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Cytochemical Profiles and Quantitative Analysis of Fiber Types in Trunk Muscle of Tigerperch, *Terapon jarbua*

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Shueh-Fen Chen and Bao-Quey Huang (2000) Cytochemical profiles and quantitative analysis of fiber types in trunk muscle of tigerperch, *Terapon jarbua*. *Zoological Studies* **39**(1): 28-37. Fiber types in trunk muscle of the tigerperch, *Terapon jarbua*, were classified by cytochemical profiles and image analysis techniques. Serial cross-sections of trunk muscle were examined by evaluating myosin ATPase (mATPase) to categorize the muscle fiber types, by measuring the oxidative enzyme activities of SDH, NADH-TR, and LDH to identify the metabolic patterns, and by determining the glycogen and lipid contents to specify the energy sources. The study revealed 3 muscle patterns (white, red, and pink muscles) distinguished by their enzyme activities. The different fiber types differed in their mATPase activities after preincubation in different pH solutions. Oxidative enzyme activities were quantitatively analyzed and correlated to their gray-level and fiber size. In white muscle only 1 histochemical fiber type (resembling type IIb fiber in mammals, with a large cross-sectional area, CSA) could be identified. Red muscle consisted of abundant type IIc fibers (with a smaller CSA) and a few type I fibers (with a medium CSA) sparsely interposed among IIc fibers. Fibers in a mosaic arrangement of pink muscle, with different histochemical reactions and of various sizes, were classified as type I, IIb, and IIc fibers. Type IIb fiber had larger cross-sectional areas (2969.30 ± 1237.27 ~ 3045.66 ± 1088.87 μm^2), type IIc had smaller (318.77 ± 105.02 ~ 353.20 ± 122.18 μm^2), and type I had medium areas (708.65 ± 142.62 ~ 1192.09 ± 308.28 μm^2).

Key words: Morphometry, mATPase, Oxidative enzyme, Gray-level, Typing fiber.

The axial musculature in most teleosts has been generally classified as white, red, and pink with reference to muscle color in living animals. The conventional notions of “white”, “pink”, and “red” muscle fiber types in teleosts have to be an over-simplified basis for classification. Further subdivision of muscle fiber types has been rarely reported (Korneliussen et al. 1978, van Raamsdonk et al. 1980, te Kronnie et al. 1983). Studies on the myotomal complex of fish have demonstrated that these muscles consist of muscle fibers possessing different biochemical, histochemical, immunohistochemical, and physiological characteristics (Barnard et al. 1971, Gill et al. 1982, Mascarello et al. 1986, Pai-Silva et al. 1995, Swank et al. 1997).

The over-simplified classification of fish muscle fibers has led to confusion between the terms “muscle” and “fiber”. According to histochemical staining profiles, myofibrillar distribution, as well as lipid, glycogen, and mitochondria contents, a muscle is commonly composed of more than 1 fiber type (Korneliussen et al. 1978, Kilarski 1990). Since the heterogeneity of muscle fiber types is highly variable among species, it is not advisable to generalize their classification simply into white or red muscle fiber types (Johnston et al. 1972, Nag 1972, Patterson et al. 1975, Carpené et al. 1982). Due to the heterogeneity shown by use of different techniques, discrepancies in the nomenclature have frequently occurred (Mosse and Hudson 1977, Jasra et al. 1991, Zhang et al. 1996).

Histochemical examinations designed to identify fiber types rely on the premise that different fiber types have different metabolic functions (Bass et al.

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1969). Johnston et al. (1972) distinguished various fiber types in many teleosts on the basis of their concentration of calcium-activated mATPase which generates energy via hydrolysis of ATP to ADP. The activity of mATPase presents a good correlation with the speed of shortening of the muscle fibers (Barany 1967). Therefore, the calcium methods for mATPase demonstration, employing solutions of different pH values, have been used primarily to distinguish muscle fiber types of skeletal muscles in mammals categorized as type I or type II. Type I fibers are slow, have more blood capillaries, and stain positively with various oxidative enzymes. Type II fibers are fast, have fewer capillaries, and stain lightly with various oxidative enzymes. Type II fibers are further subdivided into 3 subtypes: types IIa, IIb, and IIc according to their mATPase reactions after preincubation in different acidic solutions (Brooke and Kaiser 1970). These categories exhibit remarkable correlations with the fiber types in animals as defined by physiological characteristics (Dubowitz 1985).

The histochemical staining used in muscle biopsy (Dubowitz 1985) was modified and applied to fish muscle in the present study. We employed the histochemical nomenclature of Dubowitz (1985) to provide a detailed map of the quantity and composition of different fiber types in the trunk muscle of tigerperch (*Terapon jarbua*). Furthermore, we analyzed the optical density (gray-level) of the oxidative enzymes (e.g., SDH, NADH, and LDH) with an image analysis system. The properties of energy source supply (e.g., storage of glycogen, lipid) in fibers of each type were investigated by use of the periodic acid Schiff (PAS) and oil red O (ORO) stains.

MATERIALS AND METHODS

Tigerperch (*Terapon jarbua*), ranging from 15-20 cm in fork length were obtained from the northeastern coast of Taiwan by hook and line and held in the aquarium of the Fisheries Science Department, National Taiwan Ocean University. Fish were killed by severing the spinal cord. Muscle samples from the lateral muscle of the lateral line near the caudal region were dissected (Fig. 1A) and quickly immersed in isopentane in liquid nitrogen. Blocks of white, red, and pink muscles from 1 side were cut in serial 10-μm transverse sections in a cryostat (BRIGHT OTF/AS-001, Cambridge, U.K.) at –20 °C and were mounted on slides.

Histological stains

The morphology of muscle fibers and the noncontractile elements was demonstrated using Harris hematoxylin and eosin (H&E) stain (Sheechan and Harpchak 1980), and by use of a modified Gomori's trichrome stain (Engel and Lunningham 1963).

Histochemistry

Serial cross-sections were preincubated at alkaline (pH 10.2-10.7) or acidic (pH 4.5-5.0) medium at room temperature before applying the calcium method for adenosine triphosphatase (ATPase) activity to differentiate fiber types (Dubowitz and Brooke 1973). The activities of succinic dehydrogenase (SDH), NADH-tetrazolium reductase (NADH-TR), and lactate dehydrogenase (LDH) were also demonstrated (Nachlas et al. 1957, Nachlas et al. 1958, Pearse 1972, Chayen et al. 1973). The presence of glycogen was demonstrated by use of the periodic acid Schiff (PAS) (McManus 1948). Oil red O (ORO) (Lillie and Ashburn 1943) was used to demonstrate lipid distribution.

Quantitative analysis

All slides were viewed and photographed with an Olympus photomicroscope B201. The photographs (×100) were digitized with a video image-analysis system (JAVA soft, Corte Madera, CA. USA) in conjunction with a computer. The outlines of cross-sectional areas were accurately traced and measured with a mouse and computerized by an image-analysis system. The gray-level of each fiber was automatically detected based on light intensity. Relative gray-level = (gray-level of each fiber/maximum gray-level in the same picture) × 100. The relative gray-level histograms were used to show the intensities of oxidative enzyme activities in fibers of each type. In addition, statistical differences among the mean cross-sectional areas of fibers were established by *t*-test.

RESULTS

Three types of muscle (white, pink, and red) were identified in the lateral trunk muscle of tigerperch, *Terapon jarbua*. White muscle was present in the deep layers and constituted the major fraction of the myotomal muscle mass. Red muscle was observed in a V-shaped region, was sandwiched between pink muscles (Fig. 1B), and constituted only a small proportion of the total myotome. Along the horizontal septum, a few red muscle blocks were

also found near the spinal column. Underneath the skin of the lateral line, pink muscle was present as an indistinct band, a few cells thick, that merged into the white muscle from the periphery of the red muscle block (Fig. 1B).

Various sizes and histological characteristics of muscle fibers were observed to form a pattern in each muscle (Fig. 2). There were multiple nuclei distributed in the red and pink muscle fibers along the periphery of the muscle (Fig. 2A). The red muscle had distinctive boundaries of perimysium (Fig. 2B), but pink muscle showed an indistinct anatomical transition from red muscle towards white muscle.

Table 1 summarizes the histochemical profiles of the fibers in the white, red, and pink muscles of tigerperch. These 3 muscle fiber types showed various mATPase activities after preincubation in alkaline (Fig. 3A) and acidic solutions (Fig. 3B, C). A gradient of decreasing SDH (Fig. 3D), NADH-TR (Fig. 3E), and LDH (Fig. 3F) activities from red, pink, to white muscle was shown. Using gray-level assessment, the intensities of enzyme activities (SDH, NADH-TR, and LDH) in these muscles exhibited significant differences, although there was some overlap. One, 2, and 3 levels of different response intensities were found in the white (Figs. 6A, 7A, 8A), red (Figs. 6B, 7B, 8B), and pink (Figs. 6C, 7C, 8C) muscles, respectively. The gray-level histograms of muscle fibers were helpful in distinguishing enzyme activities.

The detailed descriptions for different fiber types in the 3 muscle patterns are as follows:

White muscle

The fibers of white muscle had larger cross-sectional areas (CSA) (2969.30 ± 1237.27 μm^2, n = 215, Table 1), fewer nuclei (Fig. 2A), and less glycogen and lipid than did those of red muscle (Table 1). This muscle displayed a homogeneous, non-mosaic appearance (Fig. 3) when stained with mATPase and oxidative enzymes.

Fibers of white muscle possessed alkali-stable mATPase activity (Fig. 3A) and low SDH (Figs. 3D, 6A), NADH-TR (Figs. 3E, 7A), and LDH (Figs. 3F, 8A) activities. On the basis of mATPase and oxidative enzyme activities, the fibers of white muscle were classified as type IIb fiber (Dubowitz 1985).

Red muscle

Most of the fibers were small in size (318.77 ± 105.02 μm^2, n = 183, Table 1) and round in cross-section with many nuclei (Fig. 2A) and a rich intramyofibrillar network (Fig. 4D, parallel lines shown in each fiber). There were rich reactions for glycogen and lipid in red muscle (Table 1), thus confirming high glycogen and lipid concentrations used as an energy source.

Fibers in red muscle were variable in size and heterogeneous in histochemical responses. Most smaller fibers exhibited alkali- and acid-stable mATPase activities (Fig. 4A-C) after preincubation in alkaline and acidic solutions typical of type IIc fiber (Dubowitz 1985). The staining of these fibers for SDH, NADH-TR, and LDH activities was intense (Figs. 4D-F, 6B, 7B, 8B). Some medium-size (708.65 ± 142.62 μm^2, n = 18, Table 1) fibers were sparsely distributed among the smaller IIc fiber and showed different mATPase activities (Fig. 4A-C) when compared with IIc fiber. Thus, these medium-size fibers possessing alkali-labile and acid-stable mATPase activities (Fig. 4A-C) and high activities of SDH, NADH-TR, and LDH (Figs. 4D-F, 6B, 7B, 8B) were classified as type I fiber (Dubowitz 1985).

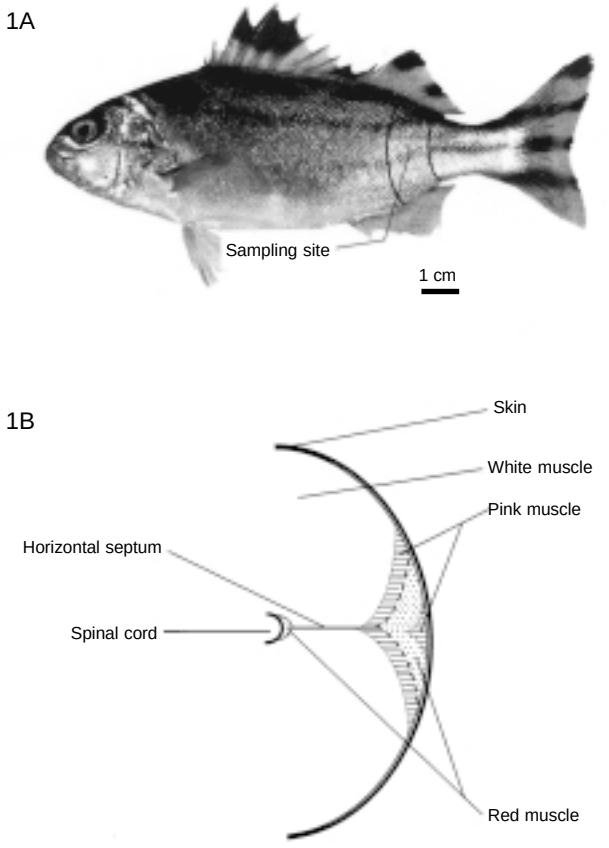

Fig. 1. Diagrammatic illustrations showing (A) sample site of removed muscle for study, and (B) distribution of muscle patterns in trunk musculature of *Terapon jarbua* at the sampling site.

Pink muscle

The composition of pink muscle is more complex than that of white or red muscles. Fibers in the pink muscle layers displayed a mosaic arrangement of different sizes (Table 1) and with various mATPase (Fig. 5A-C), SDH (Figs. 5D, 6C), NADH-TR (Figs. 5E, 7C), and LDH (Figs. 5F, 8C) activities. The glycogen and lipid contents were intermediate as compared with those of white and red muscles (Table 1). From the mATPase activity (Fig. 5A-C) and the gray-level histograms of oxidative enzymes activities (Figs. 6C, 7C, 8C), we showed that the mosaic layers consisted of at least 3 types of fibers in pink muscles. A few (less than 10% of pink muscle fibers) larger-sized (3045.66 ± 1088.87 μm^2, n = 36, Table 1) fibers exhibited alkali-stable mATPase activities (Fig. 5A), an intermediate reaction to mATPase stain after preincubation in pH 5.0 (Fig. 5B), and no reaction to mATPase stain after preincubation in pH 4.8 (Fig. 5C). These fibers were similar to type IIb fiber (Dubowitz 1985), and their size was not significantly different ($p > 0.05$) from that of IIb fiber in white muscle (Table 1). The smaller-sized (353.20 ± 122.18 μm^2, n = 196, Table 1) fiber showed alkali- and acid-stable mATPase activities (Fig. 5A-C) and was classified as type IIc fiber (more than 50% of pink muscle fibers) (Dubowitz 1985), whose size again revealed no significant difference ($p > 0.05$) from that of IIc fiber in red muscle (Table 1). The 3rd fiber of intermediate size (1192.09 ± 308.28 μm^2, n = 158, Table 1) was demonstrated to have alkali-labile and acid-stable mATPase (Fig. 5A-C) activities and could be classified as type I fiber (Dubowitz 1985). In contrast to IIb and IIc fibers, type I fiber (about 30%-40% of pink muscle fibers) was larger in size than type I fiber in red muscle ($p \leq 0.05$, Table 1).

DISCUSSION

Three different fiber types, i.e., I, IIb, and IIc, were identified in the trunk muscle of *Terapon jarbua* in the present study. Based on their distribution sites and histochemical characteristics, the "white" fibers in white muscle should correspond to type IIb fiber; the "red" fibers in red muscle should be classified as type I, and IIc fibers, and the "pink" fibers in pink muscle should represent a combination of type I, IIb, and IIc fibers. Our review of the literature disclosed no research applying this taxonomic method to typing fish trunk muscle fibers (Carpené et al. 1982, Pai-Silva et al. 1995, Zhang et al. 1996). A detailed measure of the distribution of the fiber types with distinctive classification of the trunk muscle of *Terapon jarbua* provided in the present report is more meaningful than in previous studies on fish muscle fibers (Eichelberg 1976, Carpené et al. 1982, Meyer-Rochow et al. 1994, Pai-Silva et al. 1995).

The white muscle fiber of *Terapon jarbua* responded homogeneously to various histochemical stains. Most of its fibers were large, had low oxidative enzyme activities and alkali-stable mATPase

Table 1. Summary of morphometric and histochemical characteristics of fiber types in white, red, and pink muscles of tigerperch, *Terapon jarbua*. There are 1 fiber type (IIb) in white muscle, 2 (I, IIc) in red, and 3 (IIb, I, IIc) in pink

Muscle	White	Red		Pink		
Fiber type	IIb	I	IIc	IIb	I	IIc
Composition[a]	> 99%	< 10%	> 90%	< 10%	30%-40%	50%-60%
CSA (μm^2)[b]	2,969.30 ± 1,237.27$^{\#\#\#\#}$	708.65 ± 142.62$^{\#\#}$	318.77 ± 105.02$^{\#}$	3,045.66 ± 1,088.87$^{\#\#\#\#}$	1,192.09 ± 308.28$^{\#\#\#}$	353.20 ± 122.18$^{\#}$
	(n = 215)	(n = 18)	(n = 183)	(n = 36)	(n = 158)	(n = 196)
mATPase 10.4[c]	+++	(+)	++	+++	(+)	(+++)
mATPase 5.0[c]	+	(+++)	+++	+	++	(+++)
mATPase 4.8[c]	0	(+++)	+++	0	++	++
SDH[c]	+	(+++)	+++	+	++	(+++)
NADH-TR[c]	+	(+++)	+++	+	(+++)	+++
LDH[c]	+	++	(+++)	+	++	(+++)
Glycogen[c]	+	++	++	+	++	++
Lipid[c]	0	+	++	0	+	++

[a]Composition: constituent percentages of fiber types in 1 muscle pattern (e.g., white, red, or pink muscles).
[b]CSA: mean and standard deviation of cross-sectional areas of each fiber type. Differing numbers of superscripted "#" symbols indicate statistical difference at $p \leq 0.05$.
[c]Staining intensity: 0 = no staining; + = light staining; ++ = moderate staining; +++ = heavy staining.
n: numbers of randomly measured fibers of each fiber type for CSA.

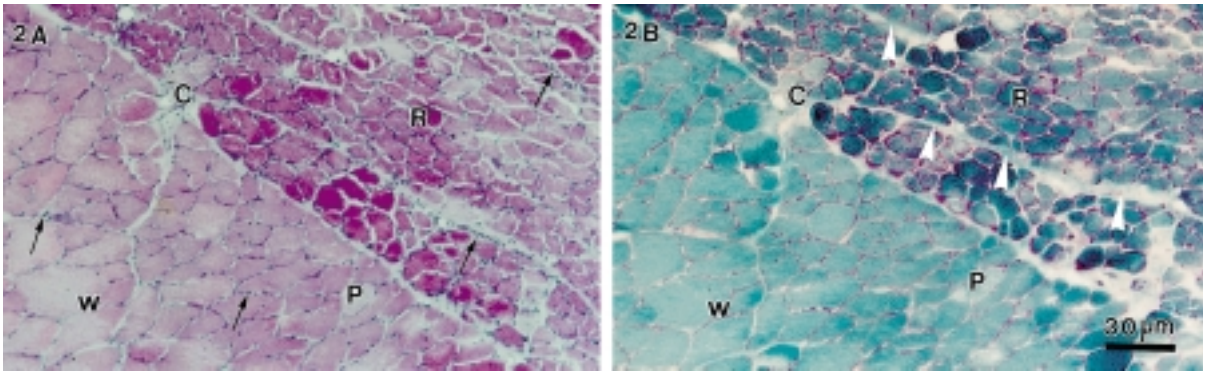

Fig. 2. Serial cross-sections of trunk muscle from *Terapon jarbua* stained with H&E (A) and modified Gomori trichrome stains (B). Cross-sectional areas illustrate fiber variability in white (W), red (R), and pink (P) muscles. Capillaries (C) are present among the muscle fibers. Sarcolemmal nuclei (A, black arrow) of the muscles are peripherally located. The red muscle blocks are separated by perimysium (B, white arrow).

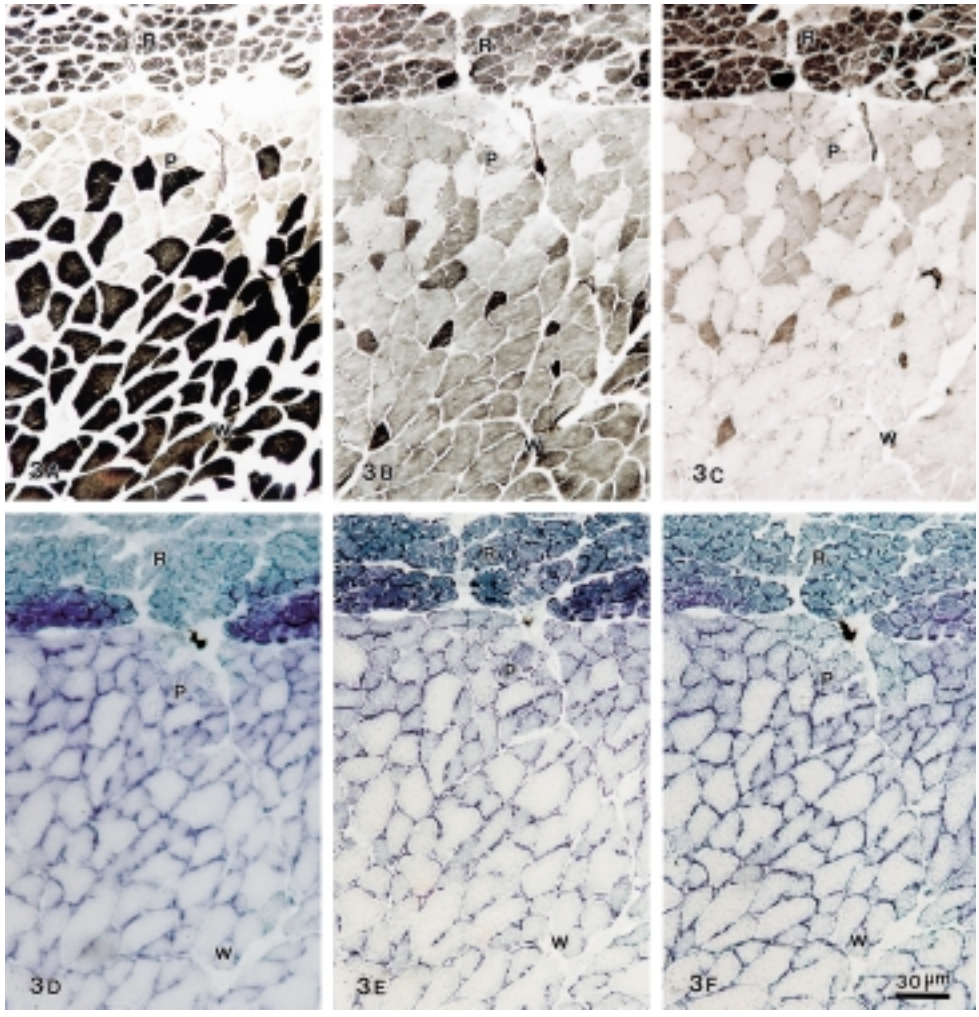

Fig. 3. Trunk muscle of *Terapon jarbua* from serial cross-sections with mATPase (A-C), SDH (D), NADH-TR (E), and LDH (F) staining. Fibers of white (W) muscle are strongly stained after alkaline preincubation at pH 10.4 (A), slightly stained after acid preincubation at pH 5.0 (B), but unstained at pH 4.8 (C). Staining with SDH (D), NADH-TR (E), and LDH (F) indicates that fibers of white (W) muscle are slightly stained, fibers of red muscle (R) are strongly stained, and fibers of pink muscle (P) have a mosaic appearance.

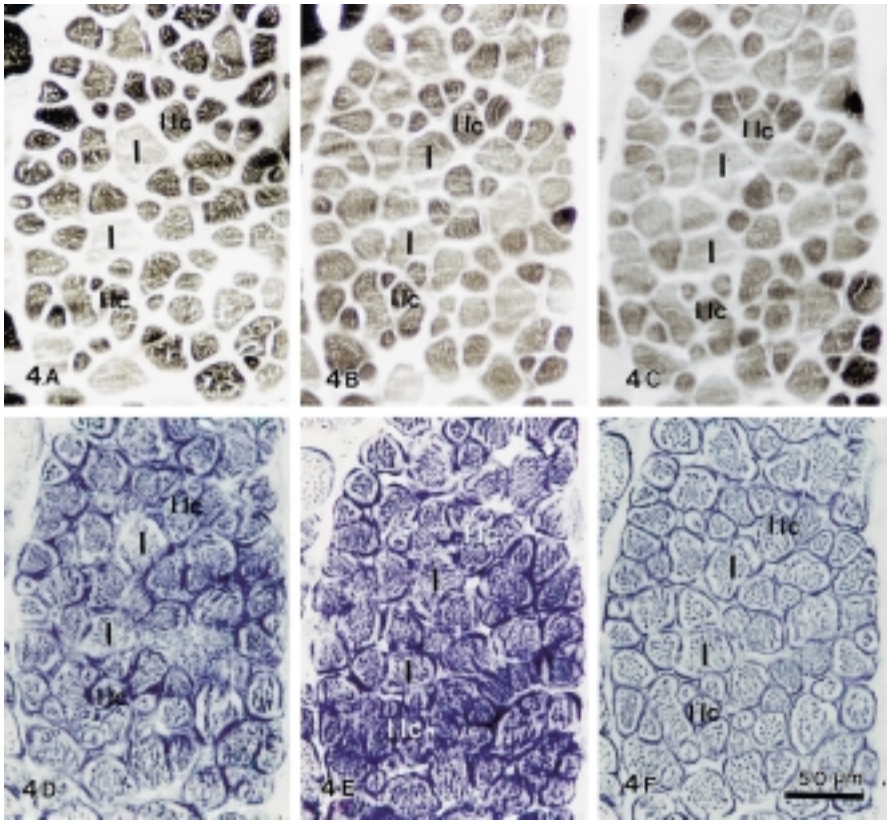

Fig. 4. Fiber types of red muscle of *Terapon jarbua* from serial cross-sections stained with mATPase (A-C), SDH (D), NADH-TR (E), and LDH (F) stains. IIc (IIc) fibers are strongly stained after both alkaline and acid preincubation at pH 10.4 (A), and pH 5.0 (B) and 4.8 (C), respectively. I (I) fibers are strongly stained after acid preincubation at pH 5.0 (B) and 4.8 (C), but very slightly stained after alkaline preincubation at pH 10.4 (A). Staining of IIc and I fibers with SDH (D), NADH-TR (E), and LDH (F) produced strong staining.

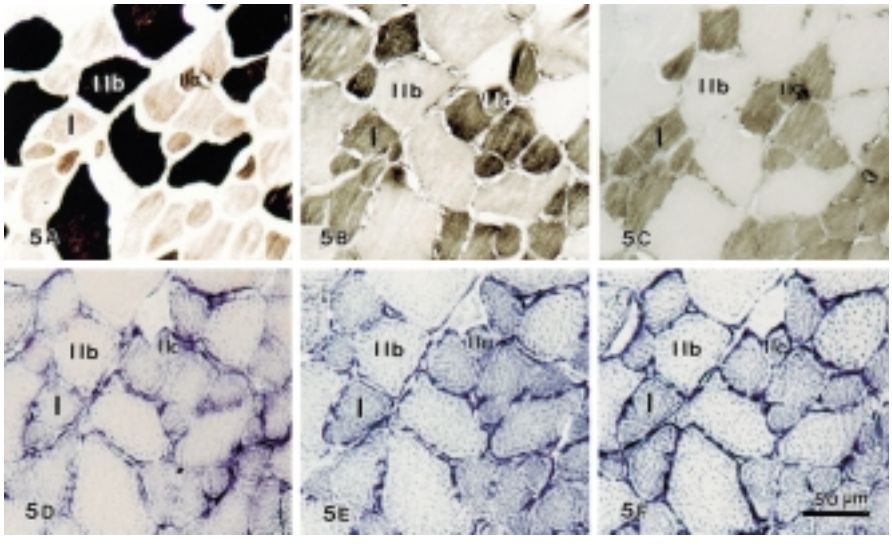

Fig. 5. Fiber types of pink muscle of *Terapon jarbua* from serial cross-sections stained with mATPase (A-C), SDH (D), NADH-TR (E), and LDH (F) stains. IIb (IIb) fibers are strongly stained after alkaline preincubation at pH 10.4 (A), slightly stained after acid preincubation at pH 5.0 (B), but unstained at pH 4.8 (C). IIc (IIc) fibers are strongly stained after both alkaline and acid preincubation at pH 10.4 (A), and pH 5.0 (B) and 4.8 (C), respectively. I (I) fibers are strongly stained after acid preincubation at pH 5.0 (B) and 4.8 (C), but very slightly stained after alkaline preincubation at pH 10.4 (A). Staining of IIc and I fibers with SDH (D), NADH-TR (E), and LDH (F) produced strong staining, but IIb fibers are slightly stained.

activity, and could be classified as type IIb fiber (Dubowitz 1985) i.e., with characteristics of having fast-twitch, oxidative metabolism, and being fatigue sensitive, rendering white muscle especially suitable for rapid swimming (Awan and Goldspink 1970, Gonyea 1980). In the present study, IIb fiber had relatively small concentrations of lipid and glycogen and low densities of oxidative enzymes, suggesting that white muscle possesses a predominantly anaerobic metabolism and lacks the ability to oxidize lipid as an energy source for ATP. Therefore, utilization of glycogen in glycolysis and lactate formation appears to be the probable source of ATP for IIb fibers in white muscle metabolism (Meyer-Rochow et al. 1994).

Heterogeneous responses of red muscle fibers suggest that red muscle is less specialized than white muscle. The predominant fibers in red muscle are small, have high oxidative enzyme activities and both alkali- and acid-stable mATPase activities, and are classified as type IIc fiber (Dubowitz 1985). These fibers may be primitive fibers which differentiate into type I, IIa, or IIb fibers (Brook et al. 1971, Dubowitz 1985). In our previous study, we also found IIc fiber presents in the sonic muscle of *Terapon jarbua* (Chen et al. 1998), but the exact functions of IIc fiber in fish muscle still needs further study. Some type I fibers scattered in muscle are slow fibers, i.e., with characteristics of slow-twitch and oxidative metabolism, making this muscle suitable for prolonged swimming and for maintaining a state of muscular tension (Awan and Goldspink 1970, Carpené et al. 1982). In each muscle pattern, therefore, metabolic differentiation of fiber types is related to the enzymatic organization of different energy-supplying modes (Bass et al. 1969).

The highly variable responses to mATPase staining after alkaline and acid preincubations in pink

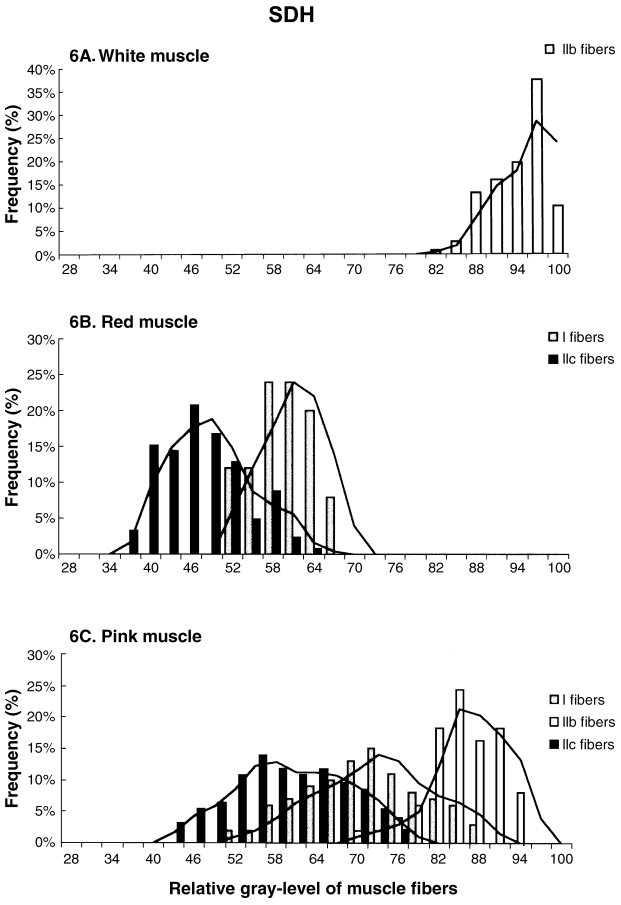

Fig. 6. SDH staining. Distributions of relative gray-level frequency (%) of type I, IIb, and IIc fibers in white (A), red (B), and pink (C) muscles in the trunk musculature of *Terapon jarbua*. A dark gray color results in a low number on the x-axis.

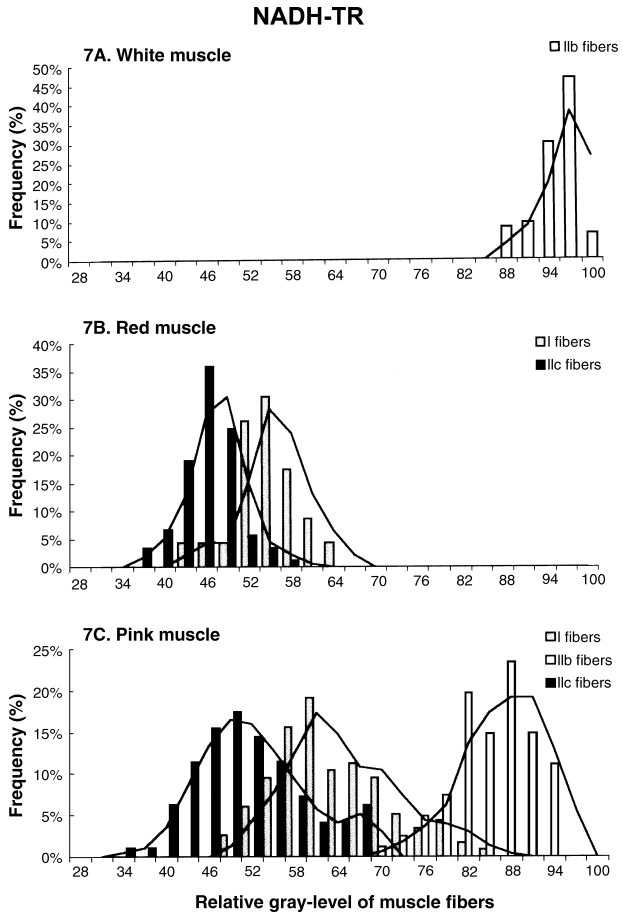

Fig. 7. NADH-TR staining. Distributions of relative gray-level frequency (%) of type I, IIb, and IIc fibers in white (A), red (B), and pink (C) muscles in the trunk musculature of *Terapon jarbua*. A dark gray color results in a low number on the x-axis.

muscles indicate the presence of several fiber types (types I, IIb, and IIc), which makes this muscle suitable for performing a wider range of functions than red muscle (Carpené et al. 1982). Application of image analysis to the intensity of responses of enzyme activities (gray-level histograms) support our differentiation of fiber types. The heterogeneous histochemical patterns of pink muscle revealed polymorphism of myosins, implying their properties for different function in swimming activity (van Raamsdonk et al. 1980, Carpené et al. 1982, Rowlerson et al. 1985, Mascarello et al. 1986). Note, also, that the heterogeneity of the fiber populations may be enhanced during the growth of muscle resulting from both addition of new fibers and hypertrophy of pre-existing fibers (Mosse and Hudson 1977, Weatherley et al. 1988). Previous studies found that the distribution of pink muscle is not clearly isolated from surrounding white or red muscle, and thus pink muscle is probably a growth stage of other muscle patterns (Mosse and Hudson 1977, Carpené et al. 1982, Mascarello et al. 1986).

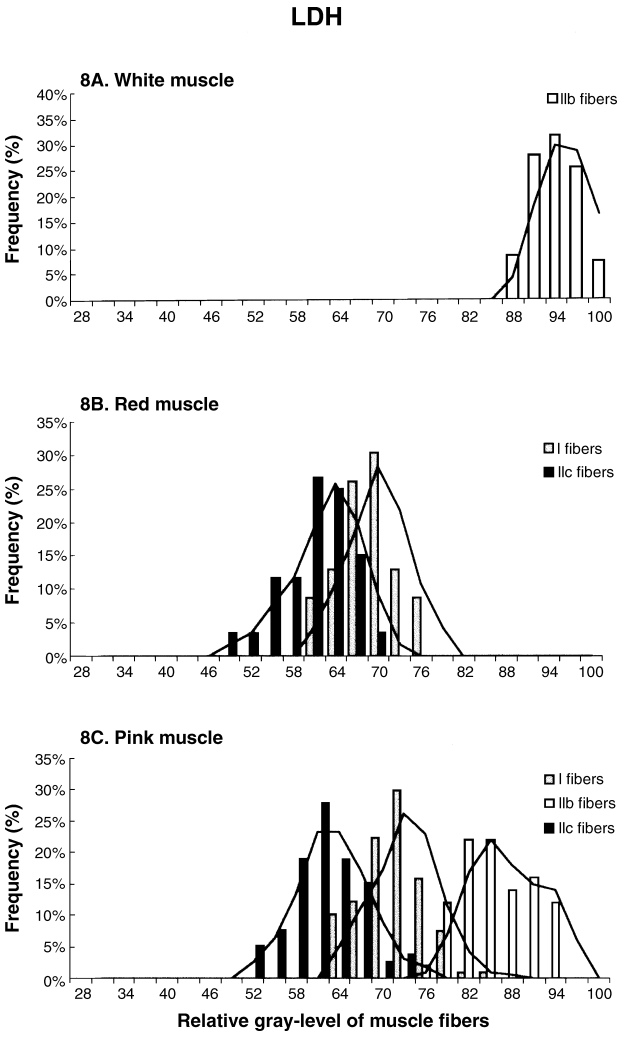

Fig. 8. LDH staining. Distributions of relative gray-level frequency (%) of type I, IIb, and IIc fibers in white (A), red (B), and pink (C) muscle in the trunk musculature of *Terapon jarbua*. A dark gray color results in a low number on the x-axis.

In teleosts, a functional interpretation of the different fiber types is more difficult, since correlation between enzymatic patterns and dynamic properties of the motor units has not yet been demonstrated (Carpené et al. 1982). Furthermore, the muscle fiber types appear to have different physiological and metabolic functions (Barnard et al. 1971, Burke et al. 1973, Dubowitz 1985). Therefore, further clarifying the innervation patterns, metabolic features, and physiologic properties of the different fiber types of fish muscle pattern are needed in future work.

In conclusion, modern histochemical techniques were good methods to localize enzyme systems and other chemical constituents at a cellular level, and this has opened the way for a direct correlation of the functional activity of individual fibers with their morphology. In the present study, cytochemical profiles provide a more detailed and intelligible but easier classification system for typing muscle fibers in teleosts, and the gray-level histogram is a valuable approach for the further quantitative differentiation of intensities of enzyme activities of histochemical responses.

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花身雞魚(*Terapon jarbua*)軀幹肌之肌纖維分類：細胞化學圖譜及定量分析之研究

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本研究係以連續組織切片之細胞化學圖譜及影像分析技術對花身雞魚(*Terapon jarbua*)軀幹肌之肌纖維做形態學上的分類。首先以 mATPase 的反應做為肌纖維型鑑別之依據，再以 SDH 、NADH-TR 及 LDH 之反應探討肌纖維之代謝型式，最後再檢定肌纖維中肝糖及脂質的含量以了解能量利用之來源。根據上述組織化學染色反應之結果，可將軀幹肌區分為白肌、紅肌及粉紅肌三種，又根據肌纖維的面積大小及氧化酵素反應的活性(以灰度值做定量分析)，發現每一種肌肉是由一種以上不同類型之肌纖維所構成。白肌主要是由一種肌纖維所構成，此肌纖維相當於哺乳類之 type IIb fibers 並具有較大之橫切面積；紅肌則是由大量的 type IIc fibers（面積最小）及少量散布於 IIc fibers 周圍之 type I fibers（具中等面積）所構成。此外，由上述反應結果發現，粉紅肌則是軀幹肌中肌纖維組成最複雜的，其至少是由 types I 、IIb 及 IIc 三種肌纖維以鑲嵌狀(mosaic)排列方式所構成。

關鍵詞：形態測定學， mATPase ，氧化酵素，灰度值，肌纖維分類。

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