Absence of Troponin in Foot Muscle of Surf Clam *Pseudocardium sachalinensis*^{*1}

Satoru Chiba,^{*2} Takao Ojima,^{*3} and Kiyoyoshi Nishita^{*3,4}

(Received March, 23 1992)

A search for troponin in surf clam foot muscle was made. The muscle, however, seemed to lack troponin, judging from SDS-polyacrylamide gel electrophoresis and immunoblotting of myofibril, native thin filament, and so-called troponin fraction in addition to their effects on Ca^{2+} -sensitivity of Mg-ATPase activity. In contrast, a 160 kDa actin-binding protein was found, though its effects on Ca^{2+} -regulation in muscle contraction are unknown.

Molluscan muscles have some characteristic properties. For instance, their actins are easily removable by washing the muscle with low salt buffer solution,¹⁾ and smooth adductor muscles and byssus retractor muscles show an energy-saving prolonged state of contraction called "catch".²⁻⁴⁾ Further, the muscles possess two Ca^{2+} -regulatory systems of contraction, namely, the myosin-linked system which is mediated by myosin light chains⁵⁻¹¹⁾ and the actin-linked system which is mediated by troponin-tropomyosin.¹²⁻¹⁷⁾ On the other hand, vertebrate skeletal muscles possess the actin-linked system alone,^{18,19)} in contrast to vertebrate smooth muscles which have been revealed to possess a myosin-linked regulatory system distinct from that of mollusca.^{20,21)}

Recently, there have been found many actin-binding proteins in vertebrate smooth muscle and non-muscle cells such as leiotoxin,²²⁻²⁴⁾ caldesmon,^{25,26)} and calponin^{27,28)} which are thought to be candidates for actin-linked Ca^{2+} -regulatory proteins. These studies suggest that even muscle with the myosin-linked regulatory system possesses actin-linked regulatory protein(s) distinct from troponin.

Since functional troponins of shellfish have been isolated from only the striated and smooth

adductor muscles of scallops,^{16,17)} we investigated their existence in another molluscan muscle species, the foot muscle from the surf clam.

Materials and Methods

Living surf clam was obtained from a local fish market. The foot muscle was cut out and the digestive tract was removed immediately and carefully. The preparation of troponin from the surf clam foot muscle was carried out by the method for akazara scallop *Chlamys nipponensis akazara* troponin.^{16,17)} Foot muscle (100 g) was homogenized with 40 mM KCl, 10 mM potassium phosphate (pH 7.0), 1 mM MgCl_2 , 5 mM 2-mercaptoethanol, 0.2 $\mu\text{g/ml}$ leupeptin, 0.2 $\mu\text{g/ml}$ pepstatin, 0.2 mM PMSF, and 1 mM NaN_3 (300 ml) in a Waring blender. The homogenate was mixed with the same solution (700 ml), and centrifuged at $10,000 \times g$ for 10 min. After the washing procedure was repeated twice more, the muscle residues were suspended into 0.4 M LiCl (pH 5.0) (1 l) and stirred, gently at 4°C for 1 h, maintaining pH 5.0 with 0.1 N HCl. The suspension was centrifuged at $10,000 \times g$ for 10 min and the supernatant was subjected to $(\text{NH}_4)_2\text{SO}_4$ fractionation (40-60% saturation). The precipitates formed were collected by centri-

^{*1} This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

^{*2} Present address; Central Research Laboratory, Nippon Suisan Co. Ltd., Kitano, Hachioji, Tokyo 192, Japan (千葉 智: 日本水産株式会社中央研究所).

^{*3} Department of Fisheries Chemistry, Faculty of Fisheries, Hokkaido University, Minato, Hakodate, Hokkaido 041, Japan (尾島孝男, 西田清義: 北海道大学水産学部水産高分子化学講座).

^{*4} To whom correspondence should be addressed.

Abbreviations: TnT, troponin-T; TnI, troponin-I; TnC, troponin-C; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis(β -aminoethylether)-*N,N,N',N'*-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; Mr, molecular weight; IgG, immunoglobulin G.

fugation at $10,000\times g$ for 10 min, dissolved in and dialyzed against 0.4 M LiCl, 1 mM NaHCO_3 , and 5 mM 2-mercaptoethanol. The dialyzate was adjusted to pH 4.5 to precipitate tropomyosin. Tropomyosin was further purified by repeating isoelectric precipitation at pH 4.5 and $(\text{NH}_4)_2\text{SO}_4$ fractionation. On the other hand, the supernatant was fractionated by 40–60% $(\text{NH}_4)_2\text{SO}_4$ saturation at pH 7.0 and the precipitates, which were expected to contain troponin, were dissolved in and dialyzed against 1 mM NaHCO_3 and 5 mM 2-mercaptoethanol.

Rabbit myosin and actin were prepared by the methods of Perry²⁹⁾ and Spudich and Watt,³⁰⁾ respectively. Native thin filaments were prepared from surf clam foot muscle by the method of Goldberg and Lehman.¹²⁾ Akazara scallop troponin and tropomyosin were prepared as reported previously.^{16,17)} SDS-PAGE was carried out by the method of Porzio and Pearson.³¹⁾ Molecular weight was calibrated using a marker protein kit (Mr 30,000–200,000, Sigma). Gels were stained with Coomassie Brilliant Blue R250, and the staining density was measured with a Shimadzu Chromatoscanner CS-9000. Akazara scallop troponin subunits were prepared from SDS-PAGE gels and their antibodies were raised against rabbit by injecting troponin antigens subcutaneously. Immunoblotting was carried out by the method of Towbin *et al.*³²⁾ The rabbit antibody bound to the antigen was allowed to react with peroxidase-labeled goat anti-rabbit IgG (Kent Lab.), and detected by coloring reaction with 4-chloro-1-naphthol as reported by Hawks *et al.*³³⁾ Actomyosin Mg-ATPase activity was assayed at 15°C in a medium containing 300 mM KCl, 20 mM Tris maleate (pH 6.8), 2 mM MgCl_2 , 1 mM ATP, and 0.2 mM EGTA or 0.2 mM EGTA plus 0.3 mM CaCl_2 . Inorganic phosphate was measured by the method of Youngburg and Youngburg.³⁴⁾ Protein concentration was determined by the biuret method using bovine serum albumin fraction V as a standard protein.³⁵⁾

Results and Discussion

Attempts to Prepare Troponin

The troponin fraction was prepared as described in "Materials and Methods". When the fraction was dialyzed against 1 mM NaHCO_3 and 5 mM 2-mercaptoethanol, a large amount of precipitate was formed unexpectedly. Therefore, the precipitate was collected by centrifugation at $10,000\times g$

Fig. 1. SDS-polyacrylamide gel electrophoresis of proteins from surf clam foot muscles. Electrophoresis was carried out with 10% polyacrylamide slab gels containing 0.1% SDS. a, myofibrils; b, native thin filaments; c, S-fraction; d, P-fraction; e, troponin of akazara scallop striated adductor muscle; HC, myosin heavy chain; PM, paramyosin; A, actin; TM, tropomyosin; LCs, myosin light chains; 300 K, the molecular mass of 300 kDa.

for 10 min. The precipitate comprised about 80% of the total amount of proteins and was named P-fraction. Meanwhile, the supernatant was named S-fraction. These fractions were separately dissolved in 50 mM KCl, 1 mM NaHCO_3 , and 5 mM 2-mercaptoethanol.

Figure 1 shows the SDS-PAGE patterns of various preparations from surf clam foot muscle. The myofibrils showed many faint bands along with those of subunits of myosin, paramyosin, actin, and tropomyosin. However, the native thin filaments and S- and P-fractions showed several bands with those of actin and tropomyosin subunits. Thus, the native thin filaments and the two fractions showed different combinations of subunit bands from those of the akazara scallop striated (52, 40, and 20 kDa) and smooth adductor (40, 21, and 20 kDa) troponins,¹⁶⁾ and other troponins.³⁶⁾

Further, the myofibrils, native thin filaments, and S- and P-fractions of surf clam foot muscle were subjected to SDS-PAGE (see Fig. 1), followed by immunoblotting using the antisera against akazara scallop troponin subunits which had

Fig. 2. Effects of S-fraction and P-fraction on the actomyosin Mg-ATPase activity. The Mg-ATPase activity of reconstituted actomyosin with either S-fraction (○, ●), P-fraction (△, ▲), or akazara troponin (□, ■) was measured at 15°C in a medium containing 30 mM KCl, 20 mM Tris maleate, pH 6.8, 2 mM $MgCl_2$, 1 mM ATP, 0.1 mg/ml rabbit myosin, 0.05 mg/ml rabbit F-actin, 0.025 mg/ml surf clam tropomyosin, and S-fraction, P-fraction or akazara scallop troponin (0–0.025 mg/ml) in the presence of either 0.2 mM EGTA (○, △, □) or 0.2 mM EGTA plus 0.3 mM $CaCl_2$ (●, ▲, ■).

been confirmed to cross-react with TnIs, TnTs, and TnCs of ezo-giant scallop *Patinopecten yes-soensis*, blue mussel *Mytilus edulis*, ark shell *Scapharca broughtonii*, and oyster *Crassostrea gigas*.^{*5} However, no cross-reactive protein was detected in these preparations (data not shown).

Effect of Surf Clam Proteins on Actomyosin Mg-ATPase Activity

Since the existence of troponin subunits could not be demonstrated in the S- and P-fractions and native thin filaments of surf clam foot muscle by SDS-PAGE and immunoblotting, effects of S- and P-fractions on the actomyosin Mg-ATPase activity were examined. In these experiments, rabbit reconstituted actomyosin was used instead of surf clam foot actomyosin because surf clam myosin itself has Ca^{2+} -regulatory ability. The S- and P-fractions were separately added to rabbit actomyosin along with surf clam foot tropomyosin, and then Mg-ATPase activity was measured in the presence and absence of Ca^{2+} . As shown in Fig. 2, the S-fraction slightly enhanced the activity irrespective of the presence or absence of Ca^{2+} , but the P-fraction did not. Further, no

Fig. 3. Effect of surf clam native thin filament with and without akazara troponin on the Mg-ATPase activity of rabbit myosin. The Mg-ATPase activity was measured at 15°C in a medium containing 30 mM KCl, 20 mM Tris maleate (pH 6.8), 2 mM $MgCl_2$, 1 mM ATP, and the following protein mixture in the presence of either 0.2 mM EGTA (○) or 0.2 mM EGTA plus 0.3 mM $CaCl_2$ (●). (A) 0.1 mg/ml rabbit myosin and 0–0.1 mg/ml surf clam NTF; (B) 0.1 mg/ml rabbit myosin, 0.1 mg/ml surf clam NTF, and 0–0.05 mg/ml akazara scallop striated adductor troponin. NTF, native thin filaments.

*5 Unpublished data by K. Nishita *et al.*, Hokkaido Univ.

Fig. 4. Binding of protein component in P-fraction with rabbit F-actin. The P-fraction (0.1 mg/ml) dissolved in 50 mM KCl, 10 mM Tris-HCl (pH 7.6), and 1 mM $MgCl_2$ was mixed with rabbit F-actin (0.1 mg/ml) and incubated at 0°C for 30 min. The mixture was centrifuged at $100,000 \times g$ for 1 h. The pellet obtained was dissolved in 10 M urea, 1% SDS, 0.05 M Tris-HCl (pH 8.9), and 0.6 M 2-mercaptoethanol and subjected to SDS-PAGE. a, P-fraction; b, rabbit F-actin; c, the pellet.

Ca^{2+} -sensitivity was recovered even when the supernatant of the muscle homogenate or the tropomyosin-free supernatant obtained during preparation was added to the S- and P-fractions (data not shown). On the other hand, when akazara scallop troponin was added to the rabbit actomyosin-surf clam tropomyosin, high Ca^{2+} -sensitivity was observed.

In addition, native thin filament of surf clam foot muscle was also incapable of conferring Ca^{2+} -sensitivity when complexed with rabbit myosin (Fig. 3A). However, this myosin-native thin filament showed high Ca^{2+} -sensitivity by the further addition of akazara scallop troponin (Fig. 3B).

From these results together with those of SDS-PAGE and immunoblotting patterns, it appeared that surf clam foot muscle lacks troponin. Moreover, it is indicated that surf clam tropomyosin retains troponin-binding sites.

Existence of Actin-Binding Protein

It is well known that vertebrate smooth muscles lack troponin but some of them contain another species of Ca^{2+} -regulatory actin-binding protein. Caldesmon is one of them and is known to cause turbidity formation when bound to F-actin.^{25,26} Recently, caldesmon-like proteins of 140 kDa were isolated from scallop and cuttlefish³⁷ and those of 120 kDa from oyster and edible mussel.³⁸

Therefore, the existence of actin-binding protein in the P-fraction was investigated, since the protein precipitates of the fraction occurred from clear solution during dialysis and comprised actin and some other proteins. Thus, the P-fraction was dissolved in 50 mM KCl, 10 mM Tris-HCl (pH 7.6), 1 mM $MgCl_2$, and 0.2 mM EGTA. The solution was centrifuged at $100,000 \times g$ for 1 h, and the supernatant obtained was mixed with rabbit F-actin at a ratio of 0.7:1 (w/w), where the precipitates were formed maximally. The precipitates were then collected and subjected to SDS-PAGE. As seen in Fig. 4, a 160 kDa protein was shown to bind to F-actin.

Whether or not this protein is caldesmon or another species of Ca^{2+} -regulatory protein is still unknown, but further studies on its characterization are now underway.

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