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Tissue specificity of tropomyosin isoform in the mussel, *Mytilus galloprovincialis*Atsuko Itoh¹ and Masakatsu Fujinoki²

SUMMARY

In the mussel, *Mytilus galloprovincialis*, there were three kinds of tropomyosin isoforms, which were designated as TM1, TM2 and TM3, respectively. It had been reported that TM1 was contained as only tropomyosin isoform and major component in the muscle tissues such as adductor and cardiac muscles, it was confirmed as the major isoform in adductor, cardiac and anterior pedal retractor muscles, and in the mantle and gills. In this present study, meanwhile, TM2 and TM3 were detected as two new isoforms in the mantle and gills. TM2 and TM3 existed only in the mantle and in both the mantle and gills, respectively. Both TM2 and TM3 were minor isoforms compared to TM1. TM2 had almost the same molecular weight as TM1 whereas TM3 had lower molecular weight than TM1. Moreover, TM3 had more basic isoelectric point than TM1 and TM2.

Key words: mussel, tissue specificity, tropomyosin, isoform.

INTRODUCTION

Tropomyosin is an actin-binding protein that exists in muscle and non-muscle cells. The physiological function of tropomyosin is known in skeletal muscle of vertebrate, where tropomyosin in association with troponin complex (i.e. troponin I, T, and C) functions as a co-regulator of muscle contraction¹⁻⁴. Generally, there are multiple tropomyosin isoforms that expressed by multiple genes and by alternative splicing of transcripts of those genes in many animals²⁻⁹. Moreover, the isoforms show a tissue-specific distribution in many animals; for example, human¹⁰, chicken¹¹, salmonid fish¹², lobster¹³, bivalve¹⁴⁻¹⁶, sea urchin¹⁷, and sea anemone¹⁸. Although it is likely that tissue-specific distribution is associated with the roles of the isoforms in the tissues, the functions of tropomyosin itself in the absence of troponin are not well known.

The heterogeneity and tissue specificity of tropomyosin isoforms has been examined in several species of bivalves¹⁴⁻¹⁶. In *Patinopekten yessoensis* (scallop) and *Mimachlamys nobilis* (senatorial scallop), three tropomyo-

sins have been isolated from adductor muscle and cardiac muscle^{15, 16}. One isoform was striated-muscle-specific and the other two existed in adductor smooth muscle and cardiac muscle. In six other species [*Scapharca broughtonii* (ark shell), *Atrina pectinata* (surf clam), *Crassostrea gigas* (Pacific oyster), *Macra chinensis* (Chinese macra), *Tresus keenae* (Trough shell) and *Meretrix lammarckii* (orient clam)^{14, 16}], only two tropomyosin isoforms have been found in muscle tissues. In *A. pectinata*, *M. chinensis*, *T. keenae* and *M. lammarckii*, one of the two isoforms was adductor-muscle-specific and the other existed in both adductor and cardiac muscles^{14, 16}. In *S. broughtonii* and *C. gigas*, however, both isoforms existed in adductor and cardiac muscles, although the localization in adductor muscle differed between the two species¹⁴. In *M. galloprovincialis* (mussel), interestingly, only one tropomyosin had been found in both adductor muscle and cardiac muscle¹⁴.

Although tropomyosin found from many bivalves showed tissue specific distributions¹⁴⁻¹⁶, only mussel tropomyosin did not show it¹⁴. In order to examine whether mussel tropomyosin does not show tissue specific distribution, in the

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Abbreviations: TCA, trichloroacetic acid; Tris, Tris (hydroxymethyl) aminomethane; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF, poly (vinylidene difluoride); PBS, phosphate-buffered saline; IgG, immunoglobulin G; CBB, Coomassie brilliant blue.

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present study, we tried to find tropomyosin from other muscle and non-muscle tissue.

MATERIALS AND METHODS

Preparation of tropomyosin extracts

The mussel, *M. galloprovincialis* was purchased from a fish market and tropomyosin extracts were prepared as described previously^{14, 18}. Samples of the anterior pedal retractor muscle, translucent and opaque portions of adductor muscle, cardiac muscle, gills and mantle were suspended in 50 volumes of acetone, minced with scissors, washed seven times with acetone, air dried, and used as acetone powder, which was treated with 100 volumes of 5% (w/v) trichloroacetic acid (TCA) for 1 hour at room temperature to prevent endogenous proteolysis¹⁹. The TCA-treated acetone powder was washed three times with a barbital buffer containing 0.1 M NaCl and 0.035 M sodium barbital-HCl (pH 8.0), washed six times with acetone, and air dried. Tropomyosin was extracted from the TCA-treated acetone powder using 20 volumes of a KCl buffer containing 1 M KCl, 0.1 M KH₂PO₄-KOH (pH 7.0) and 1% (v/v) 2-mercaptoethanol. After incubation for 14 hours at 4°C, the KCl extraction product was centrifuged at 20,000×g for 1 hour at 4°C. The supernatant was dialyzed against the barbital buffer, and then re-dialyzed against a solution containing 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS and 5% (v/v) 2-mercaptoethanol. After the supernatant was centrifuged at 20,000×g for 1 hour at 4°C, it was used as the tropomyosin extract.

Sodium dodecyl sulfate gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to the method of Laemmli²⁰ using a separating gel of 10% or 15% (w/v) polyacrylamide containing 0.1% (w/v) SDS or 0.1% (w/v) SDS and 5 M urea.

Two-dimensional urea-shift gel electrophoresis

Two-dimensional urea-shift gel electrophoresis was carried out according to the method of Carmon et al.²¹ with some modification. Electrophoresis was performed on 10% (w/v) polyacrylamide separating gel containing 0.1% (w/v) SDS and 5 M urea in the first dimension and 0.1% (w/v) SDS in the second dimension with the Tris-buffer system²⁰.

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoreses with isoelectric focusing (IEF) and SDS gels, and with IEF and SDS/Urea gels were carried out according to the method of Hirabayashi²² with some modifications¹⁴. First dimension IEF was performed on cylindrical agarose gel at 4°C with a 1:2 mixture of two types of Ampholines (pH 4–6 and pH 3.5–10, GE Healthcare UK Ltd., Buckingham, UK) as carrier ampholyte. Second dimension SDS and SDS/Urea

gel electrophoresis was done with 10% polyacrylamide gels containing 0.1% (w/v) SDS with the Tris-buffer system²⁰, and 0.1% (w/v) SDS and 5 M urea²¹, respectively.

Immunoblotting

Immunoblotting was based on the method of Towbin et al.²³. After electrophoresis, proteins were transferred to PVDF sheets (Immobilon-P, Millipore Co., Bedford, MA, USA). The blotted membrane was blocked with 5% (w/v) skim milk in Tween-PBS containing 0.2 M NaCl, 7.2 mM Na₂HPO₄, 2.8 mM NaH₂PO₄, and 0.1% (w/v) Tween-20 for 1 hour at room temperature, and incubated with either anti-chicken tropomyosin antiserum (Sigma-Aldrich Co., St Louis, MO, USA) (1:100 dilution with 5% (w/v) skim milk in Tween-PBS) or anti-*Minachlamys nobilis* tropomyosin antiserum¹⁵ (1:20,000 dilution with 5% (w/v) skim milk in Tween-PBS) for 1 hour at room temperature. A horseradish-peroxidase-conjugated anti-rabbit IgG antibody (Wako, Osaka, Japan) (1:20,000 dilution with 5% (w/v) skim milk in Tween-PBS) was used as the second antibody. Color reaction was carried out using ECL-Plus Western blotting detection reagents (GE Healthcare UK Ltd, Buckingham, UK).

Limited proteolysis with V8 protease

Limited proteolysis of tropomyosin was carried out according to the method described by Cleveland et al.²⁴. Each tropomyosin isoform was isolated by excising the protein band from the gels after SDS-PAGE with 5 M urea. The protein band and 40 ng of V8 protease (Wako) were subjected to electrophoresis, and the proteolysis reaction was performed by stopping the electrophoresis for 1 hour at room temperature.

Amino acid sequencing

After limited proteolysis of tropomyosin, fragments of tropomyosin were transferred to PVDF sheets (Immobilon-P^{SQ}, Millipore Co.) and after CBB staining, they were subjected to amino acid sequencing (Procise Model 491cLC, Applied Biosystems Inc., Foster City, CA, USA). Amino acid sequences were identified using FASTA (<http://fasta.genome.jp>).

RESULTS

Tissue-specific distribution of tropomyosin

When tropomyosin is extracted from TCA-treated muscle sources, it is detected as a dominant band¹⁹. In our previous study¹⁴, we found one tropomyosin in the translucent and opaque portions of adductor muscle and in cardiac muscle. In the present study, one tropomyosin was detected as the dominant band from samples of the same muscle tissues (Fig. 1 lanes b–d), as well as in anterior pedal retractor muscle (Fig. 1 lane a). In the mantle and gills, however, there was the tropomyosin that corresponded to the domi-

Fig. 1. Tropomyosin obtained from tissues of the mussel, *Mytilus galloprovincialis*.

Tropomyosin extracts were prepared from TCA-treated acetone powder of (a) anterior pedal retractor muscle, (b) the translucent portion of adductor muscle, (c) the opaque portion of adductor muscle, (d) cardiac muscle, (e) mantle and (f) gills, and subjected to 10% SDS-PAGE. In (a–c), (d), (e) and (f), 4 μ L, 17.5 μ L, 100 μ L and 70 μ L of tropomyosin extract were applied, respectively. Arrow indicates tropomyosin.

nant band detected in the other muscle tissues (Fig. 1 lanes a–d) and some new minor bands (Fig. 1 lanes e and f).

In the next step, mussel tropomyosins were subjected to two-dimensional urea-shift gel electrophoresis (Fig. 2). It has been shown that the electrophoretic mobility of tropomyosin on SDS-PAGE is retarded when urea is included in the gel system²⁵. Therefore, we can detect tropomyosin as a spot that is separated from the diagonally arranged spots of other proteins on the two-dimensional urea-shift gel electrophoresis. As shown in Fig. 2a–d, only one isoform was detected in anterior pedal retractor muscle, the translucent and opaque portions of adductor muscle and in cardiac muscle, whereas in the mantle and gills (Fig. 2e and f), there was one major spot, which corresponded to the tropomyosin detected in the other muscles, and three or four minor spots.

Fig. 3. Immunoblotting of tropomyosin.

Tropomyosin obtained from the translucent portion of adductor muscle was used for immunoblotting with anti-*Min-achlamys nobilis* tropomyosin antiserum (a) and anti-chicken tropomyosin antiserum (b). Lane (c) shows CBB-stained gel. In (a–c), 4 μ L of 20-fold dilution of tropomyosin extract were applied. Arrow indicates tropomyosin.

Immunoblotting of tropomyosin

The band of tropomyosin detected in several muscle tissues (Figs. 1 and 2) reacted strongly to the anti-*M. nobilis* tropomyosin antiserum (Fig. 3 lane a), but reactivity against the anti-chicken tropomyosin antiserum was very weak (Fig. 3 lane b).

In the samples from the mantle, the major tropomyosin spot and the two minor spots reacted against the anti-*M. nobilis* tropomyosin antiserum (Fig. 4a and b). The three spots identified as tropomyosin isoforms by immunoblotting were designated TM1, TM2 and TM3. TM1 was the dominant spot and corresponded to the tropomyosin found in retractor muscle, adductor muscle and cardiac muscle. TM2 was the same molecular weight as TM1, and was largely retarded by urea. TM3 had a lower molecular weight than TM1 and TM2. In the samples from the gills, one major spot and one minor spots reacted against the anti-*M. nobilis* tropomyosin antiserum (Fig. 4c and d). The major spot corresponded to TM1 and the minor spot with a low molecular weight corresponded to TM3.

Characterization and amino acid sequencing of mussel tropomyosin isoforms

As shown in Fig. 5a and b, TM1 and TM2 of mussel tropomyosins had the same molecular weight. Their molecular weight was estimated to be approximately 38,000 Da from mobilities of those components on SDS gels with a phos-

Fig. 2. Two-dimensional urea-shift gel electrophoresis of mussel tropomyosin.

Tropomyosin extracts were prepared from TCA-treated acetone powder of (a) anterior pedal retractor muscle, (b) the translucent portion of adductor muscle, (c) the opaque portion of adductor muscle, (d) cardiac muscle, (e) mantle and (f) gills, and subjected to two-dimensional urea-shift gel electrophoresis. In (a–c), (d), (e) and (f), 5 μ L, 20 μ L, 50 μ L and 75 μ L of tropomyosin extract were applied, respectively. Arrows and white arrowheads show the tropomyosin spot and other spots, respectively, separated from the diagonally arranged spots of proteins. Dotted lines show the diagonal line of the electrophoretogram.

Fig. 4. Immunoblotting of tropomyosin obtained from mantle and gills.

Tropomyosin extracts prepared from the mantle (a, b) and gills (c, d) were subjected to two-dimensional urea-shift gel electrophoresis, and then immunoblotted using anti-*M. nobilis* tropomyosin antiserum (b, d). Photographs (a) and (c) show CBB-stained gels. Arrows and arrowheads show the spots separated from the diagonally arranged spots of proteins. Arrows also show one major spot corresponding to the major tropomyosin from muscle tissues. Arrows and arrowheads indicate spots that reacted with anti-*M. nobilis* tropomyosin antiserum, designated as TM1, TM2 and TM3, respectively. White arrowheads indicate spots that did not react with anti-*M. nobilis* tropomyosin antiserum. Dotted lines show the diagonal line of the electrophoretogram.

phate buffer system²⁶⁾ (data not shown) and with a Tris-HCl buffer system. On the other hand, apparent molecular weight of TM3 was lower than TM1 and TM2 and was estimated to be approximately 31,000 Da from mobilities of those components on SDS gels with a phosphate buffer system²⁶⁾ (data not shown) and with a Tris-HCl buffer system. In the next step, mussel tropomyosins were subjected to two-dimensional gel electrophoresis with isoelectric focusing (IEF) and SDS gels, and with IEF and SDS/Urea gels (Fig. 5c, d, e and f). TM1 and TM2 were same pI values, whereas pI value of TM3 was a little basic rather than TM1 and TM2 (Fig. 5d, f). Their pI values were estimated to be approximately 4.5 from mobilities of those components on IEF gels^{14, 22)} (data not shown).

Because TM1 had a large volume component, it was purified from adductor muscle using electrophoresis, and digested by limited proteolysis with V8 protease. As shown in Fig. 6A, five fragments were obtained (Fig. 6A) and analyzed for their amino acid sequences (Fig. 6B). EIQLTD-KXSXLEEDXXXSEE and EAQLXAEED for fragments 2 and 5, respectively (Fig. 6B), were the only sequences obtained, and using a database (FASTA) search (Fig. 6C) they were identified as the deduced amino acid sequence 85–105 and 150–159 of mussel tropomyosin (GenBank/

Fig. 5. Two dimensional gel electrophoresis of mussel tropomyosin isoforms.

Tropomyosin extracts prepared from the translucent portion of adductor muscle (a, c, e) and mantle (b, d, f) were analyzed with two-dimensional urea-shift gel electrophoresis (a, b) and two-dimensional gel electrophoresis with IEF and SDS gels (c, d), and with IEF and SDS/Urea gels (e, f). The numbers on the left of electrophoretogram (a) and (b) represent the molecular weight of TM1, TM2 and TM3, respectively. Arrows and arrowheads on each electrophoretogram show TM1, TM2 and TM3, respectively.

EMBL/DBJ accession no. [AB00907](#)).

DISCUSSION

Several previous studies investigated that bivalve tropomyosin showed heterogeneity and tissue-specificity^{14–16)}. Of the three tropomyosins found in two scallop species, *P. yessoensis* and *M. nobilis*^{15, 16)}, one is a striated-muscle-specific isoform located in adductor striated muscle and the other two isoforms exist in the opaque portion of adductor smooth muscle. One of those two isoforms also exists in the translucent portion of adductor smooth muscle and the other also exists in cardiac muscle. Of species from the Veneroida, two tropomyosins have been found in *M. lamarkii*, *T. keenae* and *M. chinensis*¹⁶⁾; one isoform exists in the translucent and opaque portions of adductor muscle and in cardiac muscle, and the other is opaque-portion-specific. Two tropomyosin isoforms have also been found in species from the Arcoida (*S. broughtonii*), the Pterioidea (*A. pecti-*

Fig. 6. Amino acid sequences of mussel tropomyosin.

Tropomyosin from adductor muscle was subjected to 15% SDS-PAGE for limited proteolysis with V8 protease. Five proteolytic fragments, designated 1, 2, 3, 4 and 5, were obtained (A) and analyzed for their amino acid sequences (B). Only the sequences of fragments 2 and 6 could be obtained and they were EIQSLTDKXSXLEEDXXXSEER and EAQLXAEED, respectively, which corresponded to *M. galloprovincialis* tropomyosin (C). The numbers in (C) show the position from the N-terminal amino acid sequence of mussel tropomyosin. Colons and dots respectively show the amino acid residues of the fragments that completely or partially identified with mussel tropomyosin.

nata) and the Ostreoida (*C. gigas*)¹⁴. In *S. broughtonii*, one of two isoforms exists in the translucent portion of adductor muscle and the other in the opaque portion, and both forms are located in cardiac muscle. In *A. pectinata*, both isoforms exist in the translucent portion of adductor muscle, and one of them exists in both the opaque portion and in cardiac muscle. In *C. gigas*, both isoforms exist in the adductor muscle and cardiac muscle, but one of them is major isoform in adductor muscle and another is major isoform in cardiac muscle. Although it seems that the distribution of the tropomyosin isoforms in the two species of scallops is related to different muscle types and/or character, this is not the case for the other types of bivalves. If anything, the findings suggest that the distribution of tropomyosin isoforms in bivalves is associated with phylogenetic evolution¹⁴. In bivalves, it was reported that mussel might be a close prototype²⁷⁻²⁹. Interestingly, only one tropomyosin was found in adductor muscles and cardiac muscle of mussel in the previous study¹⁴ and in the present study (Fig. 1 and 2). Because several tropomyosin isoforms were found in many bivalves¹⁴⁻¹⁶, we examined whether mussel had only one tropomyosin. Although only one tropomyosin was found in major muscle tissue such as adductor muscle, cardiac muscle and retractor muscle, new two tropomyosin isoforms were found in mantle and gills (Fig. 2 and 4). Three mussel tropomyosins were designated TM1, TM2 and TM3, respectively. TM1 was major component in all tissue and may be the common isoform in mussel. TM2 was minor component and was mantle specific component. TM3 also was minor component and was mantle and gills specific component. Moreover, the molecular weight of TM3 was

Table 1. Qualities of each mussel tropomyosin isoform.

	Retardation by Urea	Detected Tissues	Molecular Weight	pI
TM1	+	retractor muscle, adductor muscle, cardiac muscle, mantle, gills	38,000	4.5
TM2	++	mantle	38,000	4.5
TM3	+	mantle, gills	31,000	4.5

lower than that of TM1 and TM2. Because TM3 was a low molecular weight type and included in gills (Figs. 4 and 5), it is also likely that it is a non-muscle type isoform. In many cases, the non-muscle type of tropomyosin has a lower molecular weight because the COOH-terminal is shorter than that of the muscle-type isoforms^{1,2}. The COOH-terminal of tropomyosin is involved in actin binding and polymerization^{1,2} and in the sea urchin the actin-binding ability of a non-muscle-type tropomyosin isoform was lower than that of a muscle type³⁰. Thus, the three tropomyosin isoforms in the mussel might differ in their actin-binding ability. Qualities of each mussel tropomyosin isoform found in the present study are shown in Table 1. From different qualities of each isoform, therefore, it is likely that mussel tropomyosin also show heterogeneity and tissue-specific distribution. In the present study, we could not suggest relationships between functions and structures of each isoform. A previous study has cloned one tropomyosin gene³¹, but as we have found three tropomyosin proteins in the mussel, we will screen for other genes in future studies.

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