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Structure and Function of Myosin Isoforms in Adult Chicken Hindlimb Muscles

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Although large accumulation of sequence data is published for a variety of myosin heavy chain (MHC) isoforms, the meaning of heterogeneity among the amino acid sequences remains unclear as to the key contractile and biochemical properties of muscle fibres. In the present study, we studied on MHC isoforms in three adult chicken hindlimb muscles: *ilio-tibialis*, *gastrocnemius* and *femori-tibialis*) and *pectoralis* muscle, by means of *in vitro* motility assay and measurement of ATPase activity. The motility speed of myosins and ATPase activities of myofibrils extracted from the hindlimb muscles were significantly lower than those from the *pectoralis* muscle consisting of a homogeneous MHC (P-type). ATPase activity of *femori-tibialis* myofibril was remarkably lower than those of *ilio-tibialis* and *gastrocnemius* myofibrils. We found the differential expression of MHC isoforms in these muscles by northern blot analysis. Furthermore, we determined the amino acid sequences of the 23kDa, 50kDa and 20kDa fragments from a major MHC isoform (G-type) found in the three hindlimb muscles. There was approximately 4.3% amino acid difference between G-type and P-type, however the characteristically methylated amino acids were recognized in the G-type at the same residues as in the P-type. In the course of sequencing the 20kDa fragment from *femori-tibialis* muscle myosin, we found another MHC isoform (F-type). Content-ratios of P-type, G-type and F-type were about 3 : 7 : 0 in *ilio-tibialis*, 2 : 7 : 0 in *gastrocnemius*, and 1 : 6 : 3 in *femori-tibialis*, respectively. All these data suggest that the motility speed of myosin and ATPase activity of myofibril correlate with the content-ratio of the MHC isoforms in each muscle.

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Key Words: myosin; isoforms; amino acid sequence of S-1; *in vitro* motility assay; ATPase activity of myofibril

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Introduction

Myosin, a major contractile protein within thick filament of myofibril, is composed of two heavy chains and four light chains. Myosin heavy chain (MHC) contains the adenosine triphosphatase (ATPase) activity providing energy that is the driving force for contractile processes, and numerous MHC isoforms exist to carry out this function. Contractile properties of the various muscle fibres are considered correlating with the myosin ATPase activity. MHC heterogeneities found at the protein level were due to different gene or transcriptional expression, or due to post translational modifications such as methylation[1-3]. The MHC isoforms are encoded by diverse multigene family. By extensive genomic study of chicken muscle, at least 31 unique MHC genes were identified, and seven of them were expressed in the fast muscle fibres[4]. The nucleotide sequences of the 5'-untranslated region through to the end of the fifth exon of the seven genes have been reported[5].

Myosin from adult chicken pectoralis muscle consists of a homogeneous MHC (P-type) sequence of which well corresponds to the genomic DNA, N116 (M13512)[6-9]. The purpose of this study is to examine whether myosin isoforms in fast muscles have different contractile functions. In the present study, we found the functional differences in the myosin motility and the myofibrillar ATPase activity among pectoralis and three hindlimb (*ilio-tibialis*, *gastrocnemius*, *femori-tibialis*) muscles from adult chicken. Northern blot analysis with MHC isoform specific probes revealed MHC isoform expression pattern in pectoralis and the hindlimb muscles. Furthermore, we clarified that a major MHC isoform in the three hindlimb muscles was G-type, and that a new MHC isoform (F-type) was expressed in femori-tibialis muscle, by the amino acid sequence analysis. We discuss the relation between the structure and function of the MHC isoforms.

Materials and Methods

Materials

The sources of materials used for this study were as follows: collodion 2% (Nissin EM co., Tokyo, Japan), Texas Red-X phalloidin (Molecular Proves, OR, USA), methyl cellulose 400 cP (Sigma Chemical co., St Louis, MO), α-chymotrypsin (Sigma), trypsin (sigma), lysyl endopeptidase (Wako Pure Chemical Industries, Osaka, Japan), *Staphylococcus aureus* V8 protease (Wako), acylamino-acid-releasing enzyme (Takara Shuzo Co. Ltd., Kyoto, Japan), digoxigenin-11-dUTP (Boehringer Mannheim, Mannheim, Germany), DIG wash and block buffer set (Rosh Diagnostics, Basel, Switzerland), CSPD (Rosh Diagnostics). All other chemicals were analytical grade commercial products.

Preparation of muscle proteins

We prepared myosins and myofibrils from adult (8 weeks) chicken pectoralis and three hindlimb muscles, femori-tibialis connecting femur to tibia or patella, ilio-tibialis connecting ilium to tibia or patella, and gastrocnemius connecting the Achilles' tendon to femur or patella. Myosin was extracted and purified from the muscles by the method of Hynes *et al*[10], and was then frozen in 0.5M KCl, 0.5mM dithiothreitol and 20mM Tris-HCl (pH7.5) with liquid nitrogen, and stored at -80℃. Actin was purified from chicken pectoralis muscle by the method of Spudich and Watt[11], and a portion of actin was fluorescently labeled with texas red-phalloidin by the method of Kron and Spudich[12].

In vitro motility assay

Motility assay was carried out according to the method of Kojima *et al.*[13]. To remove non-functional myosin molecules that could bind actin filaments without ATPase function, myosin preparations were centrifuged at 100,000g for 20 min in the presence of unlabelled F-actin and 2mM ATP in the standard assay buffer (25mM KCl, 3mM $MgCl_2$, 20mM HEPES, pH7.8). Myosin at concentration of 40 μg/ml was infused into a flow cell, to bind to a nitrocellulose coated glass surface for 5min. 25mg/ml BSA was then immersed into the flow cell to wash unbound myosin molecules and blocking for 15 min, followed by a wash with the standard assay buffer. A solution of 2.6 μg/ml texas red-phalloidin-labeled F-actin in 100mM KCl, 10mM HEPES (pH7.8) was infused into the flow cell. Motility buffer (standard assay buffer + 2mM ATP, 0.5% methylcellulose as a viscosity-enhancing reagent, and 4.5mg/ml glucose, 216 μg/ml glucose oxidase and 36 μg/ml catalase as an oxygen scavenger system) was then added to initiate motility. The movement of fluorescence-labeled actin filaments was observed with a fluorescence microscope, Olympus BX 50 (Olympus, Tokyo, Japan) at 30℃. Actin movements were recorded with a SIT camera, C2400 (Hamamatsu Photonics, Japan) on to a videotape with video recorder, HR-VXG 300 (JVC, Yokohama, Japan). The motion of actin filaments was converted to a TIFF file by a Macintosh computer with a Scion LG3/PCI video capture board. The velocity of each actin filament was calculated from the x and y locations in 750 successive frames at an acquisition rate of 30 frame/sec. From each muscle, fifty actin filaments moving with constant speed were selected for speed analysis. Statistical analysis was made by *Mann-Whitney* U test.

Measurement of myofibrillar ATPase activity

Myofibrils were prepared from the hindlimb and pectoralis muscles[14]. ATPase assay was carried out by a modified method of Itaya and Ui[15]. Myofibrils, 0.2mg/ml, were allowed to react with 1mM ATP for 0-1 min in the presence of 0.1M KCl, 20mM imidazole-HCl (pH 7.0), 5mM $MgCl_2$, 0.2mM $CaCl_2$. 100 μl of reaction medium was quickly mixed with 900 μl of ice-chilled 0.27M perchloric acid (PCA) and centrifuged at 20,000g for 5min at 4℃. An aliquot of the supernatant, 700 μ l, was incubated in a thermo unit at 25℃ for 10min after mixing with an equal volume of Malachite Green reagent, and absorbance was measured at 650nm. Myofibrils were analyzed five times for each muscle. Statistical analysis was made by *Mann-Whitney* U test.

Northern blot analysis

We designed a synthetic probe (P-type, *ggctgcgctcaca caggaagcacagtcagaagag*) to detect a major MHC isoform in pectoralis muscle from clone N116[5], which was identical to the major MHC cDNA sequence of chicken pectoralis muscle (our unpublished data). We also designed a synthetic probe (G-type, *ggctgctgtttatgctgttcagc gcacgaggggg*) for a MHC isoform in gastrocnemius muscle from clone N125 (M13516)[5]. A synthetic probe for a MHC mRNA (common type) as a control was designed from the cDNA sequence in myosin isoforms[5], *cggtaggccaacaccacctccgggttgtacaccggcagcc*. They were successively modified with DIG system[16]. Total cellular RNA from 1g of the chicken pectoralis and three hindlimb muscles was isolated by the methods of Chomczynski and Sacchi[17]. Total RNA (20 μg) of each

sample was electrophoresed on 1% formaldehyde agarose gels and transferred to nylon membranes. After cross-linking of the RNA to the positively charged nylon membrane (Boehringer Mannheim) with UV crosslinker UVC 500 (Amersham Pharmacia Biotech, Buckinghamshire, England), filters were prehybridized for 1h and hybridized (50% formamide, 5x Denhardt's solution, 0.5% SDS, 4x SSPE and herring sperm DNA) for 20h at 47℃ (N116 and N125 probes) or at 52℃ (common probe), and detected with anti-digoxigenin-ALP and with CSPD. Filters were detected on x-ray film.

Amino acid sequence analysis of S-1

Myosin was digested with α-chymotrypsin at an enzyme/substrate ratio of 1:100 (w/w) in 20mM imidazole-HCl (pH 7.0) containing 120mM NaCl and 1mM EDTA at 20℃ for 20min. The digestion was stopped by adding 0.1mM PMSF, and the digestion mixture was centrifuged at 100,000g for 1h. The supernatant was collected as S-1. S-1 was digested with trypsin at an enzyme/substrate ratio of 1:100 (w/w) in 20mM Tris-HCl (pH 7.8) at 20℃ for 60min. The digest was denatured in 0.5M Tris-HCl (pH8.3) containing 6M guanidine-HCl and 0.2% EDTA, and S-carboxymethylated with iodoacetic acid. The 23, 50 and 20kDa fragments were isolated from the digest by gel filtration on a Sephadex G-100 column and subsequently by ion exchange chromatography on a CM52 column. The purified 23, 50 and 20kDa fragments were further fragmented with lysyl endopeptidase, α-chymotrypsin, V8 protease, or cyanogen bromide, and the resulting peptides were purified by using a reverse-phase HPLC column. The fragmentations with lysyl endopeptidase, α-chymotrypsin, V8 protease, cyanogen bromide were carried out in 25mM Tris-HCl (pH 8.1) containing 4M urea at 37℃ for 8h, in 25mM Tris-HCl (pH 8.5) at 37℃ for 4h, in 50mM ammonium acetate (pH 4.0) at 37℃ for 20h, in 90% formic acid at 25℃ for 16h, respectively. The amino acid analysis of peptides were performed by a JCL-200A analyzer with a sodium citrate buffer system after hydrolysis with 5.7M HCl at 110℃ for 22h. Automated sequencing was performed by using an Applied Biosystems 477A protein sequencer with an Applied Biosystems model 120A PTH analyzer on-line system.

Results

Motility speed of myosin and ATPase activity of myofibril

Motility assay has been an established method to characterize function of myosins. To detect a possible functional profile of myosin from hindlimb muscles, we carried out the motility assay of purified myosins. Motility speeds of pectoralis, ilio-tibialis, gastrocnemius and femori-tibialis myosins were 3.7 ± 0.6, 1.9 ± 0.3, 1.9 ± 0.4 and 1.7 ± 0.5 μm/s, respectively. Myosin from hindlimb muscle could move actin filaments with significantly lower speed than those from the pectoralis muscles (Fig.1). We then determined the ATP hydrolysis of muscle fibre with using myofibrillar preparation. The ATPase activities of myofibrils from the three muscles were summarized in Table 1. Myofibrillar

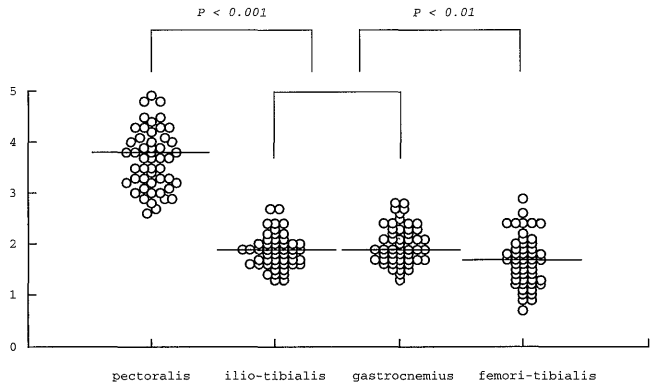

Figure 1. *In vitro* motility assay of myosins from chicken pectoralis and hindlimb muscles. Movements of actin filaments on myosin attached to a nitrocellulose-coated glass were recorded, and velocities of 50 actin filaments for each muscle were analyzed. Median of motility speed on pectoralis, ilio-tibialis, gastrocnemius and femori-tibialis myosins were 3.7, 1.9, 1.9 and 1.7 μm/s, respectively. P value was deduced by *Mann-Whitney* U test.

Table 1. ATPase activities of myofibrils from pectoralis and the three hindlimb muscles. The mesurement of ATPase activity was carried out by Malachite Green method described in Methods.

	Myofibrillar ATPase activity mol(Pi)/mol/min	
pectoralis	466 ± 65	$P < 0.01$
ilio-tbialis	361 ± 22	
gastrocnemius	345 ± 57	$P < 0.01$
femori-tibialis	190 ± 41	

ATPase activity on pectoralis, ilio-tibialis, gastrocnemius and femori-tibialis myosins were 466 ± 65, 361 ± 22, 345 ± 57and 190 ± 41 mol (Pi) / mol / min, respectively. Hindlimb myofibril had significantly lower ATPase activity than pectoralis muscles. The ATPase activity of femori-tibialis myofibril was lower than those of ilio-tibialis and gastrocnemius myofibrils. The result of actomyosin super precipitation was consistent with the result of myofibrillar ATPase activity, i.e., pectoralis > ilio-tibialis ≒ gastrocnemius > femori-tibialis (data not shown).

Northern blot analysis

Next we examined the possibility of differential expression of MHC isoforms in the four muscles by northern blot analysis with two synthetic oligo-DNA probes (P-type and G-type) for the isoform as described in *Materials and Methods*. As shown in Fig 2, P-type MHC isoform was clearly detected but not G-type MHC isoform in pectoralis muscle. On the other hand, G-type MHC isoform was higher expressed than P-type MHC isoform in other three hindlimb muscles, indicating the differential expression of MHC isoforms in the four muscles.

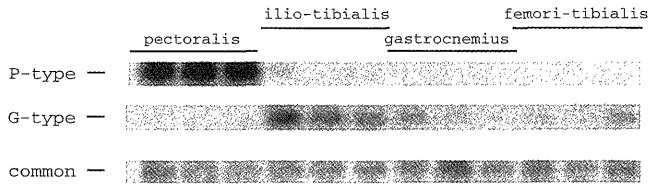

Figure 2. Northern blot analysis showing the expression of selected members of the MHC isoform in chicken pectoralis and three hindlimb muscles. Twenty μg of total RNA isolated three times independently from each muscles was hybridized with the specific probes for the P-type, G-type and common type mRNAs.

Amino acid sequence of S-1 heavy chain

We determined amino acid sequences of the 23kDa, 50kDa and 20kDa fragments from a major isoform (G-type) in ilio-tibialis muscle. The strategies and results of sequence analysis are shown in Fig.3 to Fig.7. Although some heterogeneous minor peptides were detected (Fig.4), the major peptides with relatively higher yields were considered to be derived from the major isoform. The major peptides could be aligned in the respective fragments as shown in Fig.5 to Fig.7. Most of the minor peptides were considered to be derived from the pectoralis type isoform (P-type) because their sequences were identical to those of P-type. This indicates that ilio-tibialis muscle contained at least two types of MHC isoforms. A ratio of P-type and G-type in ilio-tibialis muscle was calculated to be about 3:7, from yields of the peptides (Table 2).

Fig.8 shows a comparison of the S-1 heavy chain sequence of G-type with that of P-type. The characteris-

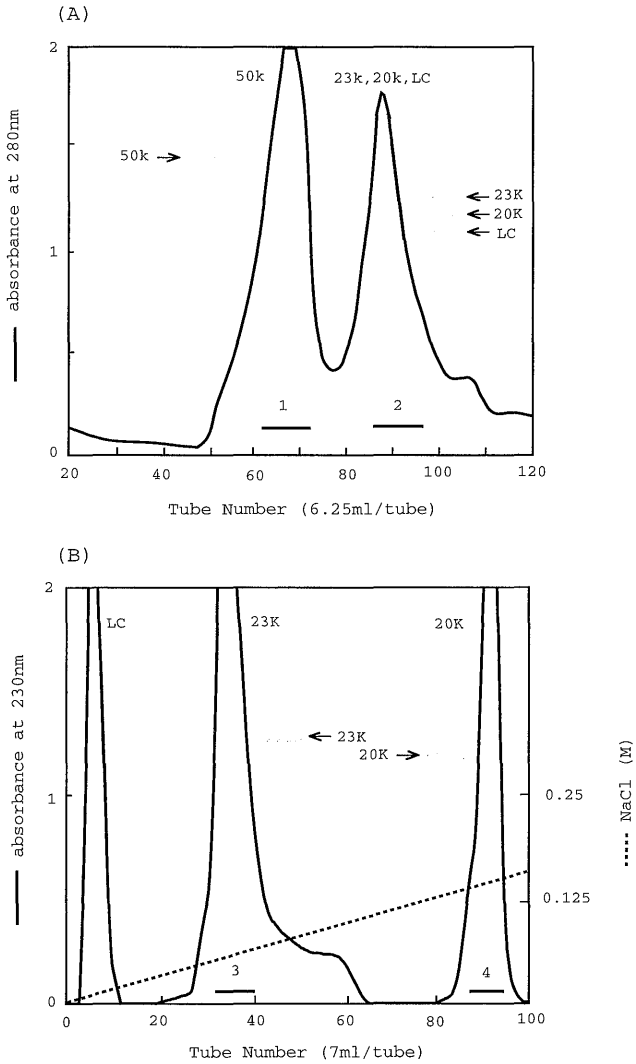

Figure 3. Isolation of the 23kDa, 50kDa and 20kDa fragments from S-1 of ilio-tibialis myosin. (A) A tryptic digest of S-1 (see Methods) was applied to a Sephadex G-100 column (ϕ 3.2 x 140cm). Peptide fragments were eluted with 20mM Tris-HCl (pH 7.5) containing 5M guanidine-HCl and 5mM EDTA. The eluate indicated by the bars were collected, desalted, and finally lyophilized. The 50kDa fragment was obtained from the fraction 1. (B) Peptide fragments in the second fraction in (A) were chromatographed on a CM52 column (ϕ 2.4 x 20cm) equilibrated with 20mM sodium phosphate buffer, pH 6.0 containing 8M urea. Elution was carried out with a linear gradient of NaCl. The 23kDa and 20kDa fragments were obtained from the fractions 3 and 4, respectively. Purity of the isolated fragments was checked by SDS-polyacrylamide gel electrophoresis.

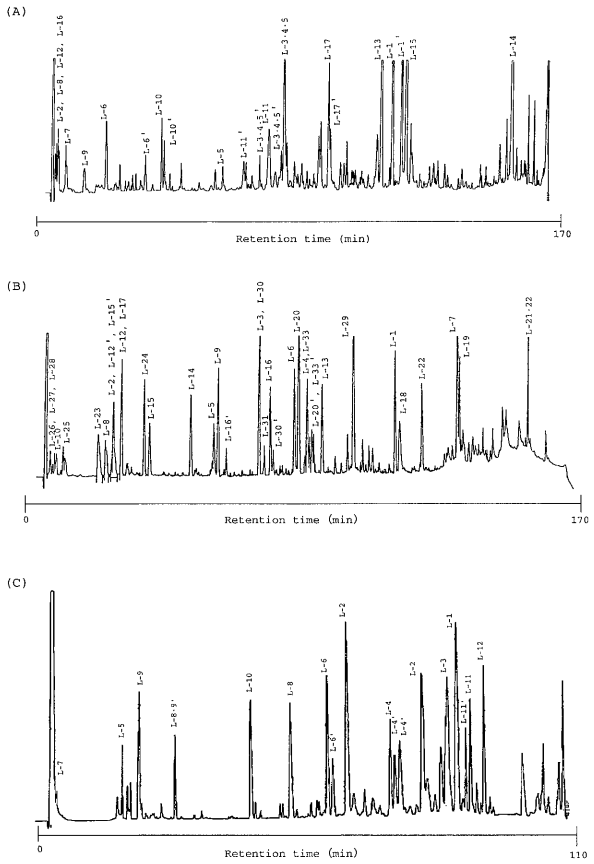

Figure 4. Separations of lysyl endopeptidase peptides of the 23kDa, 50kDa and 20kDa fragments from ilio-tibialis muscle myosin. (A) A digest of the 23kDa fragment with lysyl endopeptidase was applied on a reverse-phase HPLC column of Wakosil 5C18. Peptides were eluted with a linear gradient of acetonitrile concentration obtained by mixing 0.1% trifluoro acetic acid (TFA) and 90% acetonitrile containing 0.09% TFA. Amino acid compositions of peptides in each peak were analyzed, and some of them were sequenced. L-1 to L-17 refer the major peptides, and peaks indicated by L-X' contained the minor peptides. (B) An elution profile of lysyl endopeptidase peptides of the 50kDa fragment. Conditions of the chromatography were the same as (A). (C) An elution profile of lysyl endopeptidase peptides of the 20kDa fragment. Conditions of the chromatography were the same as (A).

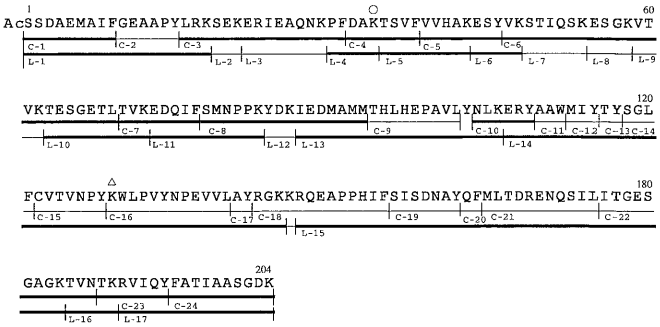

Figure 5. Strategies for sequence analysis of the 23kDa fragment of G-type. To align the major lysyl endopeptidase peptides (L-1 to L-17), chymotryptic pepides (C-1 to C-24) of the 23kDa fragment from ilio-tibialis muscle myosin were separated by almost the same methods described in Fig.4. Peptides shown by — were sequenced, and — were analyzed only composition, respectively. L-1 and C-1 were digested with acylamino-acid-releasing enzyme, because their α-amino groups were blocked. Acetylserine (AcS) was detected by reverse-phase HPLC in the both digests, and the resulting deacetylserine-peptides were sequenced. K (○) at position 34, and K (△) at position 129 represent ε-N-monomethyl-lysine and ε-N-trimethyl-lysine, respectively.

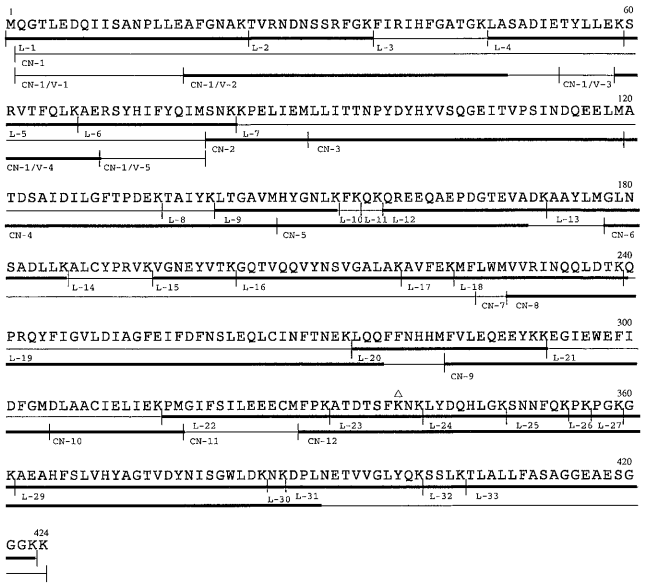

Figure 6. Strategies for sequence analysis of the 50kDa fragment of G-type. To align the major lysyl endopeptidase peptides (L-1 to L-33), cyanogen bromide peptides (CN-1 to CN-12) of the 50kDa fragment from ilio-tibialis muscle myosin were isolated by successive chromatographies on Sephadex G-50 and reverse-phase HPLC columns. CN-1/V-1 to CN-1/V-5 indicate V-8 protease peptides of CN-1. K (△) at position 338 represents ε-N-trimethyllysine.

tic methylated amino acids were recognized in G-type at the corresponding positions to P-type, and the N-terminal α-amino group of G-type was acetylated same as that of P-type. There exists approximately 4.3% amino acid difference between G-type and P-type including a deletion at N-terminus.

We further isolated lysyl endopeptidase peptides of the 23kDa, 50kDa and 20kDa fragments from gastrocnemius muscle myosin. Most of the isolated peptides were identified to the peptides derived from G-type or P-type by their compositions and/or sequences. A ratio of P-type and G-type in gastrocnemius muscle was about 2: 7 (Table 2). In the experiment we found curious peptides corresponding to residues 35-42 of the 23kDa fragment, SSVFVVHAK (P·G-type, a hybrid of P and G type sequences) and TSVFVVHPK (G · P-type), other than SSVFVVHPK (P-type) and TSVFVVHAK (G-type). The yields of P·G-type and G·P-type were lower than that of P-type. In the amino

Figure 7. Strategies for sequence analysis of the 20kDa fragment of G-type. To align the major lysyl endopeptidase peptides (L-1 to L-12), V-8 protease peptides (V-1 to V-7) of the 20kDa fragment from ilio-tibialis muscle myosin were isolated, and sequenced. H (▲) at position 115 represents 3-N-methyl-histidine.

Table 2. Yield of lysyl endopeptidase peptides from ilio-tibialis, gastrocnemius and femori-tibialis muscles.

	ilio-tibialis			gastrocnemius			femori-tibialis		
peptide	P-type	G-type	F-type	P-type	G-type	F-type	P-type	G-type	F-type
L-1 of the 23kDa fragment	0.16	0.44	—	0.11	0.41	—	—	—	—
L-6 of the 23kDa fragment	0.23	0.66	—	0.20	0.65	—	—	—	—
L-16 of the 50kDa fragment	0.21	0.65	—	0.20	0.70	—	—	—	—
L-2 of the 20kDa fragment	0.20	0.51	ND	0.19	0.63	ND	0.08	0.49	0.21
L-4 of the 20kDa fragment	0.19	0.61	ND	0.16	0.57	ND	0.07	0.33	0.25
ratio	3	: 7		2	: 7		1	: 6	: 3

Yield of the each peptide was presented by mol of the isolated peptide / mol of the digested fragment.
ND : not detected.

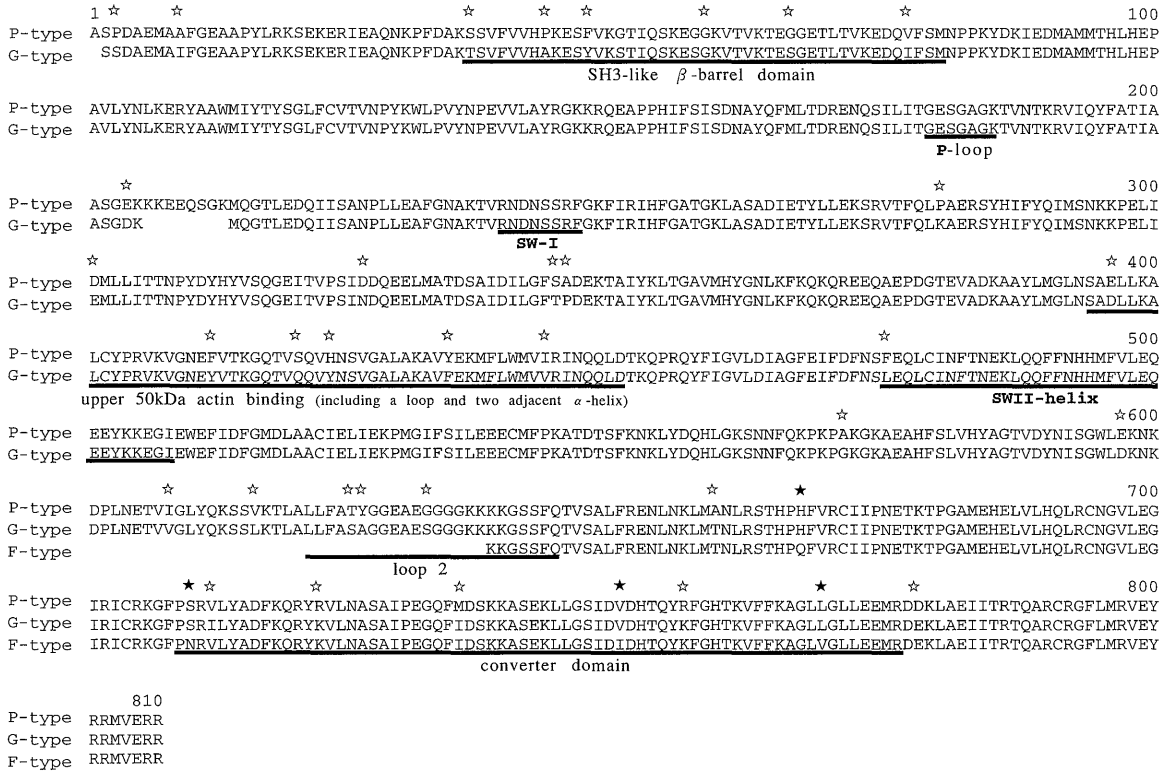

Figure 8. Comparison of the S-1 heavy chain sequence of P-type with those of G-type and F-type. Residues were numbered according to P-type sequence[9]. The sequence of G-type was deduced from the sequences of the 23kDa (residues 1-205), 50kDa (residues 214-637) and 20kDa (residues 635-810) fragments. N-terminal S in G-type and A in P-type stand for N-acetyl serine and N-acetyl alanine, respectively. K (○) at residues 35, K (△) at residues 130 and 551, and H (▲) at residues 755 stand for ε-N-monomethyllysine, ε-N-trimethyllysine and 3-N-methylhistidine, respectively. Open stars show different amino acids among the three types. Closed stars show different amino acids only found in F-type. Some functional domains shown by ⎯, SH3-like β-barrel domain at residues 36-80, ATP binding P-loop at residues 179-185, SW-I at residues 239-246, SWII-helix at residues 475-508, upper 50kDa actin binding (including a loop and the two adjacent α-helix) at residues 394-450, actin binding loop 2 at residues 621-644, and converter domain residues 709-776.

terminal region, we similarly found another P·G-type peptide, SSDAEEMAAFGEAAPYLRK.

We also isolated and sequenced the lysyl endopeptidase and V-8 protease peptides of the 20kDa fragment from femori-tibialis muscle myosin. Another isoform in addition to P-type and G-type was found in femori-tibialis muscle. Isoform found only in femori-tibialis muscle was named as femori-tibialis type (F-type). Its sequence was shown in Fig.8. A ratio of P-type, G-type and F-type in femori-tibialis muscle was about 1 : 6 : 3 (Table 2).

Discussion

It has been reported that the pectoralis muscle contains a homogenous MHC, P-type[6-9]. In this study, we found a major isoform (G-type) in the three hindlimb muscles, and determined the sequences of its 23kDa, 50kDa and 20kDa fragments. The x-ray crystal structure of S-1 from chicken pectoralis myosin included, SH3-like β-barrel domain, ATP binding P-loop, SW-I, SWII-helix, actin binding loops, and converter domain[18,19]. The differences between the P-type and the G-type amino acid sequence were evidently recognized at the actin binding loops in the upper 50kDa (including a loop and two adjacent α-helix, residues 394-450) and in the lower 50kDa (loop 2, residues 621-644) and the converter domain, rather than the P-loop or sites related to the ATPase function, as shown in Fig.8. The converter domain is considered to function as a socket for the C-terminal region of S-1 heavy chain, or to amplify rotational movements during ATP hydrolysis with moving a lever arm[19]. The structure of G-type may be suitable to modulate the actin binding and the amplification, and for complicated fine movements of legs and feet.

Infact, the motility speed of myosin and the ATPase activity of myofibril from the hindlimb muscles were significantly lower than that from the pectoralis muscle. The activities of myofibrils from ilio-tibialis and gastrocnemius were significantly higher than that from femori-tibialis. Content-ratios of P-type, G-type and F-type isoform were about 3 : 7 : 0 in ilio-tibialis muscle, 2 : 7 : 0 in gastrocnemius muscle, and 1 : 6 : 3 in femori-tibialis muscle, respectively. These results indicate that the ATPase activities of myofibrils from the hindlimb muscles may be related with the compositions of the isoforms in the respective muscle. Femori-tibialis muscle exists in deep region of the femur, whereas the other two exist in superficial region. G-type fibre in the hindlimb muscle may be suitable to work as fast-twitch fibre together with P-type fibre, on the other hand, F-type fibre in femori-tibialis may work as slow-twitch-like fibre to keep skeletal positions.

In the course of sequencing work of gastrocnemius muscle myosin, we found two specific-sequence peptides (G·P-type, a hybrid of G and P type sequences) at the amino terminal region of MHC. The presence of such peptides may suggest a new type of MHC or rather a new splicing site in between residues in the amino terminal region. In comparison of several skeletal myosin sequences, heterogeneity of the heavy chains is evident in the amino terminal eighty amino acid residues. Though the structural diversity in the amino terminal region is yet characterized in relation to myosin function, the present finding may account for the possible alteration of myosin function.

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