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Seasonal changes in atrophy-associated proteins of the sonic muscle in the big-snout croaker, *Johnius macrorhynus* (Pisces, Sciaenidae), identified by using a proteomic approach

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Abstract In most sciaenids, males possess sonic muscles and produce sound through the contraction of these muscles and amplification of the swim bladder. The sonic muscles in some fishes exhibit seasonal changes in size. For example, they are hypertrophic in the spawning season, and atrophic in the non-spawning months. The protein profiles of the sonic muscle, red muscle, and white muscle in the *Johnius macrorhynus* were shown by two-dimensional electrophoresis (2-DE) and were compared to reveal differential protein expressions. About 80 up-regulated protein spots in the sonic muscle, and 30 spots related to six contractile proteins (fast muscle myosin heavy chain, skeletal alpha actin, alpha actin cardiac, tropomyosin, myosin light chain 2, and myosin light chain 3), four energy metabolic enzymes (enolase, acyl-CoA synthetase, creatine kinase, and cytochrome P450 monooxygenase), and two miscellaneous proteins (DEAD box protein and cyclin H) were identified. Seasonal hypertrophy and atrophy of the sonic muscles related to the reproductive cycle were verified in male big-snout croaker. The contents of some proteins were significantly different in the muscles under these conditions. The levels of cytochrome P450 monooxygenase, fast muscle myosin heavy chain, DEAD box proteins, isocitrate dehydrogenase, and creatine kinase were up-regulated in the hypertrophic muscle, but the levels of alpha actin cardiac, myosin light 2, and myosin light 3 were lower than in the atrophic muscle. Potential reasons

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for these differences in protein expression related to physiological adaptation are discussed.

Keywords Proteomics · Sonic muscle · Somatic muscle · Hypertrophy · Atrophy

Introduction

Big-snout croaker produces sound through the use of highly specialized sonic muscles, which is commonly classified as red muscle due to its color. With few exceptions, it is adapted for a high contraction rate and is considered a superfast muscle (Loesser et al. 1997; Rome 2006). The sonic muscle of the carapid fish contracts slowly (Parmentier et al. 2006), but they contain characteristics of both red and white fibers (Parmentier et al. 2003), as in the case of fast sonic muscles (Ladich and Fine 2006; Parmentier and Diogo 2006). The presence of abundant glycogen provides the muscle fibers of the fast sonic muscle with potential fuel to use as an energy source and therefore supports the designation of the sonic muscle as fast oxidative glycolytic (Fine et al. 1986). Sonic muscle fibers that have been examined by staining myosin ATPase (mATPase) have shown that the majority of their muscle fibers are type IIa fibers, which suggest that they are metabolically adapted for rapid contraction and fatigue resistance (Fine and Pennypacker 1988; Chen et al. 1998).

Two-dimensional electrophoresis (2-DE) provides a highly effective way of separating proteins, and it has recently become an approach used to understand muscle physiology that depends on global changes in protein expression. This approach has been successfully utilized for the analysis of skeletal and sonic muscles. In the studies by Huriaux et al. (1983) and Parmentier et al. (2003) on fish sonic muscle, several contractile proteins were identified, but the rarity of identifiable proteins may have been due to a lack of suitable technical support and adequate databases for protein identification available at that time. Mass spectrometry instruments (matrix-assisted laser desorption ionization time of flight, MALDI-TOF) and peptide mass fingerprinting (PMF), which are now broadly applied to proteomic research, are very useful for analyzing the expression levels of many proteins (contractile, metabolic enzymes, and so on) simultaneously.

The male big-snout croaker (*Johnius macrorhynus*) emits loud sounds (Lin et al. 2007); the females, on the other hand, lack sonic muscles. Seasonal hypertrophy and atrophy in the sonic muscles have been reported in sciaenids (Connaughton and Taylor 1994, 1995). The mass and thickness of the sonic muscles of big-snout croaker became approximately 1.6- and 1.9-fold larger in the spawning season than in the non-spawning season (Lin 2008). Details of the structural and chemical changes that occur during such transformations in these muscles are rare. For mammals, hypertrophy of skeletal muscle may occur as a result of resistance exercise and deletion of the myostatin gene (Bouley et al. 2005; Folland and Williams 2007; Hamelin et al. 2006; Isfort et al. 2002; Seo et al. 2006). Muscle atrophy, on the other hand, may occur because of losses in nerve supply, micro-gravity, disuse, and vitellogenesis (Isfort et al. 2000, 2002; Li et al. 2005; Seo et al. 2006; Salem et al. 2006a). Muscle hypertrophy is characterized by increases in muscle mass and size or the cross-sectional area (CSA) of the myofibers. In contrast, muscle atrophy is associated with a reduction in muscle mass and CSA in the myofibers. The rate of protein synthesis is much higher than the rate of degradation for muscle contractile proteins during hypertrophy, which leads to an increase in the size or volume of the muscle. The reverse is true for the atrophic muscle (Boonyarom and Inui 2006). In most cases, muscle hypertrophy and atrophy are usually associated with changes in the contractile apparatus proteins, such as the thick (myosin) and thin (actin) filaments (Pette 1998; Tajsharghi 2008). Due to the difference in the power produced, the energy metabolism enzymes are also altered during this transformation (Li et al. 2005; Seo et al. 2006).

The objectives of this study were (1) to analyze and compare the big-snout croakers' sonic muscle, red muscle, and white muscle to reveal differences in protein expression; (2) to elucidate the functional characteristics of the sonic muscle based on its proteomic characteristics; (3) and to identify the major muscle proteins of the sonic muscle to clarify how the potential proteomic modifications and the process of muscle size increment are related to the muscle contractile alterations under hypertrophic and atrophic conditions.

Materials and methods

Sample collection

The spawning season of the big-snout croaker lasts from March to October, with a peak appearing from June to September (Lin 2008). Specimens of male big-snout croakers were caught on the southwestern coast of Taiwan in August 2007 (peak spawning season), November 2006 to January 2007, March 2007, and November 2007 to January 2008 (non-spawning season). To avoid protein degradation, the specimens were stored on ice when caught, and then, they were transported to the laboratory to remove the muscle samples from the animals. Sonic muscle, white muscle, and red muscle were sampled in five mature big-snout croakers in March 2007 (170 ± 4 mm total length; gonado-somatic index, GSI $= 0.43 \pm 0.08\%$ total weight) to compare the differences between sonic muscle and somatic muscles. Due to the small amount of red muscle and the unobvious distinction of red and pink muscle, the samples of red muscle included red and pink muscle types. To examine the differences in protein concentration in hypertrophic and atrophic sonic muscles, big-snout croakers were obtained during the peak spawning season (164 ± 4 mm total length) and non-spawning season (167 ± 10 total length). Five hypertrophic (GSI $= 0.82 \pm 0.34\%$ total weight) and five atrophic (GSI $= 0.29 \pm 0.12\%$ total weight) sonic muscles were prepared for proteomics analyses. The muscles were frozen at -70°C for protein concentration and proteomics analyses.

Protein concentration analyses

To examine the protein-content difference between hypertrophic and atrophic sonic muscle, approximately 0.1 g of muscles was homogenized with a Pro200 homogenizer (Pro Scientific Inc., Oxford, CT, USA) in 0.07 M sodium phosphate buffer (pH 7.4) (Connaughton et al. 1997). The protein concentration was determined by the Bio-Rad colorimetric protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) ran against a standard curve established with bovine serum albumin. Absorbance was measured at 595 nm.

Protein extraction

Muscle samples were prepared for proteomic analysis based on the method described by Chiu et al. (2007); they were cut into small pieces and homogenized with a Pro200 homogenizer in lysis buffer [7 M urea, 2 M thiourea, 4% CHAPS, and protease inhibitor cocktail (1 tablet per 50 ml of lysis buffer; Roche Molecular Biochemicals, Roche Diagnostics GmbH, Mannheim, Germany)]. The mixture was centrifuged at 4°C for 20 min at 16,000*g*. The supernatant solution was either used immediately or stored at -70°C. The protein concentration was measured using a Bio-Rad protein assay kit.

Two-dimensional electrophoresis (2-DE)

Immobiline Dry-Strip, with pH 4–7 (13 cm, GE Healthcare Bio-Sciences AB, Amersham, UK), was incubated with 250 µl rehydration buffer (7 M urea, 2 M thiourea; 4% NP-40; 0.01% bromophenol blue) at room temperature for 12 h. After rehydration, 650 µg of total protein mixed with 1% DTT and 2% IPG buffer was placed in sample cups, and isoelectric focusing (IEF) was performed at 20°C on an electrophoretic apparatus (Ettan IPGphor or Ettan IPGphor3; GE Healthcare). The IEF started at 300 V for 3 h, then gradually increased to 1,000 V for 3 h, followed by a gradual increase to 8,000 V for 1 h, and finally increased from 8,000 until 40,000 V-hours was reached.

The focused strips were equilibrated for 20 min in SDS equilibration buffer solution (6 M urea, 30% glycerol, 2% SDS, 0.01% bromophenol blue, and 50 mM Tris–HCl at pH 8.8) with 30 mM DTT. A second equilibration step was then performed using an equilibration solution with 135 mM IAA to replace the DTT. The second dimension separation was performed with 12.5% acrylamide gels at 25 mA constant current on a Hoefer SE600 Ruby vertical electrophoresis until the dye front was approximately 1 mm from the bottom of the gel.

Protein staining and image analysis

After the electrophoresis procedure, the gels were fixed in a mixture of 40% methanol and 10% acetic acid overnight at room temperature and stained with

colloidal Coomassie Blue G250 (0.08% Coomassie Blue G-250, 8% ammonium sulfate, 0.8% phosphoric acid, 20% methanol). Gels were imaged by an ImageScanner (Amersham Biosciences).

Proteins of the sonic muscles at two states (hypertrophy versus atrophy) from five male individuals ($n = 5$) were separated by 2-DE. Additionally, to reduce the technical variations in 2-DE, each protein sample was analyzed in triplicate, and then, the best gel was chosen for further image analysis. In total, we obtained 30 gels (two stages × five individuals × three replicates), and 10 gels (5 gels of each stage) were selected for image analysis. The images were processed with Melanie software to yield the position (isoelectric point, pI and molecular weight, Mw) and intensity information for each detected spot. The intensity levels of the protein spots were calculated as their relative volume (percentage of the total volume of protein present on the gel).

Protein identification

The protein spots were excised from the 2-DE gels and washed, destained, dehydrated, and in-gel digested with trypsin (Promega, Madison, WI, USA) at 37°C overnight. The tryptic peptides were re-extracted twice in 10 µl of a 5% formic acid and 50% acetonitrile (ACN) solution. The extracted peptide solution was concentrated by vacuum centrifuge and dissolved in 2 µl of 5% formic acid. The peptides were mixed with matrix solution [50% ACN, 0.5% trifluoroacetic acid (TFA) saturated with alpha-cyano-4-hydroxycinnamic acid (CHCA)] and allowed to dry on a sample plate. Analysis by MALDI-TOF MS was conducted using an Ultraflex™ MALDI-TOF mass spectrometer (Bruker-Daltonik). The mass spectra were processed using flexAnalysis™ and Biotools™ software (Bruker Daltonics, Bremen, Germany), and the data were subjected to a search against the MASCOT search engine (http://www.matrixscience.com) (Matrix Science, London, UK). The mass tolerance was set to 150 ppm, with one missing cleavage allowed.

Statistical analysis

Non-parametric tests, including the Kruskal–Wallis test and Mann–Whitney U test, were used. The Z test with Bonferroni's correction (Dunn 1964) was used to determine their group after the Kruskal–Wallis test. The critical α level for these analyses was 0.05.

Results

Differences between sonic and somatic muscles

2-DE gel protein spots and protein identification

A gel with a protein mixture containing equal amounts of the three muscle types was prepared as a master gel to show the profile of all the proteins in these muscle types of the big-snout the croaker. There were 512.0 ± 13.7, 427.0 ± 54.3, and 402.3 ± 68.5 detectable spots in the 2-DE gels of sonic, red, and white muscles, respectively (Fig. 1). Approximately 80 up-regulated protein spots constituted the major differently expressed proteins between sonic muscle and somatic muscle (Fig. 1 and supplementary data).

Due to the drastic difference in the number of protein spots from these muscle types, only the spots showing obvious differences in intensity among the sonic, red, and white muscles were picked for identification. Some major muscle proteins, such as actin (spot 86) and tropomyosin (spot 178), were also identified to verify protein identification procedures. A total of 130 spots were selected for in-gel digestion and analysis by MALDI-TOF MS, and 30 spots were identified as particular proteins using peptide mass fingerprinting (PMF). The identified proteins shown in Fig. 2 and the relative spot intensities of the sonic muscle and the somatic muscle of these proteins are listed in Table 1. These identified proteins were classified into muscle contractile apparatus proteins, energy metabolism proteins, and miscellaneous proteins.

The 15 spots that were associated with muscle contractile apparatus were matched to six different proteins: fast muscle MHC (MHC) (7 spots), skeletal alpha actin (spot 86), alpha actin cardiac (spot 211), tropomyosin (spot 178), MLC2 (spot 257), and MLC3 (spot 215). Excluding the skeletal alpha actin and tropomyosin, the other proteins were higher in expression in the sonic muscle than in the somatic muscles (ranging from 2.93- to 25.94-fold).

Twelve spots related to energy metabolic protein were identified. The four proteins related to energy

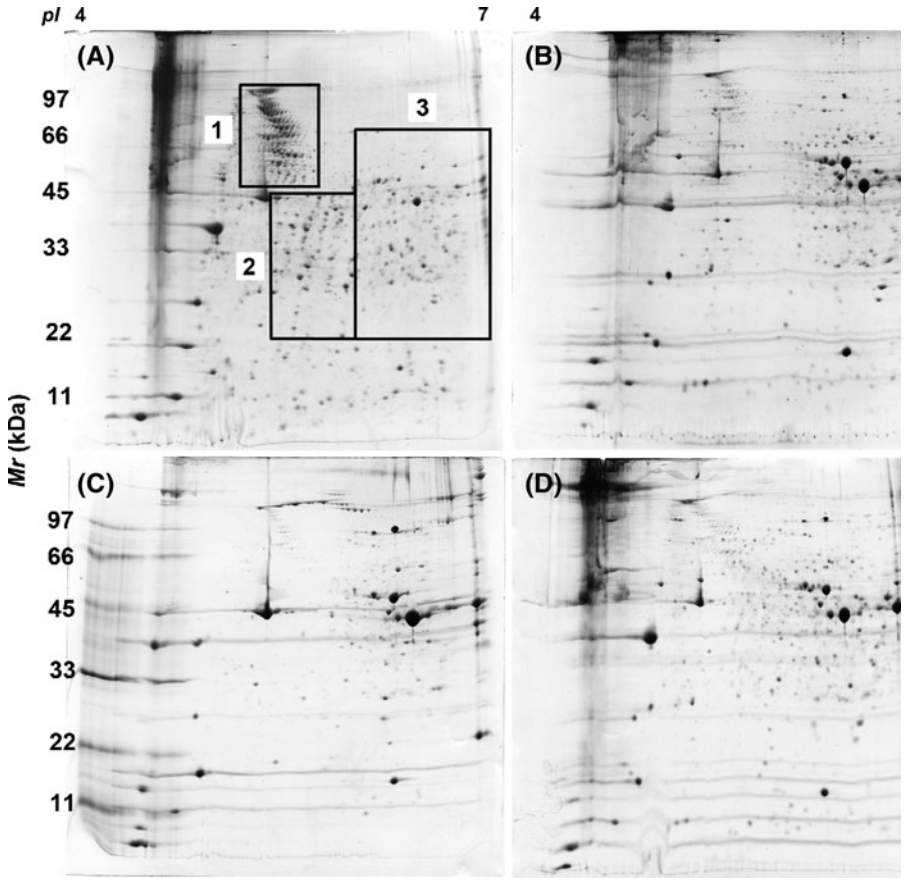


Fig. 1 2-DE of the total protein from the sonic muscle (**a**), red muscle (**b**), white muscle (**c**), and master gel (**d**) using pI 4–7 and 12.5% acrylamide gels. For convenience of interpretation, three selected discrepancy areas (1–3) were partially enlarged, as demonstrated in Supplementary Figs. 1–3. The three areas were classified as: *Area 1* pI 5.0–5.7 and Mw 60–130; *Area 2* pI 5.3–6.0 and Mw 20–50; *Area 3* pI 6.0–7.0 and Mw 20–80

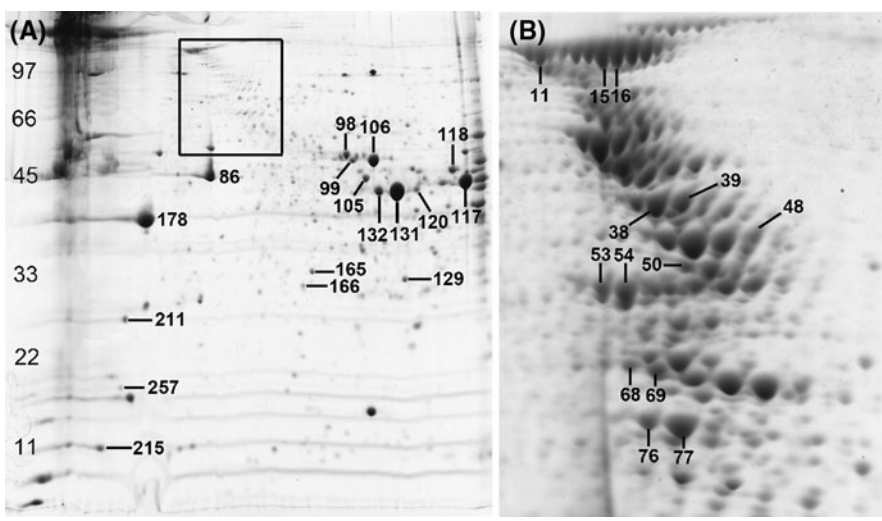


Fig. 2 The position of identified proteins in the master gel (**a**), and partially enlarged (**b**) in the sonic muscle. See Table 1 for a detailed listing of muscle proteins with changed abundance

metabolism were identified as creatine kinase (CK) (7 spots), enolase (3 spots), acyl-CoA synthetase, and cytochrome P405 monooxygenase. Two spots that were identified as CK (spots 129 and 165) showed higher expression in the sonic muscle than in the somatic muscles. Conversely, the enolase (spots 98,

Table 1 Summary of identified muscle proteins and the fold changes of the sonic muscle (SM), red muscle (RM), and white muscle (WM)

Spot no.	NCBI number	Theoretical		Estimated		Score	Petides matched	Coverage (%)	Protein identification	SM/RM	SM/WM
		pI	Mw	pI	Mw						
Contractile apparatus proteins											
16	gil29570808	5.22	94,835	5.29	124,233	94	22	26	Fast muscle-specific myosin heavy chain	3.20	3.62
38	gil29570808	5.22	94,835	5.34	92,472	106	23	27	Fast muscle-specific myosin heavy chain	11.57	6.26
39	gil29570808	5.22	94,835	5.37	93,194	94	18	23	Fast muscle-specific myosin heavy chain	4.80	5.74
48	gil29570808	5.22	94,835	5.48	87,168	56	11	13	Fast muscle-specific myosin heavy chain	3.18	5.05
50	gil8698685	5.52	48,628	5.39	80,886	68	13	29	Fast skeletal muscle myosin heavy polypeptide 1	3.21	2.93
76	gil8698685	5.52	48,628	5.33	59,043	101	13	29	Fast skeletal muscle myosin heavy polypeptide 1	6.81	11.20
77	gil8698685	5.52	48,628	5.38	58,979	81	12	27	Fast skeletal muscle myosin heavy polypeptide 1	15.02	25.94
86	gil6653228	5.28	41,843	5.27	44,345	132	12	38	Skeletal alpha actin	N. S.	N. S.
178	gil22415765	4.69	32,783	4.76	38,706	166	17	41	Skeletal muscle tropomyosin1-2	N. S.	N. S.
211	gil148695918	5.37	37,509	4.72	27,197	90	9	30	Actin, alpha, cardiac	3.91	2.95
215	gil16117351	4.37	16,639	4.54	10,766	86	5	40	Myosin light chain 3	4.55	4.24
257	gil7678730	4.6	19,038	4.67	21,327	73	5	33	Myosin light chain 2	7.68	No WM
Energy metabolic proteins											
98	gil37590349	6.16	47,044	6.12	50,714	129	15	33	Enolase 1 (alpha)	0.53	0.41
99	gil47551317	6.25	47,442	6.16	49,621	58	7	15	Enolase 3 (beta, muscle)	0.09	0.17
105	gil31980996	6.19	78,811	6.24	44,868	56	9	16	Acyl-CoA synthetase short-chain family member 2	0.20	0.19
106	gil47551317	6.25	47,442	6.28	49,532	65	10	24	Enolase 3 (beta, muscle)	0.13	0.13
117	gil21694041	6.98	43,061	6.89	45,453	110	13	31	Muscle-type creatine kinase CKM1	0.25	0.17
118	gil156028365	6.22	42,916	6.70	44,525	94	10	20	Creatine kinase 1	0.20	0.18
120	gil21694043	6.44	42,694	6.55	43,888	129	12	27	Muscle-type creatine kinase CKM2	0.24	0.13
129	gil156028365	6.22	42,916	6.50	31,840	84	8	14	Creatine kinase 1	2.83	3.17
131	gil157787181	6.29	42,815	6.43	43,514	104	13	29	Creatine kinase CKM3	0.37	0.24
132	gil157787181	6.29	42,815	6.32	43,681	119	15	34	Creatine kinase CKM3	0.24	0.19
165	gil156972295	6.86	27,207	5.90	32,190	63	6	17	Creatine kinase isoform a	3.06	3.52
Miscellaneous proteins											
15	gil114571722	6.66	64,546	5.27	124,220	36	4	11	PREDICTED: DEAD (Asp-Glu-Ala-Asp) box polypeptide 59 isoform 1	3.56	4.23
68	gil9438227	5.69	79,274	5.31	63,613	49	8	18	DEAD box RNA helicase	5.20	7.06

Table 1 continued

Spot no.	NCBI number	Theoretical		Estimated		Score	Petides matched	Coverage (%)	Protein identification	SM/RM	SM/WM
		pI	Mw	pI	Mw						
69	gil109077274	5.44	63,095	5.35	63,479	79	7	19	PREDICTED: similar to DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 isoform 1	8.01	14.19
166	gil31542350	7.04	37,665	5.87	28,988	54	6	20	Cyclin H	SM	SM
Co-located proteins											
11	Mixture			5.18	123,670	138	24			2.58	NS
	gil73948306	8.12	55,652			62	7	20	PREDICTED: similar to cytochrome P450 monooxygenase CYP2T1		
	gil29570808	5.22	94,835			62	18	20	Fast muscle-specific myosin heavy chain		
53	Mixture			5.28	74,499	149	32			2.94	2.53
	gil1339977	5.48	90,525			72	19	25	Skeletal myosin heavy chain		
	gil109077272	5.33	75,760			65	13	28	PREDICTED: similar to DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 isoform 2		
54	Mixture			5.34	74,622	149	22			3.19	3.75
	gil33088009	5.7	40,879			74	12	32	Fast skeletal myosin heavy chain 4		
	gil109077274	5.44	63,095			66	10	27	PREDICTED: similar to DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 isoform 1		

NS indicates no significant difference of intensity between the two groups. Spot 257 was not present in the white muscle (marked with No WM). Spot 166 was only revealed in the sonic muscle (marked with SM)

99, and 106), acyl-CoA synthetase (spot 105), and the 5 CK spots (spots 117, 118, 120, 131, and 132) were expressed at a lower level in the sonic muscle.

Six spots related to two proteins were included in miscellaneous proteins: The cyclin H protein (spot 166) was only revealed in the sonic muscle, and the DEAD (Asp-Glu-Ala-Asp) box proteins were expressed at a higher level in the sonic muscle than in the somatic muscles. Spots 15, 68, and 69 were identified as DEAD box proteins, and spots 53 and 54 were identified as DEAD box proteins co-located with fast muscle MHC (Table 1; Fig. 2).

Differences in protein concentration between hypertrophic and atrophic sonic muscles

The hypertrophic sonic muscle protein concentrations for specimens collected in the spawning season (i.e., August 2007) were 18.9 ± 3.3% tissue wet mass ($n = 7$). The atrophic sonic muscle protein concentrations for specimens collected in the non-spawning season (i.e., between November 2006 to January 2007 and November 2007 to January 2008) were 15.6 ± 4.5% tissue wet mass ($n = 7$) and 15.2 ± 3.1% tissue wet mass ($n = 18$), respectively. The protein content of the hypertrophic sonic muscle was significantly higher than that of the atrophic sonic muscle ($P < 0.05$).

Highly expressed proteins in hypertrophic sonic muscle

The number of detectable spots was higher in hypertrophic sonic muscle (578.3 ± 40.3 spots) than in atrophic muscle (517.8 ± 14.8 spots). The representative 2-DE gel patterns of the atrophic sonic muscle are shown in Fig. 3a. For the convenience of interpretation, the selected area of pI 5.0–5.7 and Mw 60–130 was partially enlarged, as demonstrated in Fig. 3b. The overall patterns of the electrophoretic maps were well matched, and most of the protein spots in the hypertrophic and atrophic sonic muscles were similar. However, a few distinct differences were observed (Table 2; Fig. 3c). Spot 48 was identified as fast muscle MHC, and the spot was 2.15-fold higher in the hypertrophic muscle than in the atrophic sonic muscle. Spot 11 was identified as the co-located proteins of cytochrome P450 monooxygenase and fast muscle MHC, whereas spot number 54 was the co-located proteins of DEAD (Asp-Glu-Ala-Asp) box proteins and the fast muscle MHC. These two spots showed higher expression in the hypertrophic sonic muscle than in the atrophic sonic muscle (increased 2.06- and 3.23-fold, respectively).

As for energy metabolism enzymes, the protein expression levels of isocitrate dehydrogenase (spot 116) and CK isoforms (spots 129, 131, 165) were significantly higher in the hypertrophic sonic muscle than in the atrophic sonic muscle (spots 116, 129, 131, 165: 5.99-, 3.25-, 1.81-, and 3.52-fold, respectively) (Table 2; Fig. 3c).

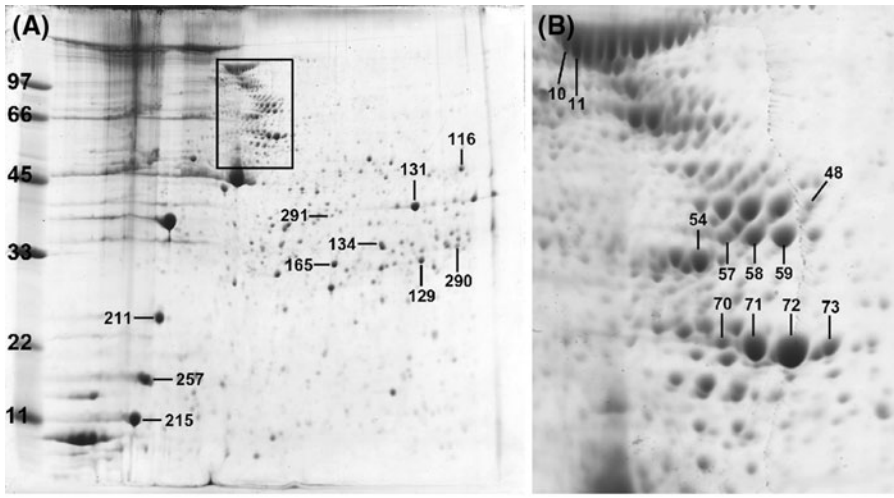
Spots 10, 57, 58, 59, 70, 71, 72, 73, 134, 290, and 291 were unknown proteins. These proteins also showed higher expression in the hypertrophic sonic muscle than in the atrophic muscle (a 2.65-, 1.67-, 1.98-, 2.88-, 2.01-, 3.27-, 2.61-, 2.99-, 6.75-, 6.48-, and 3.14-fold increase, respectively) (Table 2; Fig. 3).

Highly expressed proteins in atrophic sonic muscle

Three spots were higher in expression in the atrophic muscle compared with the hypertrophic muscle, and they were identified as alpha actin cardiac (spot 211; 2.88-fold), myosin light chain 3 (MLC3) (spot 215; 5.64-fold), and MLC2 (spot 257; 2.23-fold) (Table 2; Fig. 3c).

Discussion

The 2-DE gels in this study provide an effective protein separation, and the results show differences in the protein patterns of the sonic and somatic muscles. It provided basic information on the protein expression of skeletal muscles in the big-snout croaker. Seasonal hypertrophy and atrophy in sonic muscle have also been noted in the males of haddock (*Elanogrammus aeglefinus*) (Templeman and Hodder 1958), weakfish (*Cynoscion regalis*) (Connaughton and Taylor 1994, 1995), Atlantic cod (*Gadus morhua*) (Rowe and Hutchings 2004), and fawn cusk-eel (*Lepophidium profundorum*) (Nguyen et al. 2008). However, little study investigates and discovers the role of proteins involved in atrophy-hypertrophy cycle in fish muscles. The protein concentration in the sonic muscle of male big-snout croaker significantly increased during the spawning season, and differences in protein expression



(C)

Spot no.	No-spawning	Spawning
10.11		
48		
54		
57.58.59		
70.71.72.73		
116		
129		
131		
134		
165		
211.257.215		
290		
291		

Fig. 3 The protein spots of significantly different intensity between spawning (hypertrophy) and non-spawning (atrophy) sonic muscle. The 2-DE gel of the no-spawning sonic muscle (**a**). The selected discrepancy areas were enlarged as (**b**). The individual spots are shown in 3C. *Spot numbers* refer to numbers in Table 2

Table 2 Summary of identified muscle proteins and the fold changes in hypertrophic (spawning) and atrophic (no-spawning) sonic muscle

Spot no.	NCBI number	Theoretical		Estimated		Score	Petides matched	Coverage (%)	Protein identification	Spawning/ no-spawning
		pI	Mw	pI	Mw					
10	Unknown			5.13	113,462					2.65
11	Mixture			5.15	112,856	138	24			2.06
	gil73948306	8.12	55,652			62	7	20	PREDICTED: similar to cytochrome P450 monooxygenase CYP2T1	
	gil29570808	5.22	94,835			62	18	20	Fast muscle-specific myosin heavy chain	
48	gil29570808	5.22	94,835	5.51	78,770	56	11	13	Fast muscle-specific myosin heavy chain	2.15
54	Mixture			5.34	68,662	149	22			3.23
	gil33088009	5.7	40,879			74	12	32	Fast skeletal myosin heavy chain 4	
	gil109077274	5.44	63,095			66	10	27	PREDICTED: similar to DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 isoform 1	
57	Unknown			5.39	71,280					1.67
58	Unknown			5.44	71,571					1.98
59	Unknown			5.48	72,018					2.88
70	Unknown			5.44	59,540					2.01
71	Unknown			5.49	59,433					3.27
72	Unknown			5.50	59,414					2.61
73	Unknown			5.56	59,885					2.99
116	gil93115142	7.55	50,375	6.76	46,109	125	15	32	Mitochondrial isocitrate dehydrogenase 2-like	5.99
129	gil156028365	6.22	42,916	6.50	30,951	84	8	14	Creatine kinase 1	3.25
131	gil157787181	6.29	42,815	5.87	28,409	104	13	29	Creatine kinase CKM3	1.81
134	Unknown			6.25	33,881					6.75
165	gil156972295	6.86	27,207	5.91	31,196	63	6	17	Creatine kinase isoform a	3.52
290	Unknown			6.74	33,789					6.48
291	Unknown			5.90	39,201					3.14
211	gil148695918	5.37	37,509	4.69	24,855	90	9	30	Actin, alpha, cardiac	2.88
215	gil16117351	4.37	16,639	4.50	11,103	86	5	40	Myosin light chain 3	5.64
257	gil7678730	4.6	19,038	4.59	16,864	73	5	33	Myosin light chain 2	2.23

were also observed in the seasonally atrophic and hypertrophic sonic muscles. Proteins differentially expressed among the muscle types, and between atrophic and hypertrophic sonic muscles were discussed as follows.

Muscle contractile apparatus proteins

Earlier studies have reported that the fiber types in the sonic, red, and white muscles were different. MHCs and MLCs exist in different isoforms among these muscle fiber types. The slow-twitch muscle fiber expresses slow MHC (type I), while the fast-twitch muscle fiber expresses fast types (type IIa, IIb, IIx) (Karasinski 1993; Schiaffino and Reggiani 1994). Their MLC isoforms are also different: White muscle displays MLC1, MLC2, and MLC3, while slow-twitch red muscle includes only MLC1 and MLC2 (Crockford and Johnston 1993; Karasinski 1993; Martinez et al. 1990; Huriaux et al. 1999). These differences in the contractile apparatus proteins of the muscle types are responsible for the different contraction properties.

In the previous studies, the different MHC types were shown in the sonic, red, and white muscle (Karasinski 1993; Schiaffino and Reggiani 1994), and the MHCs not only determine the muscle fiber types, but they are also related to myosin ATPase activities and the ability to generate force (Bottinelli et al. 1991; Schiaffino and Reggiani 1994). Mainly, type IIa muscle fibers are present in the sonic muscle in toadfish (*Opsanus tau*) and *Carapus acus* (Fine and Pennypacker 1988; Parmentier et al. 2003). Type I and type IIc fibers are present in the red muscle, and type IIb fiber is present in the white muscle of tigerperch (Chen and Huang 2000). Thus, the types of MHCs may represent muscle types with different contractile characteristics, and the higher expression of fast muscle MHC in the croaker sonic muscle leads to its contraction at a higher speed.

The MLC2 in the sonic muscle of toadfish has a lower isoelectric point and higher molecular weight than that of the white muscle (Huriaux et al. 1983). Spot 212 with a lower molecular weight and higher isoelectric point than that of spot 257 was identified as MLC2; however, this spot was not identified by mass spectrometry; therefore, spot 212 may represent the MLC2. MLC3 in the sonic muscle showed a similar Mw and pI in the three muscles (Mw = 10,766; pI = 4.54); however, MLC3 of carapid, *Carapus acus*, had a higher Mw and higher pI in the sonic muscle compared with the white muscle (Parmentier et al. 2003). These differences in Mw and pI may be related to the difference in the sonic muscle contraction rate (Parmentier et al. 2006; Fine et al. 2001; Sprague 2000).

Most identified contractile proteins exhibited higher expression in the sonic muscle compared with the somatic muscle, suggesting that there proteins were involved in the fast twitch of the sonic muscle, but not in the twitch strength. Because the sonic muscle is extremely fast due to its high volume of sarcoplasmic reticulum, which provides expanded Ca^{2+} capacity (Feher et al. 1998), so that the muscle can keep contracting before fatiguing. However, the excess sarcoplasmic reticulum comes at the expense of the volume of myofibrils and therefore is likely to decrease the force of the muscle.

The skeletal alpha actin and tropomyosin, which are the major thin-filament proteins, displayed similar composition in the three muscle types. In this study, thick filament constituent proteins showed more changes than the thin-filament constituent proteins. This suggests that the differences in the contractile prosperities might be a result of the regulation between proteins in thick and thin filaments in these three muscle types. In addition, the contraction of the sonic muscle is based on the sliding of thick and thin filaments relative to each other brings about the shortening of sarcomere and myofibrillar contraction (Ono and Poss 1982; Connaughton et al. 1997).

The hypertrophic sonic muscle showed higher expression of the fast MHC, but showed lower expression in the alpha actin cardiac, MLC2, and MLC3. This suggests the need of the hypertrophic muscle to twitch at a fast rate during the spawning season. Overall, the contractile apparatus proteins of the sonic muscle do not dramatically change under these conditions. This is consistent with the histological features, where both sarcoplasmic and myofibrillar hypertrophy were found in the hypertrophic sonic muscle of big-snout croaker (Lin 2008). Sonic muscle fiber has a central core surrounded by a contractile myofibrillar cylinder region and an extremely well-developed sarcoplasmic reticulum surrounding the myofibrils (Ono and Poss 1982; Lin 2008). The average areas of sonic muscle fiber and

the contractile myofibrillar cylinder region increased approximately twice in spawning season compared with the non-spawning season. The ratios of the contractile myofibrillar cylinder region area to the fiber area in the atrophic and hypertrophic sonic muscles were insignificantly different (72.5 and 72.8%, respectively). In contrast to weakfish (Connaughton et al. 1997), the decrease in the contractile myofibrillar cyclinder cross-sectional area in the big-snout croaker was larger than the decrease in the total cross-sectional area during the non-spawning period. Based on these finding, we assume that the seasonal changes in the functional and biochemical characteristics of big-snout croaker sonic muscle might be associated with the sarcoplasmic reticulum.

The protein levels of MLC2 and MLC3 were higher in the atrophic muscle. Similar results have been observed in rats (Duan et al. 2006; Haddad et al. 2003; Isfort et al. 2000; Seo et al. 2006), where the contractile apparatus proteins are not expressed in a lower level in atrophic muscle. The major thin-filament constituent proteins displayed equal expression levels in the hypertrophic and the atrophic sonic muscles, but the thick-filament constituent proteins (MHC and MLC) exhibited significant changes in these muscle conditions. Similar results have been found when comparing the sonic muscle to red and white muscle in this study. Cohen et al. (2009) have shown that the enzyme MuRF1-dependent ubiquitylation targets degrade the thick filaments in atrophic muscle, while bypassing the thin filaments. This may explain why there was no significant difference displayed between the hypertrophic and atrophic sonic muscles in the above-mentioned major thin-filament constituent proteins.

According to Moll et al. (2006), cardiac α-actin (CAA), which is highly expressed in the atrophic sonic muscle of the big-snout croaker, appears in fetal and adult myocardium and in the fetal skeletal muscle, while adult skeletal muscle is essentially not present, except for muscle spindle myocytes and a few scattered muscle fibers with an overall reduced diameter. By contrast, CAA synthesis is markedly induced in regenerating skeletal muscle cells, in Duchenne muscular dystrophy and upon degenerative atrophy. This protein has been considered a sign of aberrant differentiation in diseased skeletal muscle and of striated muscle differentiation in rhabdomyosarcomas (Moll et al. 2006). An up-regulated synthesis of cardiac α-actin in the atrophic sonic muscle of big-snout croaker agrees well with the above inference about this protein because regeneration is expected to take place later.

Energy metabolic enzymes

The three types of muscles had different expression levels of metabolic enzymes. Four metabolic enzymes, including CK, enolase, acyl-CoA synthetase, and cytochrome P405 monooxygenase, were identified in the gels. The sonic muscle had lower amounts of two glycolytic enzymes [CK (5 spots) and enolase (3 spots)] and one oxidative enzyme (acyl-CoA synthetase) compared with the somatic muscle. However, the male toadfish (*Opsanus beta*) sonic muscle has been shown to have higher contents of citrate systhase (CS), malate dehydrogenase (MDH), and lactate dehydrogenase (LDH) than white muscle (Walsh et al. 1989). Type-I male midshipman (*Porichthys notatus*) have also shown higher CS, MDH, aspartate aminotrandferase, glucose 6-phosphate dehydrogenase, and malic enzyme in the sonic muscle (Walsh et al. 1995). More research is needed to identify other common spots in the 2-DE PAGE that are associated with glycolytic and oxidative metabolism proteins (Isfort 2002; Shishkin et al. 2004). In the big-snout croaker, the identified P450 protein was expressed at a higher level in the sonic muscle than in the somatic muscle, which leads to the accommodation for lasting contraction of the sonic muscle. Only small number of mitochondria was observed in the sonic muscle (Ono and Poss 1982; Appelt et al. 1991; Fine et al. 1990, 1993)—a conclusion that agrees with the inference of rapid contraction of sonic muscle in toadfish at 200 Hz may not require huge amount of energy (Amorim et al. 2002). Thus, these proteins involved in the sonic muscle of big-snout croaker did not provide energy for twitch strength, but prevent the muscle contraction from fatigue.

In addition, these proteins may also be involved in the oxidative degradation of various compounds, leading to detoxification (Danielson 2002). For the sonic muscle, higher muscle metabolism produces a range of cytotoxic compounds (Bloomer and Goldfarb 2004) and superfast twitching that may lead to the accumulation of these cytotoxic compounds.

The expression levels of CK, isocitrate dehydrogenase, and cytochrome P450 proteins were higher in

the hypertrophic sonic muscle, suggesting that more energy is generated by the ATP-PC (phosphate creatine) system (Bessman and Geiger 1981), citric acid cycle (Bardsley 1992), and the electron transferase system (McLean et al. 2005). A similar result showing down-regulation of the cytochrome P450 superfamily of enzymes has been noted in the muscle atrophy of rainbow trout (Salem et al. 2010). The cytochrome P450 protein identified in the present study, which was at a higher level in the hypertrophic muscle, also plays a role in cellular detoxification (Danielson 2002). Higher levels of other detoxification proteins, such as Glutathione S-transferase Pi, GST-Pi, and proteasome, have been found associated with higher metabolic activity in the ovine hypertrophied muscle (Hamelin et al. 2006).

Croaker sonic muscle has a well-developed sarcoplamic reticulum surrounding the myofibrillar cyclinder (Lin 2008), and the sarcoplasm contains glycogen granules and mitochondria (Ono and Poss 1982). The oyster toadfish has only a small number of mitochondria (Appelt et al. 1991; Fine et al. 1990, 1993), an adaptation for extreme speed at the expense of muscle fatigue (Mitchell et al. 2008), compared to the midshipman type I males who have huge banks of mitochondria but contract their muscles at slower speeds (Bass and Marchaterre 1989). In croaker sonic muscle, the seasonal increases in sarcoplasmic reticulum might be for providing expanded Ca^{2+} capacity and fatigue resistance (Feher et al. 1998).

Miscellaneous proteins

The DEAD box proteins have been implicated in a wide variety of cellular processes ranging from the initiation of protein synthesis and ribosome biosynthesis to pre-RNA splicing by means of modifying the RNA structure (Pause et al. 1993; Sowden et al. 1995). Cyclins are a family of proteins involved in the progression of cells through the cell cycle. Higher display of DEAD box proteins and cyclins in the sonic muscle may be involved in cellular growth and division related to seasonal sonic muscle changes. Type IIc muscle fibers, which represent some regenerated and immature fibers or a hybrid transition state associated with muscle plasticity (Marqueste et al. 2006; Taguchi et al. 2004), found in the sonic muscle (Chen et al. 1998; Lee 2002) may be consistent with the higher expression of these proteins.

Skeletal muscle hypertrophy is possibly a result of excess synthesis of protein over protein degradation (Boonyarom and Inui 2006; Folland and Williams 2007). The higher expression of the DEAD box proteins may be related to cellular growth and division. Down-regulation of the mRNA of the ATP-dependent RNA helicase WM6, which is a member of the DEAD box family, has also been reported in atrophying rain trout muscle identified by cDNA microarray analysis (Salem et al. 2006b). In contrast, an RNA helicase-related protein containing a DEAD box motif increased in mammalian atrophic muscle (Lecker et al. 2004). The contradiction in these differences could be due to altered mechanisms of ribosomal biosysthesis in mammals and fish (Salem et al. 2006b).

Conclusions

In this study, 80 up-regulated protein spots were found in the sonic muscle compared with the somatic muscles. In addition, protein expressions between the hypertrophic and atrophic sonic muscles were also compared. Based on the results of protein identification, up-regulated proteins in the somatic muscle may be associated with the sound-production physiology of the sonic muscle, and proteins differentially expressed between atrophic and hypertrophic sonic muscles were related to energy metabolism and DEAD box proteins and cardiac α-actin. Cardiac α-actin was higher in atrophic muscle and may be associated with the need for future muscle regeneration. These differential expressions in muscle proteins may reveal new insights into the higher activity of sound production in the spawning season and preparation for regeneration in the non-spawning season. This change is consistent with the general pattern in gain and loss of muscle mass, and it suggests that sonic muscle can be used as a model to elucidate protein modulation during the cycle of muscle degeneration and regeneration.

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