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RESEARCH ARTICLE

The occurrence of two types of fast skeletal myosin heavy chains from abdominal muscle of kuruma shrimp *Marsupenaeus japonicus* and their different tissue distribution

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SUMMARY

Shrimps belong to the class Crustacea, which forms a large, diverse group in the invertebrates. However, the physiology and biochemistry of their skeletal muscles have been poorly understood compared with those from vertebrates including mammals and fish. The present study focused on myosin, the major protein in skeletal muscle, from adult specimens of kuruma shrimp *Marsupenaeus japonicus*. Two types of the gene encoding myosin heavy chain (MHC), a large subunit of the myosin molecule, were cloned from abdominal fast skeletal muscle and defined as MHCa and MHCb. Protein analysis revealed that the MHCa isoform was expressed at a higher level than the MHCb isoform. The full-length cDNA clones of MHCa and MHCb consisted of 5929 bp and 5955 bp, respectively, which encoded 1912 and 1910 amino acids, respectively. Both were classified into fast muscle type by comparison with the partially deduced amino acid sequences of fast-type and slow-type (S_1, slow twitch) MHCs reported previously for the American lobster *Homarus americanus*. The amino acid identities between MHCa and MHCb of kuruma shrimp were 78%, 60% and 72% in the regions of subfragment-1, subfragment-2 and light meromyosin, respectively, and 71% in total. *In situ* hybridisation using anti-sense RNA-specific probes, along with northern blot analysis using different tissues from abdominal muscle, revealed the different localisation of MHCa and MHCb transcripts in abdominal fast skeletal muscle, suggesting their distinct physiological functions.

Key words: expressional patterns, *in situ* hybridisation, kuruma shrimp, *Marsupenaeus japonicus*, myosin heavy chain genes, NADH-diaphorase, northern blot analysis.

INTRODUCTION

Shrimps belong to the class Crustacea, which forms a large, diverse group in the invertebrates (Martin and Davis, 2001). Many studies on shrimps have focused on physiological features including nutritional requirements, growth, disease resistance and immune systems (Jose et al., 2010; Richard et al., 2010). However, the physiology and biochemistry of shrimp muscle, the knowledge of which is useful for a better understanding of biologically diverse muscle functions, have not been examined. The physiology and biochemistry of skeletal muscle have also been studied in other crustaceans such as lobsters, crayfish and crabs (Parnas and Atwood, 1966; Mykles, 1997; Perry et al., 2009), demonstrating the presence of fast and slow muscles as seen in vertebrates (Ogonowski and Lang, 1979). In comparison with the repertoire of diverse skeletal muscle fibres in vertebrates, the repertoire in invertebrates is not well defined (Hooper et al., 2005; Hooper et al., 2008). For example, it is well known that vertebrate skeletal muscles consist of slow-twitch oxidative, fast-twitch oxidative and fast-twitch glycolytic fibres (Spangenburg and Booth, 2003). However, the functional properties of fast- and slow-twitch fibres in invertebrates have not been studied. Thus, it is interesting to investigate shrimp myosin as a comparative model with vertebrate and other invertebrate counterparts.

Myosin is a hexamer composed of two heavy chain subunits (myosin heavy chain, MHC), each approximately 200 kDa, and four light chain subunits (myosin light chain, MLC), each approximately 20 kDa, where each MHC is associated with two MLCs (Harrington and Rodgers, 1984). The myosin molecule consists of a globular head called subfragment-1 (S1) at the N-terminal half and a coiled-coil structure of α-helices called rod at the C-terminal half. Amongst the two fragments, S1 has several physiologically important functions, such as ATP and actin binding (Lowey et al., 1969; Harrington and Rodgers, 1984; Cope et al., 1996; Bobkov et al., 1997). S1 heavy chain further consists of three subfragments of 25 kDa, 50 kDa and 20 kDa in order from the N terminus (Balint et al., 1978; Mornet et al., 1979; Rayment et al., 1993). The 25 kDa and 50 kDa subfragments are connected by loop 1 whereas the 50 kDa and 20 kDa subfragments by loop 2. Loops 1 and 2 play functional roles in the sliding of myosin on actin filaments and in actin-activated myosin Mg^{2+}-ATPase activity, respectively (Uyeda et al., 1994; Murphy and Spudich, 1998; Murphy and Spudich, 1999; Sweeney et al., 1998). The myosin rod consists of two fragments called subfragment-2 (S2) and light meromyosin (LMM) at the N- and C-terminal sites, respectively. The myosin rod functions to transduce chemical energy produced by S1 ATPase into mechanical energy used during muscle contraction (Harrington and Rodgers, 1984).

Table 1. Primer sequences to amplify myosin heavy chain (MHC) clones from kuruma shrimp

Name	Sequence (5′–3′)	Primer positions (bp)*
kF1	GGCCCTGCGCATGAARAARAARYT	MHCa, 4945–4968; MHCb, 4980–5003
kMHCa-F2	CTBACTAATCARCTBGAYGAYACH	4037–4060
kMHCb-F2	ATYGARGARYTBGARGARGAR	4180–4200
kF3	GGCGAGTCCGGCGCNGGNAARAC	MHCa, 662–685; MHCb, 691–714
kMHCa-R1	GATTTCATCATGAACCTTCCTAAGA	5041–5065
kMHCb-R1	ATCTCGGCCTGGGCCTTCTTCACC	5076–5099
kMHCa-R2	CGTCCAGCTCAACAGCCGCACGCAT	4370–4394
kMHCb-R2	TCGTCGATTTCGGTAATAATGCGTCC	4405–4430
kMHCa-R3	CGTTACCGTAAGCCTCAAGGATGG	792–815
kMHCb-R3	GCAGGTACACCTCGATATCAGCAC	929–952
AAP	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG	
AUAP	GGCCACGCGTCGACTAGTAC	

AAP, abridged anchor primer; AUAP, abridged universal amplification primer.
*Right column indicates the positions of each primer from the 5′ end of MHC.

Many MHCs have been identified from vertebrate skeletal muscles. For instance, mammals contain six fast skeletal MHCs: embryonic and perinatal MHCs are expressed during pre- and postnatal development of skeletal muscle, respectively, whereas type IIa, type IIb and type IId/x MHCs, primarily expressed in adult fast skeletal fibres, have oxidative (type IIa) and glycolytic (type IIb and type IId/x) metabolic properties (Lyons et al., 1990; Schiaffino and Reggiani, 1996). Extraocular MHC is expressed in extrinsic muscle only. Type II fibres exert quick contractions and fatigue rapidly whereas type I fibres, used primarily by mammals as slow fibres because of slow contraction owing to low ATPase activity, are associated with type I MHC (Bassel-Duby and Olson, 2006).

In the case of myosin from crustaceans inhabiting aquatic environments, there is only fragmental information available on the American lobster *Homarus americanus* (Jahromi and Atwood, 1969; Cotton and Mykles, 1993; Medler and Mykles, 2003; Medler et al., 2004), the freshwater crayfish *Procambarus clarkii* (LaFramboise et al., 2000) and the gammarid amphipod (Rock et al., 2009; Whiteley et al., 2010). Besides the information about crustacean MHCs mentioned above, it has been clearly shown that the ghost crab *Ocypode quadrata* has two types of fast skeletal MHCs in the extensor carpopodite muscle as shown by SDS-PAGE (Perry et al., 2009). Unfortunately, their complete primary sequences have not yet been determined; thus, it is still not conclusive as to whether these crustaceans contain multiple MHCs each in fast and slow skeletal muscles. Therefore, it is interesting to investigate crustacean myosins, especially MHC, to clarify the possible existence of different MHCs with altered functions not only in terms of comparative biochemistry and physiology of skeletal muscle but also in terms of the molecular evolution of myosin molecules.

The present study was undertaken to obtain fundamental knowledge about MHCs in kuruma shrimp *Marsupenaeus japonicus* (Bate 1888). We first determined the complete sequences of MHCs from the abdominal muscles of kuruma shrimp. To our knowledge, this is the first report of the full-length sequences of MHCs from crustaceans. We found two fast-type MHCs and their abundances were determined by protein analysis. In addition, histological experiments for NADH-diaphorase staining and *in situ* hybridisation were carried out to localise glycolytic or oxidative fibres and the transcripts of MHCs, respectively.

MATERIALS AND METHODS

Animals

Farmed kuruma shrimp (body mass 13.2–31.5 g) were obtained in Miyazaki Prefecture, Japan, and were instantly killed by cutting the ventral nerve cord. The abdominal muscles were collected and preserved in RNA*later* (Ambion, Austin, TX, USA) until RNA extraction. The major part of the abdominal muscles was subjected to protein preparation. Cross-sections of abdominal muscle (thickness 18 μm) were used for histochemical analysis and *in situ* hybridisation.

Designing primers for the amplification of shrimp MHCs clones

CODEHOP (COnsensus-DEgenerate Hybrid Oligonucleotide Primers) (Rose et al., 1998) was used to design degenerate primers (kF1; Table 1) from the deduced amino acid sequences of MHC from the fruit fly *Drosophila melanogaster* (Meigen 1830) (DDBJ/EMBL/GenBank accession number AAA28686) and fast- (U03091) and S_1 slow-twitch (AY232598) MHCs from the American lobster *H. americanus* (H. Milne Edwards 1837).

Cloning full-length kuruma shrimp MHCs

Total RNAs were extracted from abdominal muscle of kuruma shrimp (body mass 31.5 g) using ISOGEN solution (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. First-strand cDNA synthesis was performed using adapter primer [5′-GGCCACGCGTCGACTAGTAC$(T)_{17}$-3′] and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA).

Table 1 shows the sequence and location of PCR primers. The 3′ region of the MHC clone was amplified by 3′ Rapid Amplification of cDNA Ends (RACE) using degenerate forward primer kF1 and abridged universal amplification primer (AUAP). PCR was performed using Ex *Taq* DNA polymerase (Takara, Otsu, Japan) under the following conditions: initial denaturation at 94°C for 2 min followed by 35 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 1 min. The amplified PCR products were subcloned into a plasmid, pGEM-T (Promega, Madison, WI, USA), using *Escherichia coli* (Migula 1895) strain JM109 as a host bacterium and sequenced with an ABI PRISM DNA sequencer model 3100 using BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA).

As two types of MHCs, named MHCa and MHCb, were cloned, the full-length cDNAs of each MHCa and MHCb clone were determined as follows. The gene-specific reverse primer kMHCa-R1 was designed based on the 3′ region sequence determined by 3′ RACE. PCR was performed using primer kMHCa-R1 and degenerate forward primer kMHCa-F2 with initial denaturation at 94°C for 2 min followed by 35 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 1 min. The amplified PCR products were subcloned and sequenced as mentioned above.

In the next step, the gene-specific primer kMHCa-R2 was designed based on the sequence that was determined as described above. PCR was carried out with initial denaturation at 94°C for 2 min followed by 40 cycles at 98°C for 10 s, 55°C for 30 s and 68°C for 3 min using the degenerate primer kF3, the gene-specific reverse primer kMHCa-R2 and KOD -Plus- Neo DNA polymerase (Toyobo, Osaka, Japan). The amplified products were directly sequenced.

To determine the 5′ region of the MHC clones, 5′ RACE was performed. In brief, first-strand cDNA was purified using QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) and mixed with dCTP and TdT. The mixture was incubated at 37°C for 10 min to add poly(C) tail. The poly(C)-tailed cDNA was used as a template for PCR to amplify the 5′ region of the MHCs clones. PCR was performed using abridged anchor primer (AAP) and gene-specific primer kMHCa-R3, referring to the sequence determined as described above. PCR was performed using KOD -Plus- Neo DNA polymerase (Toyobo) with initial denaturation at 94°C for 2 min followed by 40 cycles at 98°C for 10 s, 50°C for 30 s and 68°C for 1 min. The amplified products were subcloned into pBluescript II KS (+) vector (Stratagene, La Jolla, CA, USA) using *E. coli* strain JM109 and sequenced.

The same methods were used to determine the full sequence of the MHCb clone except that primers kMHCb-F2, kMHCb-R1, kMHCb-R2 and kMHCb-R3 were used (Table 1).

Sequence similarity

A comparison of the deduced amino acid sequences was carried out using partial amino acid sequences of MHC from the kuruma shrimp, the American lobster, two fly species *D. melanogaster* and *Drosophila virillis* (Sturtevant 1916), the scallop *Argopecten irradians* (Lamarck 1819) and the squids *Loligo pealei* (Lesueur 1821) and *Loligo bleekeri* (Keferstein 1866). As only the amino acid sequence of C-terminal regions of MHC have been reported for the American lobster, the corresponding sequence of 157 amino acids was used. The deduced amino acid sequences were aligned by Clustal W (Thompson et al., 1994), and trees were constructed by the neighbour-joining method using MEGA 4.1 (Kumar et al., 2008).

Preparation of myosin and its chymotryptic digests

Myosin was prepared using a similar method to Hwang et al. (Hwang et al., 1990). Briefly, abdominal muscles of kuruma shrimp (total mass 52.2 g) were homogenised with 5 volumes of 6 mmol l^{-1} K_2PO_4 buffer (pH 7.0) and centrifuged at 3000 ***g*** for 5 min at 4°C to remove the supernatant containing water-soluble sarcoplasmic proteins. This procedure was repeated twice and the resulting precipitate was dissolved in 2 volumes of 7.5 mmol l^{-1} ATP solution (pH 6.4) containing 0.675 mol l^{-1} KCl, 7.5 mmol l^{-1} $MgCl_2$ and 0.15 mmol l^{-1} dithiothreitol (DTT). The mixture was allowed to stand on ice for 15 min and was centrifuged at 5500 ***g*** at 4°C for 10 min. The precipitate was dissolved in 1/5 volume of 0.12 mol l^{-1} Tris-maleate buffer (pH 7.5) containing 3 mol l^{-1} KCl and 0.6 mol l^{-1} DTT and was then added to 1/10 volume of 110 mmol l^{-1} ATP containing 55 mmol l^{-1} ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (pH 7.5). The solution was fractionated in the 40–55% $(NH_4)_2SO_4$ saturation and centrifuged at 5500 ***g*** for 10 min at 4°C. The precipitate was dissolved in and dialysed against 20 mmol l^{-1} Tris-maleate buffer (pH 7.5) containing 0.5 mol l^{-1} KCl and 0.1 mmol l^{-1} DTT. After centrifugation at 100,000 ***g*** for 60 min, the supernatant was used as the purified myosin.

The purified myosin at a protein concentration of 4.71 mg ml^{-1} was digested at 10°C with tosyl lysine chloromethyl ketone-treated α-chymotrypsin (Sigma Chemical Co., St Louis, MO, USA) at an enzyme-to-myosin weight ratio of 1/130 in 20 mmol l^{-1} Tris-maleate buffer (pH 7.0) containing 0.05 mol l^{-1} KCl, 1 mmol l^{-1} EDTA and 1 mmol l^{-1} DTT. After certain periods of incubation ranging from 0 min to 120 min, digestion was stopped by the addition of phenylmethylsulfonyl fluoride to achieve a final concentration of 0.5 mmol l^{-1}.

N-terminal amino acid sequencing

The chymotryptic digests prepared as described above were treated with SDS-glycerol buffer (0.1 mol l^{-1} Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 12% 2-mercaptoethanol and a small volume of Bromophenol Blue), run on SDS-PAGE using 7.5–20% gradient gels containing 0.1% SDS and transferred to polyvinylidene difluoride membranes. The fragments, which were visualised by staining with 0.05% Coomassie Brilliant Blue (CBB) R-250 in 50% methanol and 10% acetic acid, were cut and analysed for N-terminal amino acid sequences with an ABI Procise 492HT protein sequencer (Applied Biosystems).

NADH-diaphorase staining

NADH-diaphorase staining was performed to distinguish between fast-twitch glycolytic and slow-twitch oxidative fibres. The abdominal muscles of kuruma shrimp (body mass 13.2 g) were snap frozen in liquid nitrogen and cut at a thickness of 18 μm using a cryostat. The sections were incubated in 50 mmol l^{-1} Tris-HCl buffer (pH 7.6) containing 2.25 mmol l^{-1} NADH and 2.45 mmol l^{-1} nitroblue tetrazolium chloride (NBT) at 37°C for 30 min, and unbound NBT was removed by dehydration in graded acetone, washed in distilled water and mounted in glycerol.

In situ hybridisation

The probes for *in situ* hybridisation were constructed from 3′-untranslated regions whose nucleotide identity between MHCa and MHCb clones was 51%. Digoxigenin (DIG; Roche Applied Science, Mannheim, Germany)-labelled RNA probes for MHCa and MHCb clones were synthesised by *in vitro* transcription using SP6 RNA polymerase (Roche Applied Science). To confirm the specificity of probes, dot-blot analysis was conducted. The plasmid inserted with each of the 3′-ends of MHCa and MHCb clones was spotted onto nylon membranes. The membranes were pre-hybridised in Church phosphate buffer (0.5 mmol l^{-1} Na_2HPO_4, pH 7.2, 1 mmol l^{-1} EDTA and 7% SDS) at 67°C for 20 min and hybridised with DIG-labelled anti-sense probes at 67°C for 16 h. The membranes were washed in saline sodium citrate (SSC) buffer, blocked with blocking solution [0.1 mol l^{-1} maleic acid, pH 7.5, 0.15 mol l^{-1} NaCl and 1% blocking reagent (Roche Applied Science)] and incubated in a solution containing 15 mU ml^{-1} anti-DIG-AP fab fragment (Roche Applied Science) at room temperature. The DIG-labelled probes were detected by using CDP-Star (Roche Applied Science).

After the specificity was confirmed, *in situ* hybridisation was performed. Transverse sections of 18 μm thickness were made from the abdominal muscles of kuruma shrimp (body mass 17.5 g). The sections were washed in phosphate-buffered saline with 0.1% Tween 20, pre-hybridised in hybridisation buffer (5× SSC, pH 7.0, 50% formamide, 50 μg ml^{-1} heparin, 500 μg ml^{-1} torula RNA and 0.1% Tween 20) at 58°C for 1 h and hybridised with DIG-labelled RNA probes at the same temperature for 16 h. After hybridisation, the sections were washed in 2× SSCT (SSC with 0.1% Tween 20) buffer containing 50% formamide and in 0.2× SSCT buffer twice. Each wash was conducted at 58°C for 15 min. After the washing procedure, alkaline phosphatase-conjugated anti-DIG antibody

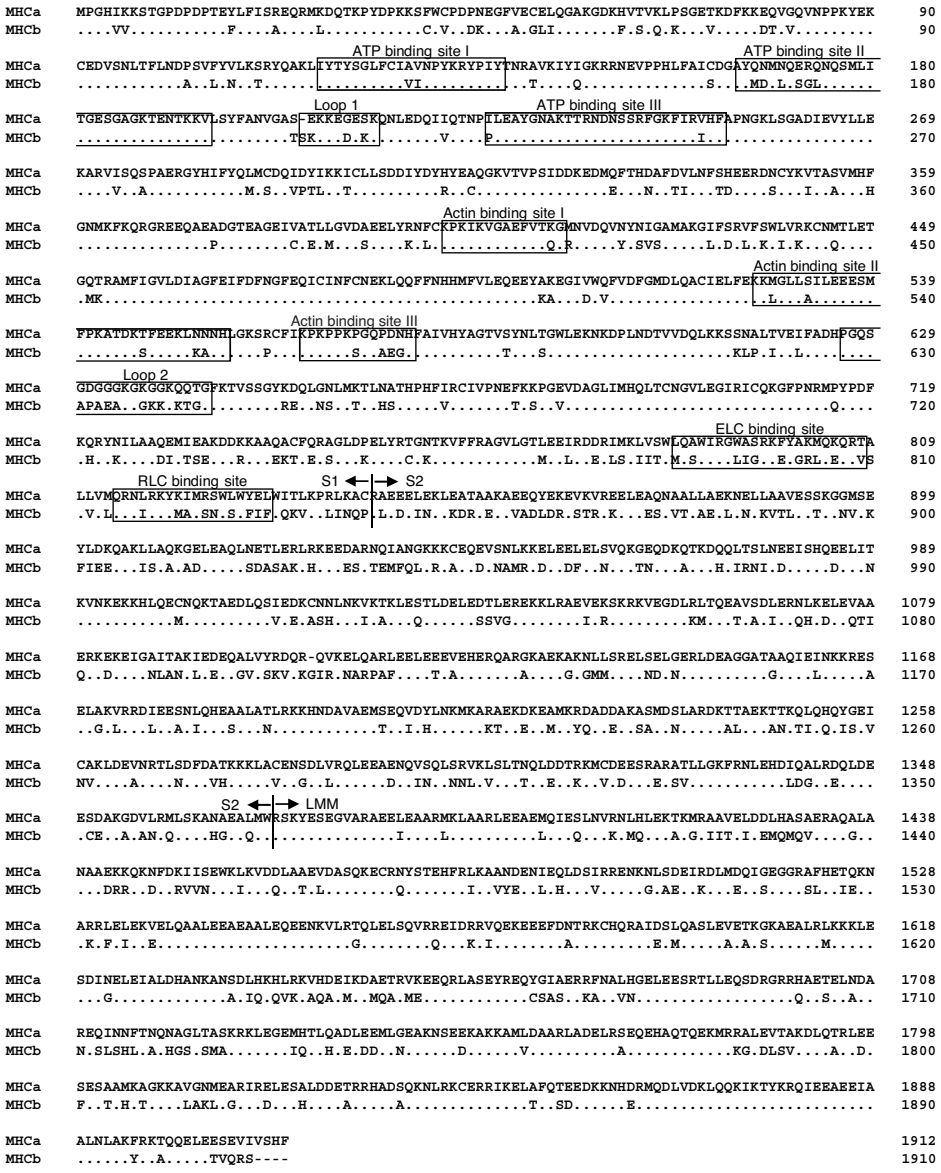


Fig. 1. The full-length deduced amino acid sequences of myosin heavy chains (MHCs) from kuruma shrimp. Amino acid residues in MHCb (AB613206) identical to those in MHCa (AB613205) are indicated by dots, and hyphens denote deletions. Putative ATP-, actin-, essential light chain (ELC)- and regulatory light chain (RLC)-binding sites, as well as loops 1 and 2, are boxed. S1, S2 and LMM indicate myosin subfragment-1 heavy chain, myosin subfragment-2 and light meromyosin, respectively. The vertical lines indicate the boundaries between S1 and S2, and between S2 and LMM shown by arrows. The DNA nucleotide sequences of MHCa and MHCb clones have been registered into the DDBJ/EMBL/GenBank databases with accession numbers of AB613205 and AB613206, respectively.

(Roche Applied Science) and NBT (0.375 mg ml^{-1})/5-bromo-4-chloro-3-indolyl phosphate (0.188 mg ml^{-1}) were used for the detection of RNA probes.

Northern blot analysis

Total RNAs were extracted from four distinct parts in the abdominal muscles of kuruma shrimp (17.8 g). 5 μg of total RNAs were electrophoresed on a 0.9% agarose gel containing 20% formamide and transferred to the nylon membranes. The membranes were pre-hybridised in Church phosphate buffer, hybridised with DIG-labelled RNA probes, washed in SSC buffer, incubated with anti-DIG-AP fab fragment and detected for the mRNAs concerned as mentioned above.

RESULTS

cDNA nucleotides and the deduced amino acid sequences of MHCs from kuruma shrimp

Two DNA fragments of 961 bp and 965 bp were amplified by 3′ RACE with primers kF1 and AUAP. The nucleotide sequences of the 3′ regions of MHC clones indicated that there were two types of MHCs, subsequently designated MHCa and MHCb. MHCa and MHCb were actually named according to their abundance in abdominal muscle (MHCa>MHCb), which will be described later.

PCR with two primer sets of kMHCa-F2/kMHCa-R1 and kMHCb-F2/kMHCb-R1 amplified cDNA fragments of MHCa and MHCb, which consisted of 1004 bp and 1178 bp, respectively. PCR performed with primer sets of kF3/kMHCa-R2 and kF3/kMHCb-R2 yielded the amplified products of MHCa and MHCb clones of 3685 bp and 3691 bp long, respectively. When PCR was carried out with primer sets of AAP/kMHCa-R3 and AAP/kMHCb-R3, the resulting amplified products of MHCa and MHCb clones were 791 bp and 928 bp long, respectively. Through sequencing of these amplified fragments, the complete DNA sequences of MHCa and MHCb were determined (DDBJ/EMBL/GenBank accession number AB613205 and AB613206, respectively). The full-length cDNAs encoding MHCa and MHCb were 5929 bp and 5955 bp long, respectively. The amino acid identity between full-length MHCa and MHCb was 71% and that of S1, S2 and LMM regions was 78%, 60% and 72%, respectively (Table 2). Thus, the identity in the S2 region was lower than that of other regions. In the S1 region,

Table 2. The nucleotide and amino acid identities between MHCa and MHCb from kuruma shrimp (%)

	S1	S2	LMM	Total
Nucleotide	76	65	74	72
Amino acid	78	60	72	71

MHC, myosin heavy chain; S1, subfragment-1; S2, subfragment-2; LMM, light meromyosin.

putative ATP-, actin- and MLC-binding sites were identified, and differences were clearly found in the MLC-binding sites between MHCa and MHCb (Fig. 1).

Marked differences were also observed in loops 1 and 2. For instance, the loop 1 region of MHCa consisted of 11 amino acids whereas that of MHCb consisted of 12 amino acids. In addition, loop 2 of MHCa contained 9 glycine residues, resulting in a low amino acid identity of 52% between MHCa and MHCb in this region. Fig. 2 shows the comparison of the sequences of loops 1 and 2 from various marine crustaceans (Holmes et al., 2002). Loop 1 from the isopods *Glyptonotus antarcticus* (Eights 1852) and *Idotea resecata* (Stimpson 1853), the amphipod *Eulimnogammarus verrucosus* (Gerstfeldt 1858) and the European lobster *Homarus gammarus* (Linnaeus 1758) each have a positive charge of +2 or +3 whereas a net charge of MHCa from kuruma shrimp was 0 and that of MHCb was +2. In contrast, loop 2 from *G. antarcticus*, *I. resecata*, *E. verrucosus* and the European lobster each have a positive charge of +2 or +3 whereas net charges of MHCa and MHCb from kuruma shrimp were +2 and +4, respectively.

Sequence similarities of MHC

Fig. 3 shows the comparison of the deduced amino acid sequence similarities of MHCs from the kuruma shrimp, the American lobster, the fruit flies, the scallop and the squids in the tree. This tree demonstrated that the two kuruma shrimp MHCs were divided

A

Loop 1

		Charge
Kuruma MHCa	-EKKEGESK	0
Kuruma MHCb	SK...D.K.	+2
G.antarcticus	TK.RGE...	+2
I.resecata	TK.RGE...	+2
E.verrucosus	TK..GE.A.	+2
H.gammarus fast	TK..GEVA.	+3
H.gammarus slow	TK.RGE.A.	+2

B

Loop 2

		Charge
Kuruma MHCa	PGQSGDGGGKGKGGK-QQTG-	+2
Kuruma MHCb	....APAEA..GKK.--K..G	+4
G.antarcticus	A....G.DAG.GK.RGKKS.G	+3
I.resecata	.....G-DAG.GK.RGKKS.G	+3
E.verrucosus	.....G.DAG....RGKKS.G	+3
H.gammarus fast	.....PVES..GR.A--KS.S	+2
H.gammarus slow	....AVADT..GR.Q--KS.S	+2

Fig. 2. A comparison of the deduced amino acid sequences of myosin heavy chain (MHC) loops 1 (A) and 2 (B). MHCs cited are from the isopods, *Glyptonotus antarcticus* and *Idotea resecata*, the amphipod *Eulimnogammarus verrucosus* and the European lobster *Homarus gammarus* (Holmes et al., 2002). Right columns indicate the net charges of respective loops.

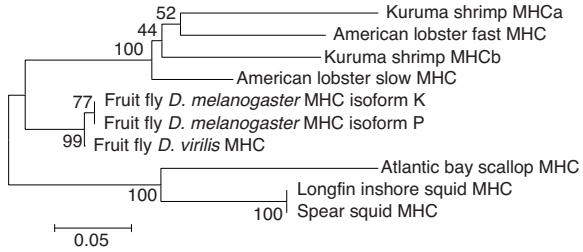


Fig. 3. The sequence similarity of myosin heavy chains (MHCs) from kuruma shrimp and other invertebrates. MHCs cited are the American lobster *Homarus americanus* (fast-twitch, U03091; S_1 slow-twitch, AY232598), the flies *Drosophila melanogaster* (isoform K, NP724008; isoform P, NP001162992) and *Drosophila virilis* (XM002051957), the scallop *Argopecten irradians* (X55714) and the squids *Loligo pealei* (AAC24207) and *Loligo bleekeri* (ACD68201).

into two clades, although the bootstrap value was low at 44. The two MHCs formed one clade with the American lobster fast-twitch MHC, clearly different from the American lobster S_1 slow-twitch MHC with a highly significant bootstrap value (100).

The abundancy of two types of MHCs in kuruma shrimp

To examine MHCs at the protein level, myosin was purified from the abdominal muscles of kuruma shrimp and digested with α-chymotrypsin, yielding partial S2 and LMM fragments (Fig. 4). The amino acid residues determined corresponded to either those of MHCa or MHCb deduced from cDNA nucleotide sequences shown in Fig. 1, except for last three residues in S2, which may be derived from the uncertainty in the N-terminal amino acid sequencing.

Localisation of MHCa and MHCb transcripts

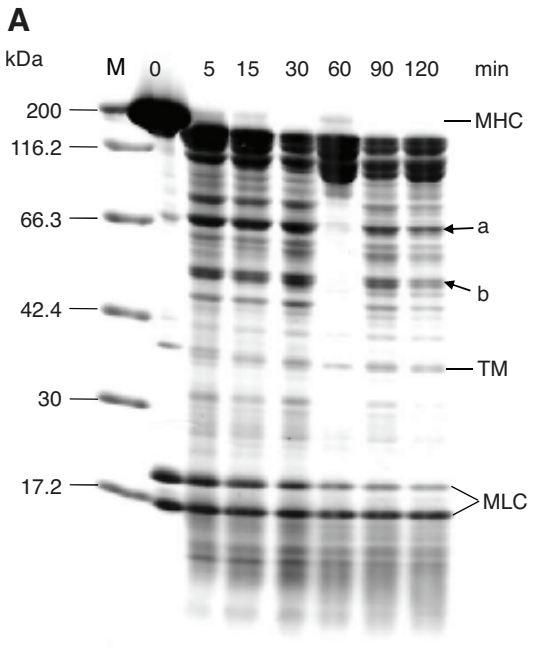
NADH-diaphorase staining, which gives positive reactions to tissues in aerobic metabolism, was performed to distinguish fibre types in kuruma shrimp (Fig. 5). With this staining, fast and slow muscles are supposed to have negative and positive reactions, respectively. While the swimmeret muscles were stained, extensor and flexor muscles were not stained, indicating that these muscle parts possess different metabolisms.

In situ hybridisation was performed to localise MHCa and MHCb transcripts. The specificity of DIG-labelled anti-sense RNA probes was confirmed by dot-blot analysis (data not shown). *In situ* hybridisation localised the transcripts of MHCb in all fibres of the abdominal muscles, including the extensor and flexor muscles (Fig. 6). In contrast, the transcripts of MHCa were not detected in the extensor muscle (Fig. 6).

In order to confirm the results obtained by *in situ* hybridisation, total RNAs were prepared from different parts of the abdominal muscles and subjected to northern blot analysis. As a result, MHCb transcripts were found to be expressed in the extensor and flexor muscles (Fig. 7E). In contrast, MHCa transcripts were not detected in the extensor muscle (Fig. 7D). Slow-type pleopod muscles did not express either MHCa or MHCb transcripts.

DISCUSSION

In the present study, the full-length cDNAs encoding two types of fast muscle MHC isoforms, MHCa and MHCb, were cloned from abdominal fast muscles of adult kuruma shrimp. The presence of the two MHCs was also confirmed by SDS-PAGE for myosin preparation isolated from the abdominal muscles (see Fig. 4). To our knowledge, this is the first report of the full-length sequences of MHC cDNAs from crustaceans.



B

Band a(S2)	SLTNQLDDTRKMCDDESKGA	20
MHCa	..............E..RAR	1325
MHCb	...T..E..K..V....RER	1327

C

Band b(LMM)	STEHFRLKAVNDENLEQLDS	20
MHCa	.........A....I.....	1496
MHCb	......I...YE....H...	1498

Fig. 4. SDS-PAGE patterns and N-terminal amino acid sequences of α-chymotryptic digests of myosin purified from the abdominal muscles of kuruma shrimp. SDS-PAGE was performed using the 7.5–20% gradient polyacrylamide gel, and numerals above each line indicate digestion time periods along with molecular weight markers (lane M) (A). Bands a and b were subjected to N-terminal amino sequencing. N-terminal amino acid sequences of band a (S2) (B) and band b (LMM) (C) obtained from myosin isolated from the abdominal muscles of kuruma shrimp were subjected to alignment with corresponding regions of MHCa and MHCb determined by cDNA cloning. Numbers in the right margin indicate amino acid residues from the N-terminus of chymotryptic digests (S2 and LMM) and those deduced from cDNAs (MHCa and MHCb). Abbreviations used are: MHC, myosin heavy chain; TM, tropomyosin; MLC, myosin light chains; S2, subfragment-2 heavy chain; LMM, light meromyosin.

MHCa and MHCb of kuruma shrimp showed the amino acid identity of 71% (see Table 2). Loops 1 and 2 in the myosin head S1 have been known to have large variations depending on MHC isoforms, and their sequences were also different between kuruma shrimp MHCa and MHCb. These loops play pivotal roles in myosin kinetics and thus regulate muscle contraction (Goodson et al., 1999). The reduction of net charge in loop 1 results in the decrease of *in vitro* motility (Sweeney et al., 1998) whereas the charge change in the range of –1 to +2 does not affect the behaviour of loop 2 (Furch et al., 1998). The net charge of loop 1 in MHCa from kuruma shrimp was 0 and lower than those of *G. antarcticus*, *I. resecata*, *E. verrucosus* and the European lobster (+2 to +3) (see Fig. 2), indicating that the motility of kuruma shrimp myosin may be lower compared with those of the above-mentioned crustacean myosins.

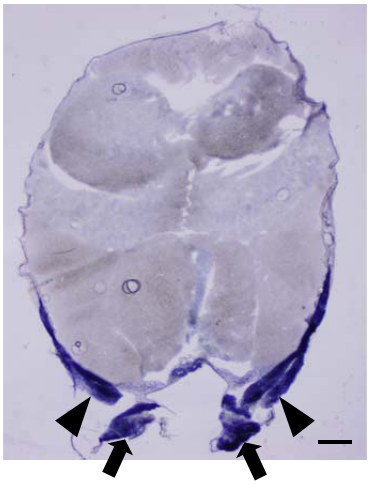


Fig. 5. Histochemical analysis of NADH-diaphorase activity in the abdominal muscles of kuruma shrimp. Slow muscles are located at pleopods (arrows) and its neighbouring areas (arrowheads), as revealed by staining. The scale bar=1.0 mm.

A comparison of the deduced amino acid sequences suggested that the two MHCs of kuruma shrimp are fast type. MHCs from kuruma shrimp did not form a cluster with the S_1 slow-twitch MHC of the American lobster, which was supported by a high bootstrap value. While kuruma shrimp MHCa was monophyletic with the fast-type MHC of the American lobster, kuruma shrimp MHCb was separated from these two MHCs, although the bootstrap value was not as high. Therefore, kuruma shrimp MHCa and MHCb were both regarded to be fast type. In this analysis only partial sequences of MHCa and MHCb were employed, because no full sequences of crustaceans MHCs have been available so far except from our present data. Hence, it is necessary to determine the full sequences of crustacean slow-type MHCs and to compare these sequences with fast-type MHC counterparts for a more detailed comparison.

It has been reported that American lobster deep abdominal muscles express MHCs in fast, slow-twitch S_1 and slow-tonic S_2 fibres, which are significantly different from the fast fibres of cutter claw muscles (Medler and Myles, 2003). Koenders et al. identified two populations of fast fibres with different Ca^{2+}-sensitivity and troponin-I isoforms in the claws of the Australian freshwater crustacean *Cherax destructor* (Koenders et al., 2004). Furthermore, SDS-PAGE on the ghost crab muscle revealed that two MHCs are co-expressed in the glycolytic fibres. These data suggest that crustacean fast muscles have multiple MHCs as in the case of vertebrates. The present study provided the most convincing evidence for the existence of distinct fast MHCs in crustacean fast muscles.

Interestingly, MHCa was expressed only in the flexor muscle of kuruma shrimp and MHCb, in both extensor and flexor muscles, as revealed by *in situ* hybridisation, with both muscles having glycolytic metabolism as shown by NADH-diaphorase staining. According to the NADH-diaphorase activity staining, swimmeret muscles were found to have slow-type oxidative muscle fibres. However, it is noted that there are several examples of oxidative fast muscle (Silverman and Charlton, 1980; Tse et al., 1983). Thus, it is important to clone MHCs from these oxidative muscles to determine whether other fast-type MHCs are present or not.

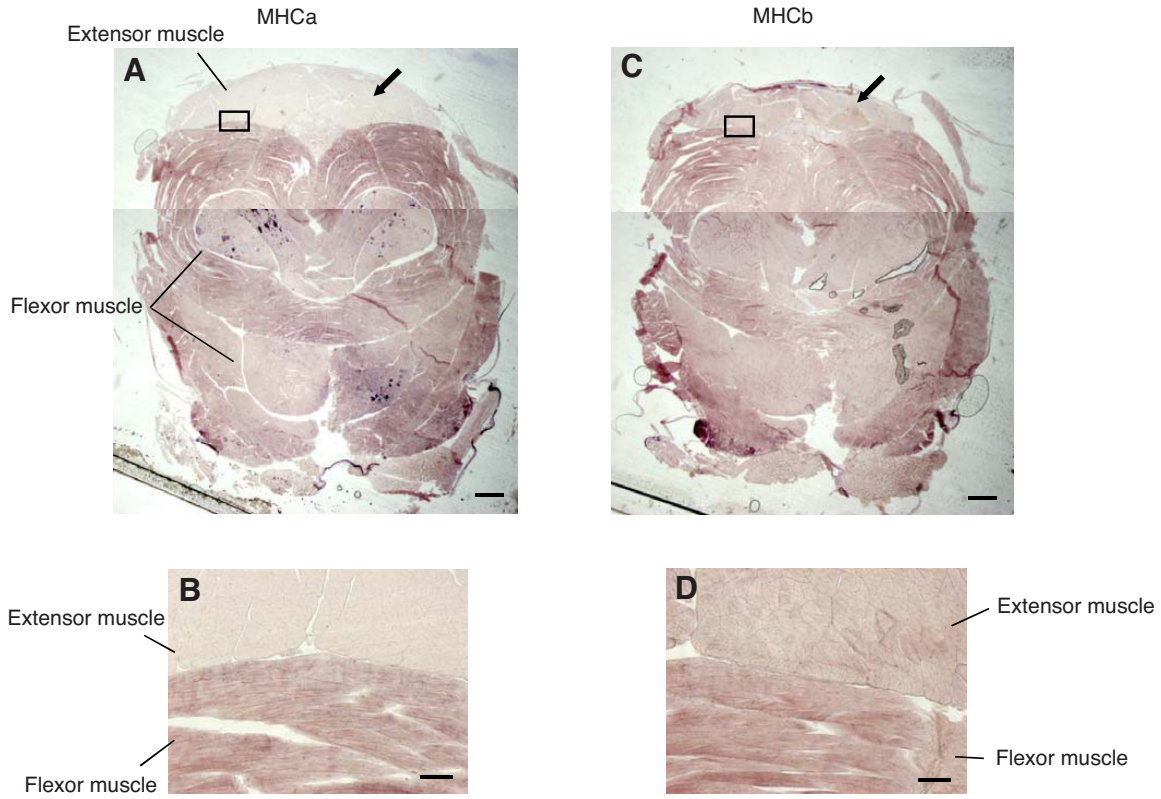


Fig. 6. Tissue distributions of MHCa and MHCb transcripts from kuruma shrimp as revealed by *in situ* hybridisation. *In situ* hybridisation was performed with probes specific to MHCa (A,B) and MHCb clones (C,D). MHCa transcripts are localised in most parts of the abdominal muscles except the extensor muscle (indicated with an arrow) (A). MHCb transcripts are localised at the whole abdominal muscle (C). Panels B and D show the magnification of the boxes indicated in panels A and C, respectively. Scale bars are 1.0 mm for panels A and C, and 100 μm for panels B and D. MHC, myosin heavy chain.

Alternatively, ATPase staining is another examination to be employed for discrimination of fast and slow muscle types (Ognonowski and Lang, 1979).

Although the detailed physiological properties of the extensor muscle in kuruma shrimp, including shortening velocity and maximum tension, have remained unknown, it is assumed that this muscle is used for flicking the tail (Parnas and Atwood, 1966). Despite a lack of MHCa expressed in the extensor muscle, N-terminal amino acid sequencing for myosin preparation indicated that MHCa was expressed dominantly in the abdominal muscles. Thus, it seems that MHCa is expressed much more abundantly than MHCb in the flexor muscle of the abdominal muscle. In addition, neither of the two MHCs was detected in pleopod muscles. These results are consistent with those from phylogenetic analysis. It is likely that pleopod muscle has MHCs of slow type, which were not cloned in the present study.

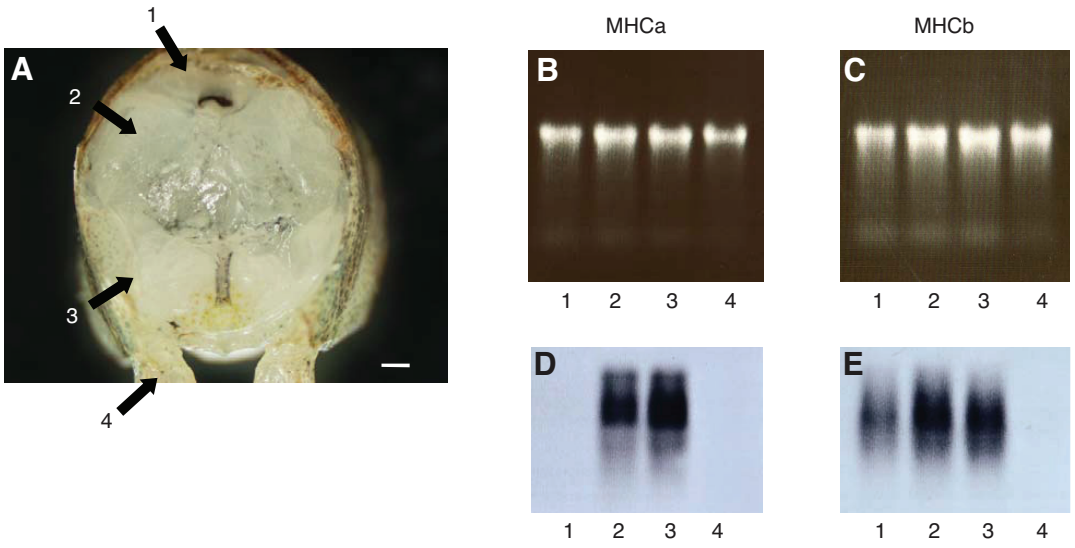


Fig. 7. Northern blot analysis for MHCa and MHCb clones of kuruma shrimp. Total RNAs were extracted from four distinct parts indicated by arrows 1–4 (A). Electrophoretic patterns are shown for total RNA (B,C) and northern blots with probes specific to MHCa (D) and MHCb clones (E). No MHCa transcripts are detected in either the extensor or pleopod muscles (D) whereas MHCb transcripts are observed in the whole abdominal muscle, but not in the pleopod muscles (E).

In this study, the complete sequences of two types of MHCs from kuruma shrimp abdominal muscle were determined. The locations of MHCa and MHCb transcripts were also examined in this shrimp. It is interesting to examine whether or not other shrimps such as black tiger *Penaeus monodon* and Pacific white *Penaeus vannamei* shrimps inhabiting tropical areas also contain different MHCs, which is now under investigation in our laboratory.

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