acid preincubation but also after alkaline preincubation, and the distinguishable mosaic pattern in these fibers was observed at both preincubations; all muscle fibers of acid stable actomyosin ATPase were also stable at alkaline preincubation. Therefore, the ordinary muscle in this fish species was composed of IIa or IIb-like subtype of white muscle and pink muscle. The same composition of muscle fiber types was observed in nine fish species

(code nos. 103, 105, 106, 107, 108, 209, 212, 213, and 214) from the Sakishima Islands and Nagasaki. However, the relative values of number and area of pink muscle fibers in all muscle fiber types exhibited a considerably different tendency among these species.

In the bambooleaf wrasse (code no. 204), the muscle fibers exhibited a remarkable mosaic pattern after both acid and alkaline preincubations, the same as the foregoing fish species. Although some fibers retained actomyosin ATPase activity after acid preincubation, these disappeared at an alkaline preincubation. Only much smaller muscle fibers were stained strongly after alkaline preincubation as well. Therefore, the ordinary muscle in this fish species was organized from IIa and IIb-like subtypes of white muscle and pink muscle. The composition of three muscle fiber types the same as this species was observed in five other species (code nos. 101, 102, 109, 110, and 111) from the Sakishima Islands and in nine species (code nos. 201, 202, 203, 204, 205, 206, 207, 211, and 215) from Nagasaki. However, the rela-

Fig. 2. Comparison of number and area of pink muscle fibers in dorsal ordinary muscle among three fish species. Indications of W-IIa, W-IIb, and P in the photographs are the same as in Fig. 1.

Table 2. Classification of dorsal ordinary muscle to four compositions of muscle fiber types in various fish species.

Waters	Muscle fiber type	Code number	Fish species
Sea adjacent to Sakishima Islands at 28°C	Ila or IIb	104	Drab large-eye bream
	Ila+IIb	112	Threestriped tigerfish
	Ila or IIb+Pink	103	Gold-lined sea bream
		105	Half-belted emperor
		106	Blue and Yellow snapper
		107	Five-barred goatfish
		108	Yellowstriped whiptail
	Ila+IIb+Pink	101	Kingfish
		102	Barred wrasse
		109	Flagtail rock-cod
		110	Black-tipped grouper
		111	A kind of rock cod
	Total	12	
Sea adjacent to Nagasaki at 17°C	Ila or IIb	—	—
	Ila+ IIb	208	Red sea bream (cultured)
		210	Dusky spinefoot
	Ila or IIb+Pink	209	Pacific mackerel
		212	Unicorn filefish
		213	Black scraper
		214	Filefish
	Ila+IIb+Pink	201	Striped mullet
		202	Round scad
		203	Horse mackerel
		204	Bambooleaf wrasse
		205	Japanese parrot fish
		206	Threeline grunt
		207	Footballer
		211	Izu scorpion fish
		215	Scribbled today
	Total	15	

Ila, IIb, and Pink denote muscle fiber types of Ila and IIb-like subtypes of white muscle and pink muscle fibers, respectively.

tive values of number and area of pink muscle fibers showed a considerably different tendency among these fish species as well.

Number and area of pink muscle fiber in dorsal ordinary muscle The remarkable differences of number and area of pink muscle fibers among the species sampled are shown in Fig. 2. The relative number among all muscle fiber types was more remarkably numerous in kingfish (code no. 101) and Pacific mackerel (code no. 209) than in round scad (code no. 202), while the relative area of these fibers to white muscle fibers was much smaller in the kingfish, but much larger in the Pacific mackerel, and similar in the round scad. Furthermore, the relative area of pink muscle fibers in the Pacific mackerel was larger than that in white muscle fibers. Thus, the relative number and area of these fibers to all muscle fiber types varied greatly among the sample fish species.

Relationship between area and number percentages of pink muscle fibers among all muscle fiber types The relationship between the area (x) and number (y) percentage values for the twenty-three fish species that interposed the pink muscle fiber is shown in Fig. 3. The relationship between the two values exhibited a significantly higher positive correlation ($r=0.804$ and $p<0.001$) with the exception of the Pacific mackerel (code no. 209). Furthermore, the calculated regression line moved parallel at the upper position distant from the $y=x$ line so that the values of both area and number are the same values. These results suggested that the size of pink muscle fiber in each species was somewhat smaller than that of white muscle fiber. However, the size in the Pacific mackerel (code no. 209) was farther below the $y=x$ line, suggesting, contrarily that the pink muscle fiber was the

Fig. 3. Relationship between area and number percentages of pink muscle fibers in dorsal ordinary muscle for various fish species. The dotted line indicates the $y=x$ line where percentages of both area (x) and number (y) are the same values. ●: fishes in sea adjacent to Sakishima Islands in Okinawa, ○: fishes in sea adjacent to Nagasaki.

larger. Moreover, in the round scad (code no. 202), it was distributed nearly at the $y=x$ line, which suggested that the two fibers were very similar in size. *Variola albiguttata* (code no. 111), kingfish (code no. 101), and yellowstriped whiptail (code no. 108) were at positions above the calculated regression line, which suggested that the pink muscle fiber was remarkably smaller than the white muscle fiber.

Thus, the area percentage of pink muscle fibers against all muscle fiber types in the dorsal ordinary muscle differed greatly, and was much higher in the Pacific mackerel (code no. 209), the blue and yellow snapper (code no. 106), and the bambooleaf wrasse (code no. 204) within the range from 53.68% to 38.10%. Other fish species fell within the range from 2.22% in the footballer (code no. 207) to 21.69% in the scribbled today (code no. 215). The numerical percentage of pink muscle fibers was from 12.90% in the horse mackerel (code no. 203) and the footballer (code no. 207) to 56.53% in the blue and yellow snapper (code no. 106).

Discussion

The pink muscle fiber is recognized in the dorsal ordinary muscle of many fish species and exhibits a mosaic pattern among the white muscle fibers (Figs. 1 and 2). Findings similar to this have been reported in the grey mullet by Carpena *et al.* (1982), the cod by Korneliussen *et al.* (1978), the roach by Kilarski (1990), and a number of fish species by both Mosse & Hudson

(1977), and our own previous studies (Jabarsyah *et al.*, 1999a, 1999b). The present study clarified that the number and area percentages of pink muscle fibers against all kinds of muscle fiber types in the dorsal ordinary muscle were considerably different among fish species, and fell within ranges of from 12.90% to 56.53% and from 2.22% to 53.68%, respectively (Fig. 3). We further recognized there was no pink muscle fiber in dorsal ordinary muscles in four of the species we sampled (Table 2 and Fig. 1). These differences were not caused by the difference of habitat temperature (Table 2 and Fig. 3), but the causative factors are still unknown. We speculate that it may be connected with not only a difference in genes but also swimming ability, because the muscle fiber type in the dorsal ordinary muscle differed between the cultured and wild specimens of the red sea bream (Jabarsyah *et al.*, 1999a), and metabolism is more aerobic in pink than in white muscle fiber.

We reported in previous studies that the histochemical actomyosin ATPase activity of pink muscle fibers in the mosaic pattern of ordinary muscle was very stable in both acid and alkaline preincubations, which was similar to that of these fibers in the intermediate part of the muscle (Jabarsyah *et al.*, 1999a), and also that the glycogen content in the pink muscle fibers in ordinary muscles was considerably higher, which was also similar to that in the intermediate part of the muscle (Yada *et al.*, 2000). Namely, the histochemical characteristics of pink muscle fibers in both ordinary and intermediate muscles were very similar. The same findings were reported in the rainbow trout (Johnston *et al.*, 1975), and we reported that the temporal change of *K*-value in the dorsal ordinary muscle of carp was accelerated by the interposition of pink muscle fibers (Yada *et al.*, 2000). It was therefore thought that the interposition of pink muscle fibers into the ordinary muscle might cause a temporal change in *K*-value (one item of the freshness index), which might be explain the difference in the increasing *K*-value rate among fish species.

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