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Characterization of Molluscan Muscle based on the Properties of Major Myofibrillar Protein Components

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Abstract

For the deeper understanding of the molluscan muscles for their effective utilization, the major protein components of molluscan mantle muscle, namely, myosin (heavy chain) and tropomyosin, were investigated from the viewpoint of structure-stability relationships. Molluscan myosin heavy chains are unique in that they have several additional residues in the rod region forming α-helical coiled-coil structure. On the other hand, molluscan tropomyosins clearly differed from other orthologous proteins, suggesting that the structural uniqueness gives rise to their characteristics, such as allergenicity. The amino acid sequence data, together with thermodynamic analysis data, could be useful to estimate the stability (or instability) of these protein components, and further the properties of the muscle *per se*.

Introduction

The phylum Mollusca (consisting of more than 110,000 species) has diverged in a variety of forms and ecological profiles, i.e., from bivalves, gastropods and further to cephalopods, which possess advanced brain and excellent locomotion system enabling their dexterous swimming at high speed, mainly by jet propulsion (Rokni and Hochner 2002, Takuwa-Kuroda et al. 2003). They are the highest class of the phylum, consisting of subclasses Coleoidea and Nautiloidae with 786 living species. They occur in all marine habitats of the world, and are great sources of protein from the sea, thus important for commercial fisheries and processing. Incidentally, the annual catch of mollusc in the world is around four million tons per year, and approximately 2.3 megatons for squids. For the effective utilization of the edible parts (mostly muscles) of molluscs, molecular approach to proteins consisting myofibril is worth intensive investigation.

Myosin and paramyosin are the two major components of molluscan muscle. Paramyosin does not show any biological activity, but takes a part in filament formation.

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However, myosin is directly involved in muscle contraction, using the chemical energy produced by ATP hydrolysis. Myosins are classified into eighteen different classes, though all the skeletal and obliquely striated muscle myosins belong to type II (Foth et al. 2006). Myosin is composed of two heavy chains of approximately 200 kDa and four light chains of approximately 20 kDa (Craig and Woodhead 2006). A globular head of molecule is called subfragment-1 (S-1), containing and N-terminal half of the heavy chain together with light chain subunits of approximately 20 kDa. Actin binding and ATP binding sites are also located in this domain. The fibrous part is referred to as a rod, consisting of a C-terminal half of the heavy chain alone. N- and C-terminal sides of the rod are called subfargment-2 (S-2) and light meromyosin (LMM), respectively. The rod portion forms a coiled-coil structure composed of two helices, which enables forming a thick filament under physiological conditions. In most cases, the properties of myosin are the major determinant for the stability of meat against heat and frozen storage. Toughness and water-holding capacity of meat are also greatly ascribed to the properties of the major proteins. For better understanding the properties of muscle, it is essential to characterize the major protein component, myosin.

Myofibrillar proteins including myosin and tropomyosin from fish and shellfish are generally less stable than the counterparts of higher vertebrates (Ogawa et al. 1993, Higuchi et al. 2002, Paredi et al. 2002, Li et al. 2003, Huang and Ochiai 2005). Myosin is also a very important factor for gel formation of fish meat (kamaboko) (Satoh et al. 2006). Myofibrillar (myosin) ATPase activity is considered to be a good quality indicator of fish meat paste (surimi) (Katoh et al. 1979). On the other hand, tropomyosin stabilizes actin filament and regulates muscle contraction (Yu and Ono 2006). Sequence data are available for molluscan myosin heavy chain and tropomyosin, while very scarce data are available for paramyosin. The rod portion of myosin has a coiled-coil structure composed of two α-helices (Root et al. 2006), and this is also true for tropomyosin, which has this structure throughout the entire molecule. The coiled-coil is considered to function as molecular motors propelled by electrostatic energy of ions (Jarosch 2005). Such unique structure makes it possible to characterize the structures of proteins, because the changes of α-helical content through thermal treatment or in the presence of denaturants can be monitored quite easily by circular dichroism analysis.

So far, most of the studies on molluscan muscle proteins have been focused on Ca^{2+}-regulated muscle contraction (Szent-Gyorgyi et al. 1999, Azzu et al. 2006), because these myosins are special in that the direct binding of Ca^{2+} to myosin light chain subunit regulates contraction. Primary structures have been revealed for some myosins and

tropomyosins (Matulef et al. 1998, Janes et al. 2000, Fujinoki et al. 2006), and collagen (Morales et al. 2000). In addition, invertebrate tropomyosins have also been identified as major allergens (Nakamura et al. 2005, Motoyama et al. 2006, Zhang et al. 2006). A few studies have dealt with the changes of biochemical characteristics of muscle proteins (Khaitlina et al. 1999, Hatzisisis et al. 2000, Inoue and et al. 2004, Kasamatsu et al. 2004).

In the present study, attempts were made to characterize the muscles from several molluscan species, taking the two representative muscle proteins, myosin heavy chain and tropomyosin as the markers for thermal stability of coiled-coil structure. These properties were discussed in relation with their amino acid sequences.

Materials and Methods

Live specimens of Japanese common squid *Todarodes pacificus*, Ommastrephidae, and common octopus *Octopus vulgaris*, Octopodidae, were purchased at a local wholesale market, immediately frozen with dry ice and transported to the laboratory. They were kept at -80ºC until used. Tropomyosin was isolated from the mantle muscle of *T. pacificus* and *O. vulgaris*. The acetone-dried muscle prepared according to the conventional method was extracted with 10 volumes of 1 M KCl. The supernatant obtained was subjected to isoelectric precipitation of tropomyosin by adjusting the pH to 4.5 by titrating 0.1 M HCl. Tropomyosin was further purified by ammonium sulfate fractionation between 40 ~ 50% saturation fractions. Protein concentration was determined by biuret method. DSC was performed using a microcalorimeter (model VP-DSC, MicroCal, Northampton, MA, USA) on the purified tropomyosin in a medium consisting of 10 mM sodium phosphate (pH 7.0), 0.1 M KCl, 1 mM dithiothreitol (DTT) and 0.01% NaN_3. The temperature range was from 5 to 80 ºC. The increment of temperature was set to at 1ºC/min. Protein concentration was in the range from 1.0-1.5 mg/mL. DSC data were analyzed for determination of melting temperature (*T*m) using a software package Origin developed by MicroCal. Amino acid sequences of myosin heavy chain and tropomyosin were aligned with the software ClustalW. Phylogenetic tree was drawn on the basis of the amino acid sequences of α-tropomyosin using the neighbor-joining method.

Results and Discussion

Characterization of cephalopod myosins

Amino acid sequences of myosin heavy chain were compared between longfin inshore squid and bay scallop (Fig. 1).

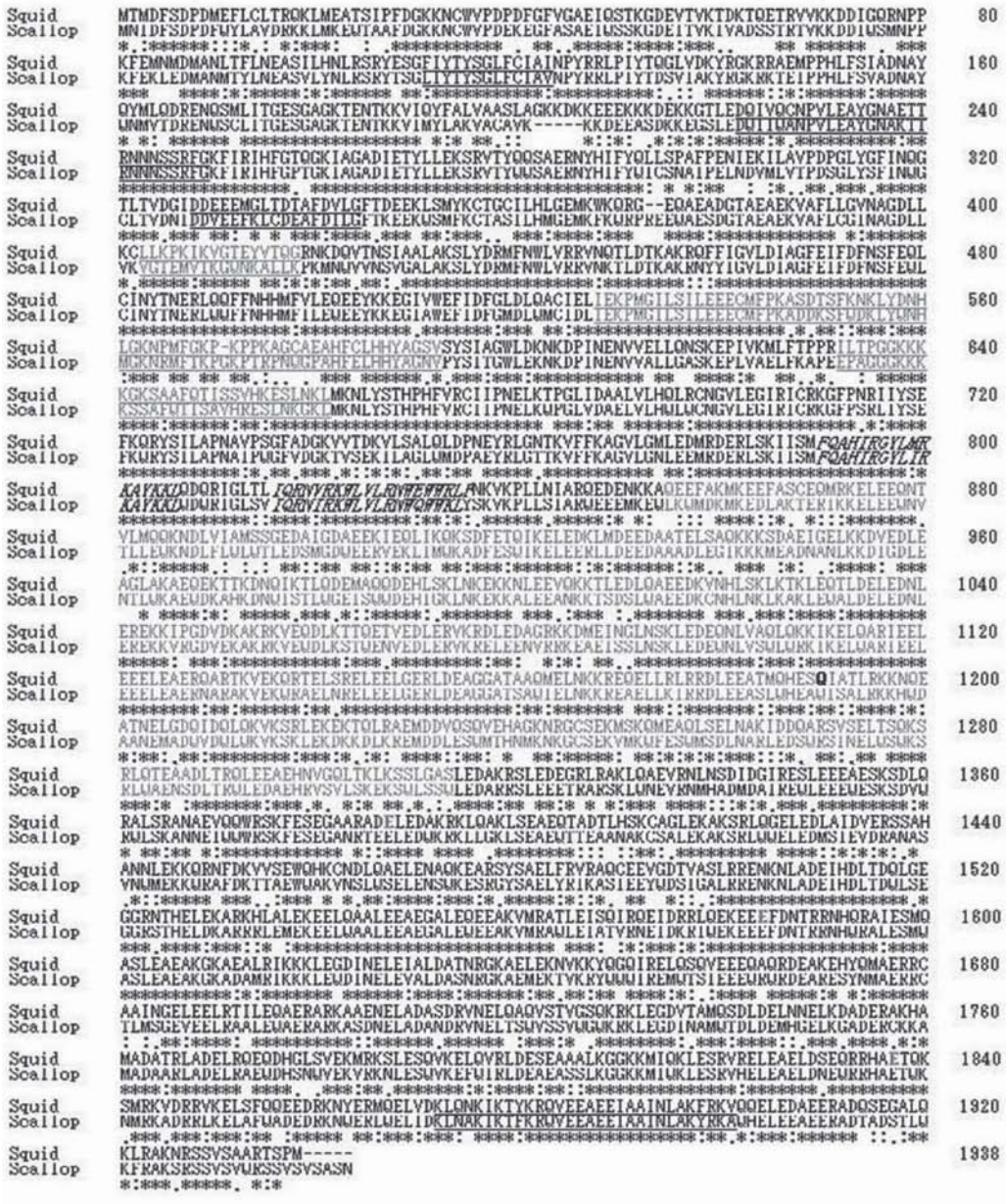

Figure 1**.** Alignment of the amino acid sequences of squid and scallop myosin heavy chains. The sequence data of longfin inshore squid *Loligo pealeii* (O44934) and bay scallop *Aequipecten irradians* (P24733) were aligned by Clustal W. The dentical residues are shown by asterisks, the conservative and semi- conservative replacements are shown by colons and semicolons. Gaps are indicated by hyphens. The residues consisting of the S1 and light meromyosin (LMM) regions are in black letters, while those of S2 region are in gray. ATP binding sites are underlined, whereas actin binding sites are in gray and underlined. The light chain binding sites are italicized. The assembly competent domain close to the N terminus is underlined. The skip residues are bold-faced. Symbols used; *, identical residues; : , conservative replacements; semi-conservative replacements, in comparison with the squid myosin heavy chain sequence.

In the figure, S-2 region connecting S-1 and LMM regions is shown by gray letters. Gaps were found in the head region of scallop heavy chain, while the tail of squid myosin heavy chain was a little shorter compared to scallop myosin. The sequence identity was about 87% throughout the molecule, though the identity varied among the regions of the molecule. In the rod regions of both myosins, four skip residues (as shown by bold-faced letters in the figure), which could perturb the regular coiled-coil structures, were specified.

The structure of the head portion (S-1) of myosin is very complicated. It is thus almost impossible to draw conclusive remarks on the sequence-stability relationship only by comparison of amino acid sequence. However, as described above, the rod portion of molluscan myosins give rise to characteristic sequence, suggesting the instability of this portion. It is established that the rod portion is involved in gel formation of meat, especially for the case of fish (Fukushima et al. 2003).

Myosin itself exists in a large amount in muscle, and thus is easily prepared. However, biochemical and thermodynamic data obtained from myosin are very complicated because this protein is a large molecule (~500 kDa) and is composed of quite different parts, namely, S-1 and rod. However, it requires a lot of labor to prepare S-1 or rod, because it is only possible after enzymatic cleavage of these portions. This is a drawback to the detailed study on structure-stability relationship of the myosin molecule. In contrast, it is much easier to prepare tropomyosin.

Characterization of cephalopod tropomyosins

As shown in Fig. 2A, tropom-yosin molecule is fibrous, forming α-helical coiled-coil structure almost throughout the entire molecule. The schematic diagram of the cross section of the coiled-coil structure is shown in Fig. 2B. In general, at the *a* and *d* positions, hydroph-obic residues tend to occupy to form a hydrophobic core to stabilize the coiled-coil (so-called 'a heptad repeat rule'). On the other hand, salt bridges tend to be formed between the *e* and *g* positions. As long as the tropomyosins so far studied are concerned, there are many exceptions to the localization of such amino acid residues, suggesting that the coiled-coil structure of tropomyosin is loosened at several regions.

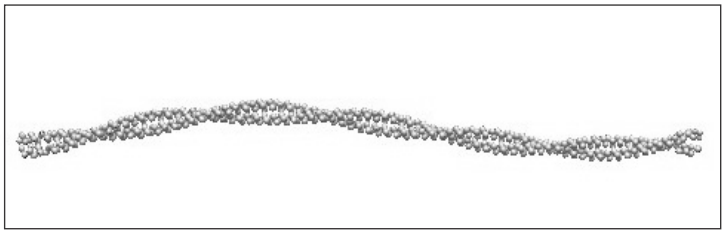

Figure 2A**.** Tertiary structure of tropomyosin molecule (PDB 2tma)

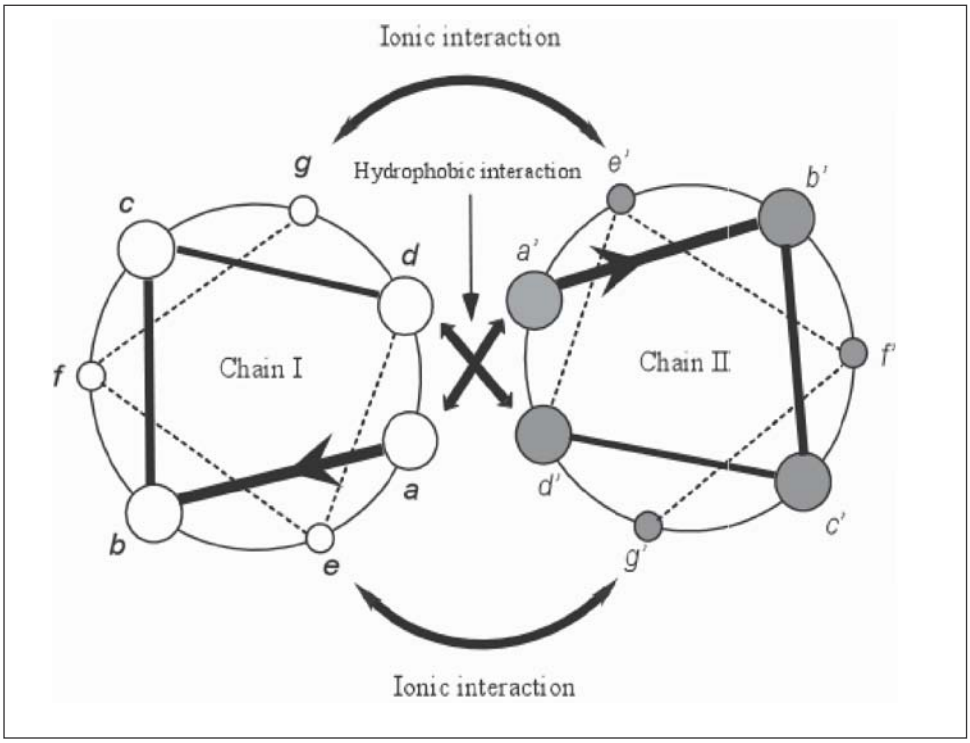

Figure 2B. The schematic diagram of the cross - section of coiled-coil structure

It is considered to be necessary for the function of this protein, namely, regulation of myosin-actin interaction during muscle contraction. In connection with this, molluscan tropomyosins tend to be extracted in water-soluble fraction, unlike vertebrate counterpart (Motoyama et al. 2006). The amino acid sequences of four molluscan tropomyosins were aligned with those from other sources (Fig. 3).

The organisms included Japanese common squid *T. pacificus*, common octopus *O. vulgaris*, and Japanese abalone *Haliotis diversicolor* (molluscs), lobster *Homarus americanus*, crab *Portunus sanguinolentus*, and prawn *Marsupenaeus japonicus*, (arthropods), amphioxus *Branchiostoma belcheri* (cephalochordate), white croaker *Pennahia argentata* and frog *Rana temporaria* (vertebrates). The sequences were headed with the heptad positions of the coiled-coil (*a* ~ *g*) corresponding to those in Fig. 2B. From Fig. 3, it is clear that there are so many amino acid replacements between tropomyosins from different phyla, though the heptad repeat rule is roughly true for all tropomyosins. Interestingly, the N-terminal eight residues are conserved for all tropomyosins, while the C-termini are not conserved so much.

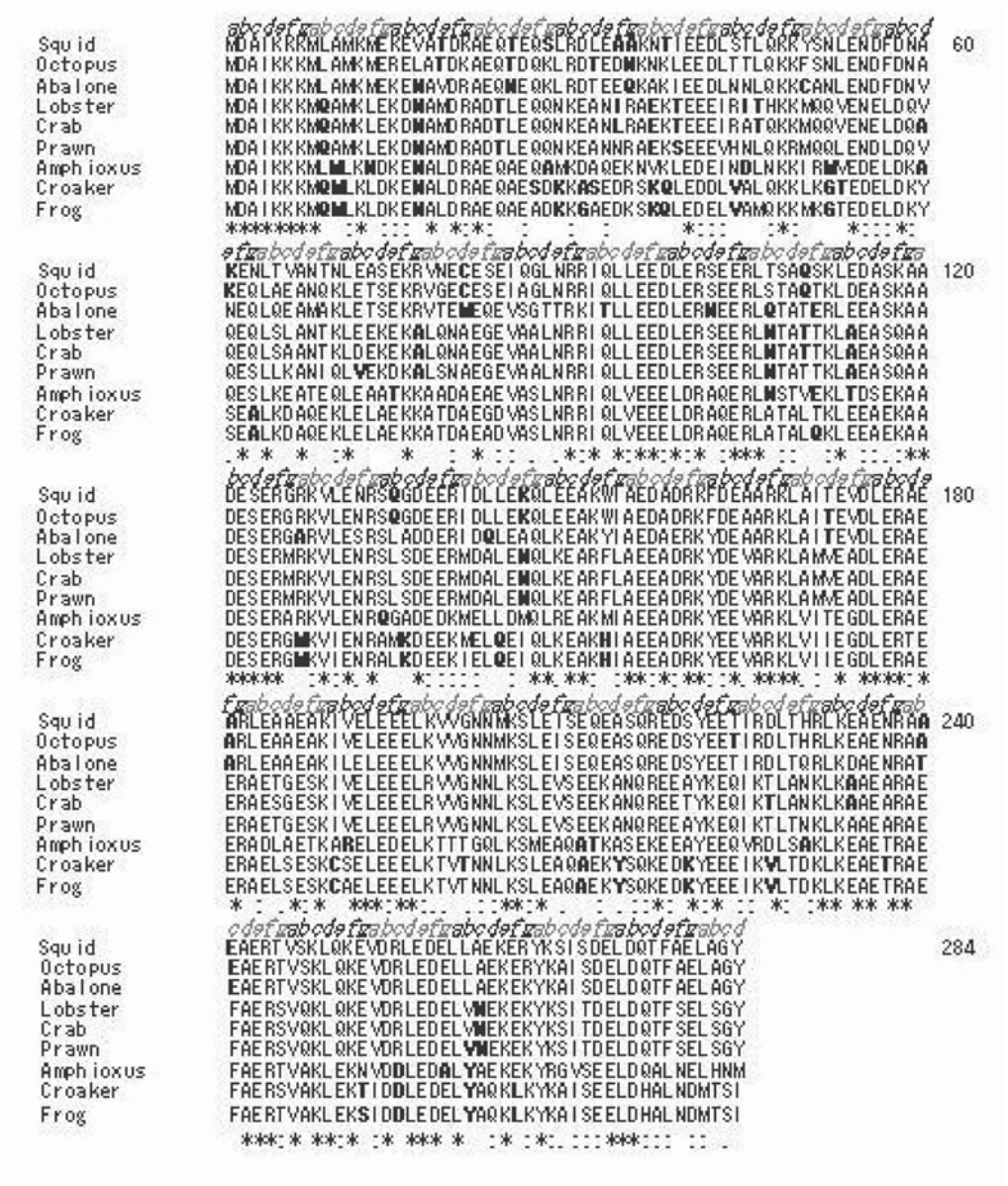

Figure 3. Alignment of amino acid sequences of four molluscan tropomyosins. Squid, Japanese common squid *Todarodes pacificus* (accession #Q2V0V2); octopus, common octopus (#Q2V0V0), Japanese abalone, *Haliotis diversicolor* (#Q9GZ71), lobster, *Homarus americanus* (#O44119), crab, blue crab *Portunus sanguinolentus* (#A1YYV6), prawn, *Penaeus japonicus* (#A2V731), amphioxus, *Branchiostoma belcheri* (#Q9NDS0), croaker, white croaker *Pennahia argentata* (#AB045645), frog, *Rana temporaria* (#P13105). The positions occupied with unique residues are boldfaced. Refer to the legend of Fig. 1 for the symbols used.

Based on the amino acid sequences of these tropomyosins, a phylogenetic tree was drawn by the neighbor-joining method (Fig. 4).

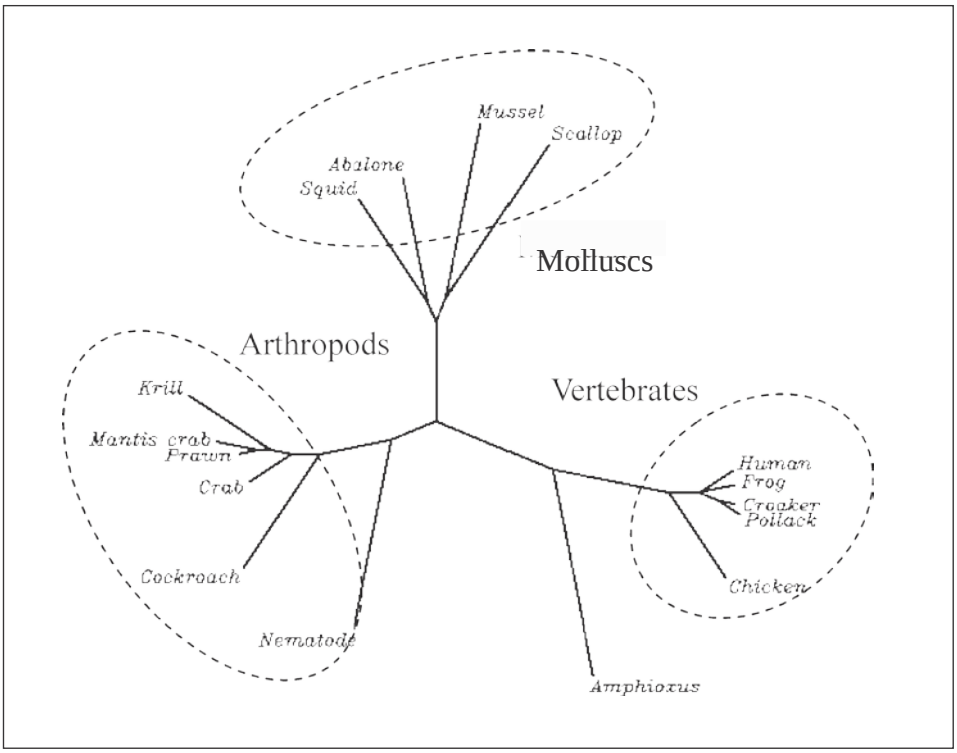

Figure 4. Unrooted phy-logenetic tree drawn based on the amino acid sequences of α tropomyosins by neighbor-joining method. The clusters of vertebrates, arthropods and molluscs are contained in circles separately. Squid, Japanese common squid *Todarodes pacificus* (accession #Q2V0V2); octopus, common octopus (#Q2V0V0), abalone, *Haliotis diversicolor* (#Q9GZ71), lobster, *Homarus americanus* (#O44119), crab, *Portunus sanguinolentus* (#A1YYV6), prawn, *Penaeus japonicus* (#A2V731), amphioxus, *Branchiostoma belcheri* (#Q9NDS0), croaker, *Pennahia argentata* (#AB045645), frog, (#P13105) *Rana temporaria*.

As a result, tropomyosins from different phyla formed clear clusters. It was suggested that molluscan tropomyosins have special structure, though it is not possible to predict the tertiary structure at present, because there is no proper template available. However, such characteristics might be related to the allergenicity of these tropomyosins.

Two species of molluscs, namely, common squid *Todarodes pacificus* and common octopus *Octopus vulgaris* were used for the preparation of tropomyosin. The sequence identities of these proteins were found to be higher than 70%. The parameter of thermal stability and DSC patterns were compared as shown in Fig 5.

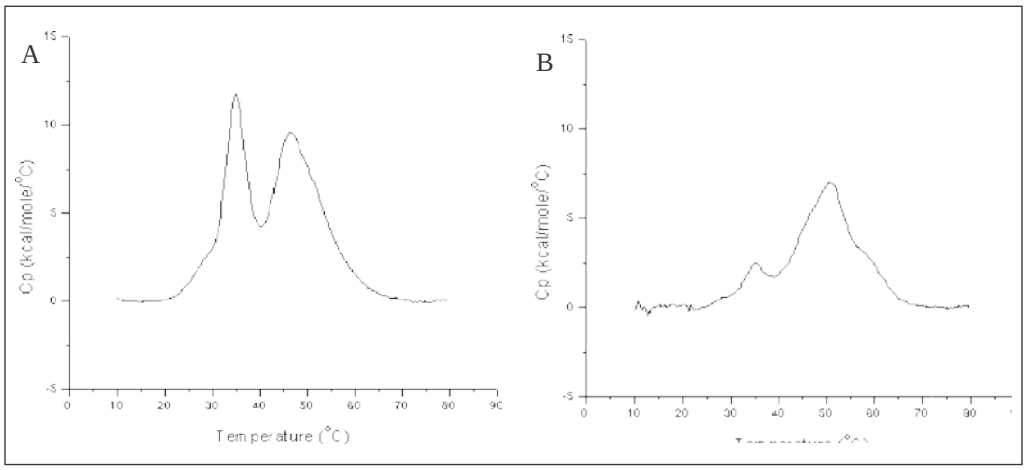

Figure 5. DSC patterns of squid *T. pacificus* tropomyosin. A, first scan; B, second scan. DSC analyses were performed in a medium consisting of 10 mM sodium phosphate (pH 7.0), 0.1 M KCl, 1 mM DTT, 0.01% NaN_3. The temperature range was set to 5-80ºC. The increment of temperature was set at 1ºC/min.

Squid tropomyosin showed a slightly higher transition temperature (*T*m) of 47.0ºC, than the octopus counterpart, whose *T*m value was 44.5ºC. The second scan of squid tropomyosin by DSC suggested that this protein was refolded even after the first thermal treatment which caused structure perturbation, though the endothermic peak at the lower temperature was not recognized in the second scan. Preliminary experiments showed that tropomyosins from scallop adductor and smooth muscles showed clearly different stability, with the *T*m values being 29.4 and 35.8ºC, respectively. Because smooth muscle (catch muscle) of the adductor has unique composition of proteins (Perreault-Micale and Szent-Gyorgyi 1996, Shelud'ko et al. 2001), tropomyosins from these two muscles seem to have adapted to respective physiological conditions.

These results suggest that molluscan muscle proteins are relatively stable. Incidentally, the *T*m values of fish tropomyosins are in the range of 26.4 ~ 46.5ºC (Huang and Ochiai 2005). Because molluscs are ectotherms, thus the stability of their proteins is greatly affected by the environmental temperature. Therefore, the proteins from cold-water inhabiting species are considered to be less stable compared to those from warm-water species. However, the stability of each protein component is to be examined for further discussion. So far several reports suggest that tropomyosin is a suitable model for the relationship between sequence, structure and function (Kluwe et al. 1995, Perry 2001, Miura-Yokota et al. 2005). This seems also to be true for molluscan counterparts.

The difference between the coiled-coils from myosin heavy chains and tropomyosins is that the former forms side-by-side filament under physiological condition, while the latter forms only head-to-tail polymerized filament and is soluble

even in water. The amino acid residues located at the surface of these proteins cause such a difference. Thermal stability is considered to depend on the stability of the hydrophobic core of these proteins.

It is very important to handle myosin during storage and processing of meat, because denaturation of this protein causes deterioration of meat as observed in decrease in solubility and water holding capacity. Filament formation ability largely affects such changes. To know the thermodynamic properties of such filamentous proteins is thus considered to be essential to optimize storage and processing conditions. Above all, the coiled-coil regions are excellent tools to monitor the structural changes of proteins.

Conclusion

Myosin heavy chain and tropomyosin from molluscan muscle, especially those from cephalopods were characterized based on their amino acid sequence and thermal denaturation profile. The sequence alignment of these proteins revealed the uniqueness of molluscan proteins. The coiled-coil regions of these proteins are excellent markers for their thermal stability. It could be helpful for optimizing the storage and processing conditions of molluscs.

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