Scientific Research Publishing

American Journal of Molecular Biology, 2019, 9, 16-27
http://www.scirp.org/journal/ajmb
ISSN Online: 2161-6663
ISSN Print: 2161-6620

Tropomyosin Isoform Expression in the Adductor Muscle of the Japanese Pearl Oyster, *Pinctada fucata*

Daisuke Funabara*, Ayaka Ohta, Jungo Sueyoshi, Satoshi Kanoh

Graduate School of Bioresources, Mie University, Tsu, Japan
Email: *funabara@bio.mie-u.ac.jp

How to cite this paper: Funabara, D., Ohta, A., Sueyoshi, J. and Kanoh, S. (2019) Tropomyosin Isoform Expression in the Adductor Muscle of the Japanese Pearl Oyster, *Pinctada fucata*. *American Journal of Molecular Biology*, **9**, 16-27.
https://doi.org/10.4236/ajmb.2019.91002

Received: November 13, 2018
Accepted: December 8, 2018
Published: December 11, 2018

Abstract

We determined the full-length primary structure of the tropomyosin (TM)-1 and -2 proteins from the adductor muscle of the Japanese pearl oyster *Pinctada fucata* (Pifuc-TM-1 and Pifuc-TM-2), and found that they are each composed of 284 amino acid residues. We predicted the gene structure of *P. fucata* TM (*Pifuc-TM*) using Splign alignment of our cDNA with genomic sequences and elucidated that *Pifuc-TM* consists of 10 exons. Exons 1 - 3 and 5 - 10 are used to transcribe *Pifuc-TM*-1 mRNA, and exons 1 - 4 and 6 - 10 are used to transcribe *Pifuc-TM*-2 mRNA. Both genes share the same start and stop codons located in exon 1 and exon 10, respectively. Using quantitative real-time PCR, we determined that the *Pifuc-TM*-1 gene was mainly expressed in adductor phasic muscle, and at a relatively weaker level in adductor catch muscle, whereas the *Pifuc-TM*-2 gene was expressed equally in both phasic and catch muscles. They were weakly expressed in gill and mantle. Immunoblot analysis using anti-Pifuc-TM-1 and anti-Pifuc-TM-2 antibodies revealed that adductor phasic muscle contained Pifuc-TM-1, while adductor catch muscle contained both Pifuc-TM-1 and Pifuc-TM-2. Differential scanning calorimetry (DSC) analysis was carried out for Pifuc-TM-1 and Pifuc-TM-2 expressed in bacteria, as well as TM purified from *P. fucata* phasic and catch muscle tissues (phasic-TM and catch-TM). The DSC data indicated that phasic-TM was mainly composed of Pifuc-TM-1, whereas catch-TM contained Pifuc-TM-1 and Pifuc-TM-2. These findings suggest that the distribution of Pifuc-TM-1 and Pifuc-TM-2 in adductor muscle is specific to the muscle fiber type, and reflects the properties of each.

Keywords

Adductor Muscle, Alternative RNA Processing, Catch Contraction, Pearl Oyster, Tropomyosin

1. Introduction

Bivalve adductor muscles are composed of two muscle fiber types: phasic and catch. The larger phasic muscle is used for quick closure of shells, whereas the smaller catch muscle is involved in the sustained closure of shells. Catch muscles can develop a long-lasting high-tension state with little energy expenditure [1]. They begin to contract following an increase in intracellular Ca^{2+} concentrations, which activates myosin and develops the tension. They subsequently enter the catch state once Ca^{2+} concentrations decrease to resting levels. In the catch state, thin and thick filaments are thought to be tethered together by a complex of myosin, actin and twitchin, a giant myosin-associated protein [2] [3]. There are currently few data to suggest that thin filament-linked regulation is involved in catch contraction. However, molluscan muscle has the thin filament-related muscle proteins troponin complex (Tn), tropomyosin (TM) and calponin, which are known as the regulators of muscle contraction in vertebrates and serve to remind us that mollusks employ a similar regulatory system to that of vertebrate muscle [4]. We recently analyzed troponin C, a subunit of troponin, of the Japanese pearl oyster *Pinctada fucata* (Pifuc-TnC), and revealed that it is distributed only in phasic muscle, suggesting that troponin does not participate in the regulation of catch contraction [5]. Therefore, if there is thin filament-linked regulation associated with Tn in molluscan muscles, it might be operational in phasic muscle instead. In fact, it was reported that TM might be involved in the regulation of scallop striated muscle (*i.e.* non-catch muscle) via movement along different domains of actin filaments in a Ca^{2+} concentration manner [6].

TM consists of two parallel α-helical polypeptides that form a coiled-coil structure and is localized along actin filaments. Muscle TM plays an important role in the regulation of skeletal muscle contraction in collaboration with Tn. While much is known about vertebrate TM, the functions of TM in molluscan muscle regulation remain obscure, although various molluscan TM proteins have been studied [6]-[14]. The elucidation of thin filament-related regulation in molluscan muscle requires characterization of molluscan TM.

In our previous study, we determined partial sequences of two TM isoforms from *P. fucata* (Pifuc-TM-1 and Pifuc-TM-2) and raised the possibility that they are expressed by alternative RNA processing from a single gene [4]. In this study, we determined the full sequences of Pifuc-TM-1 and Pifuc-TM-2, predicted the gene structure of *Pifuc-TM*, and investigated the tissue distribution of the two isoforms.

2. Materials and Methods

2.1. Animal Samples

We obtained live specimens of *P. fucata* that were cultured in Ago Bay, Mie Prefecture, Japan. The adductor muscle, gill and mantle were dissected from each oyster body, immediately frozen in liquid nitrogen, and stored at −80˚C until use.

2.2. cDNA Cloning of *Pifuc-TM*-1 and *Pifuc-TM*-2

Total RNA was extracted from the phasic part of the adductor muscle using a conventional method [15]. Partial nucleotide sequences of the *P. fucata* tropomyosin-1 and -2 genes (*Pifuc-TM*-1 and *Pifuc-TM*-2) as determined by 3' RACE were reported previously [4]. To determine the full-length sequence of each, 5' RACE was carried out using the 5' RACE system for Rapid Amplification of cDNA Ends, version 2.0 (Invitrogen, Carlsbad, CA, USA) using total RNA as a template. Primers were designed using the known sequences of *Pifuc-TM*-1 and *Pifuc-TM*-2. For *Pifuc-TM*-1, we used 5'- GATCCATTTGGCCTCAGATA-3' for synthesizing cDNA, 5'-ACTGTTTCTCCAGCACGTCT-3' for the first PCR, and 5'-GACCTCTCCTCTGATGCATT-3' for the second PCR. For *Pifuc-TM*-2 we used 5'-AACGTATTTAGCTTCTTTCA-3' for synthesizing cDNA, 5'-GTTGCTGTTCCAGGGCATCT-3' for the first PCR, and 5'-ATTCTCTCATCATCAGAGAC-3' for the second PCR. PCR was carried out using SapphireAmp Fast PCR Master Mix with the forward primers detailed above and the primers included in the kit. PCR conditions were as follows: 30 cycles of denaturation at 98˚C for 5 s, annealing at 55˚C for 5 s, and elongation at 72˚C for 10 s. The amplified DNA fragment was sequenced after insertion into a pTAC-1 vector. The determined sequences were registered in DDBJ/EMBL/GenBank (accession numbers LC431553 and LC431554).

2.3. Prediction of the *Pifuc-TM* Gene Structure

The genome sequence including the *Pifuc-TM* gene was obtained by BLAST searching the *Pifuc-TM*-1 and *Pifuc-TM*-2 nucleotide sequences against the *P. fucata* genome database [16]. The gene structure of the *Pifuc-TM* gene was predicted by analyzing cDNA and genome sequences using the Splign alignment tool (NCBI).

2.4. Gene Expression Analysis of *Pifuc-TM*-1 and *Pifuc-TM*-2 in Tissues

The gene expression patterns of *Pifuc-TM*-1 and *Pifuc-TM*-2 in the catch and phasic muscles, gill and mantle were analyzed by quantitative real-time PCR. The cDNAs were synthesized using total RNA from each tissue as templates in RiverTra Ace® qPCR RT Master Mix (Toyobo Co., Ltd., Osaka, Japan). Primers and probes were designed by Universal Probe Library Assay Design Center (Roche Diagnostics, Mannheim, Germany) using the distinct nucleotide sequences between *Pifuc-TM*-1 and *Pifuc-TM*-2. For *Pifuc-TM*-1, the primers used were: 5'-CTTGGAAAACCTGAACAATGC-3' (forward) and 5'-GGCCTCAGATAACTGTTTCTCC-3' (reverse), and a TaqMan probe 5'-CAGAGGAG-3'. For *Pifuc-TM*-2, the primers used were: 5'-AGAGAGTAGAAATGTCTCTGATGATGA-3' (forward) and 5'-TCCTCGGCAACGTATTTAGC-3' (reverse), and a TaqMan probe 5'-TGCCCTGG-3'. *P. fucata β-actin* (AF378128) was used as an internal standard. For *β-actin*, the primers used were 5'-TCGTTCCTCGGAATGGAA-3'

(forward) and 5'-TCGACATCGCATTTGAGAAT-3' (reverse), and a TaqMan probe 5'-CAGAAGGAG-3'. The PCR reaction was performed using Eagle Taq Master Mix with ROX (Roche Diagnostics).

2.5. Production of Anti-Pifuc-TM-1 and Pifuc-TM-2 Antibodies

To produce anti-Pifuc-TM-1 antibodies, a peptide with a sequence of NLNNASEERSDVLEK encoded in exon 5 of *Pifuc-TM*-1 was used as an antigen after addition of cysteine residue to its N-terminus to conjugate keyhole limpet hemocyanin as a carrier. For anti-Pifuc-TM-2 antibodies, a peptide with a sequence of SRNVSDDERIDALEQ encoded in exon 4 was used as an antigen similarly to anti-Pifuc-TM-1. Peptide synthesis and antibody production was performed by Sigma-Aldrich Japan.

2.6. Protein Expression Analysis of Pifuc-TM-1 and Pifuc-TM-2 in Tissues

Protein expression patterns of Pifuc-TM-1 and Pifuc-TM-2 in tissues of *P. fucata* were analyzed by immunoblotting using the antibodies described above. Catch and phasic muscles, gill and mantle were homogenized in phosphate-buffered saline and subjected to 10% SDS-PAGE, followed by electroblotting onto a polyvinylidene difluoride membrane. After blocking, the membrane was hybridized with anti-Pifuc-TM-1 or anti-Pifuc-TM-2 antibodies. Horseradish peroxidase-conjugated goat anti-rabbit IgG was used as a secondary antibody. Detection was carried out with Pierce ECL Western Blotting Substrate (ThermoFisher Scientific).

2.7. Bacterial Expression and Purification of Pifuc-TM-1 and Pifuc-TM-2

DNA fragments encoding Pifuc-TM-1 or Pifuc-TM-2, with codon usage optimized for expression in *Escherichia coli*, were commercially synthesized by GenScript Gene Synthesis Service (GenScript, Piscataway, NJ, USA) and inserted into the T7 expression vector pET21b (Novagen Darmstadt, Germany). To mimic acetylation, alanine and serine residues were added to the N-terminus. *E. coli* BL21(DE3) transformed with *pET-Pifuc-TM*-1 or *pET-Pifuc-TM*-2 was cultured in auto-induction media at 37˚C for 24 h [17]. The *E. coli* cultures were collected by centrifugation and suspended in a lysis buffer included in the Ez-BactYeast Crusher kit (ATTO, Tokyo, Japan). Supernatants of the lysates obtained by centrifugation containing Pifuc-TM-1 or Pifuc-TM-2 were subjected to ion-exchange chromatography on a TSK-gel DEAE-5PW column (7.5 × 75 mm) (Tosoh, Tokyo, Japan) after dialysis against 50 mM Tris-HCl (pH 7.5) containing 30 mM KCl and 0.1 mM DTT. The proteins were eluted with a linear gradient of 30 mM to 600 mM KCl.

2.8. Purification of TM from Adductor Phasic and Catch Muscles

TM was purified from the adductor phasic and catch muscles of *P. fucata* (phas-

ic-TM and catch-TM, respectively) as described in a previous report [9]. The phasic adductor muscle acetone powder was extracted with 10 volumes of 20 mM Tris-HCl (pH 7.5) containing 1 M KCl and 5 mM 2-mercaptoethanol overnight, followed by centrifugation at 10,000 g for 10 min. The supernatant was subjected to isoelectric precipitation at pH 4.5 adjusted with 1 M HCl and subjected to centrifugation at 10,000 g for 10 min. The precipitate was dissolved in the least amount of water, and the pH was adjusted to 7.6 with 1 M NaOH. Phasic-TM was fractionated with ammonium sulfate at 40% - 45% saturation. The phasic-TM pellet was dissolved in the least amount of 50 mM Tris-HCl (pH 7.5) containing 30 mM KCl and 0.1 mM DTT and then subjected to ion-exchange chromatography on a TSK-gel DEAE-5PW column (7.5 × 75 mm) equilibrated with the same buffer at a flow rate of 1 mL/min. The proteins were eluted with a linear gradient of 30 mM to 600 mM KCl. Equivalent procedures were used to purify catch-TM.

2.9. Differential Scanning Calorimetry (DSC) Analysis of Pifuc-TM-1, Pifuc-TM-2, Phasic-TM and Catch-TM

Pifuc-TM-1, Pifuc-TM-2, phasic-TM and catch-TM were subjected to DSC analysis after dialysis against 10 mM sodium phosphate buffer (pH 7.0) containing 0.1 M KCl and 0.1 mM DTT overnight. The external dialysis solution was used to adjust the protein solution to 2.0 mg/mL and served as a reference for DSC. DSC was performed using a Microcal VP-DSC (Malvern Panalytical Ltd., Malvern, UK). The thermal scanning was performed at 10˚C to 80˚C at a scanning rate of 1˚C/min.

3. Results

3.1. Molecular Characteristics of *Pifuc-TM*-1 and *Pifuc-TM*-2

We used 5' RACE to determine 365 bp of new sequence including the 5'-untranslated region of *Pifuc-TM*-1 and 362 bp of new sequence of *Pifuc-TM*-2. Combined with known sequences, the full nucleotide sequences of *Pifuc-TM*-1 and *Pifuc-TM*-2 were determined in this study (**Figure 1**). The two sequences were found to be identical except for the region from 530 - 633 from the 5' -end of the *Pifuc-TM*-1 sequence. The open reading frame of *Pifuc-TM*-1 begins at 155 from the 5'-end and encodes 284 amino acid residues (**Figure 2**). *Pifuc-TM*-2 also has a sequence encoding 284 residues from the same position. The two sequences were identical except for residues 126 - 160 from the N-terminus. The N-terminal sequences of Pifuc-TM-1 and Pifuc-TM-2 are MDAIKKKM, a well-conserved sequence in TM regardless of the species. TFAELAGY at the C-terminus is a well-conserved sequence in molluscs.

3.2. Gene Structure of *Pifuc-TM*

BLAST searching with our *Pifuc-TM*-1 and *Pifuc-TM*-2 nucleotide sequences against the genome database of *P. fucata* yielded a single nucleotide sequence of

scaffold121.1. In our previous study, we annotated a gene model (pfu_aug1.0_6509.1_67448) that was automatically predicted by the genome database to be Pifuc-TM-1, which contained a predicted sequence that was identical to the sequence of Pifuc-TM-1 determined in this study. The gene model did not contain a sequence specific to Pifuc-TM-2. We then predicted the gene structure of *Pifuc-TM* using Splign alignment of the cDNA and genome sequences (Figure 3). The *Pifuc-TM* gene consists of 10 exons. Exons 1, 2, 3, 6, 7, 8, 9 and 10 are common to *Pifuc-TM*-1 and *Pifuc-TM*-2, while exon 4 is used for *Pifuc-TM*-2 and exon 5 for *Pifuc-TM*-1. *Pifuc-TM*-1 and *Pifuc-TM*-2 use the same start and stop codons, which are located in exon 1 and exon 10, respectively.

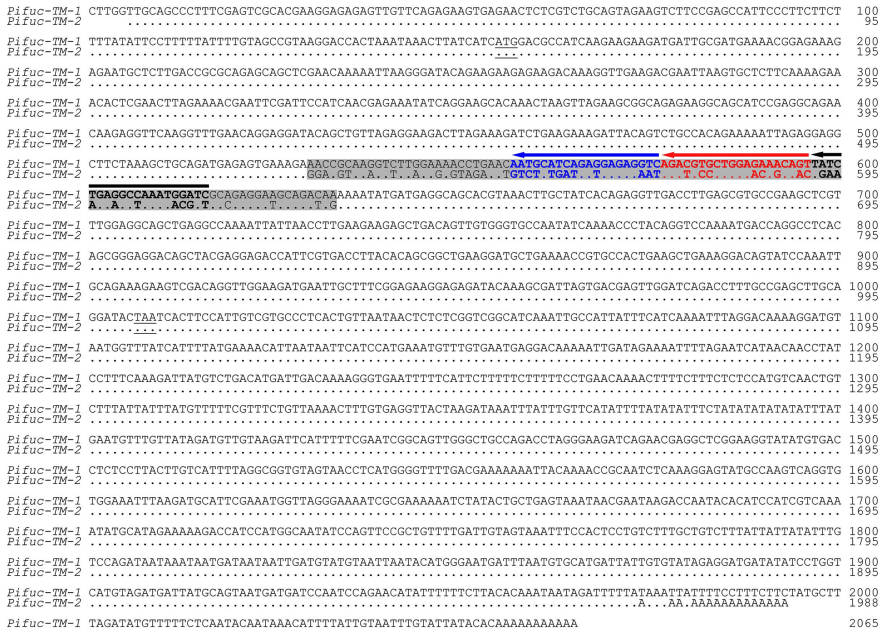

Figure 1. Nucleotide sequences of the *Pinctada fucata* tropomyosin-1 and -2 (*Pifuc-TM*-1 *and Pifurc-TM*-2) genes. Distinct sequences between *Pifuc-TM*-1 and *Pifuc-TM*-2 are shaded. The numbers on the right of the sequences denote nucleotides from the 5'-end. The start and stop codons are underlined. Positions of primers used for cDNA cloning are represented by arrows above the sequences.

Figure 2. Deduced amino acid sequences of *Pinctada fucata* tropomyosin-1 and -2 (Pifuc-TM-1 and Pifuc-TM-2). Segments encoded by the nucleotide sequences that were shaded in Figure 1 are in boldface. Solid and dashed lines above the sequences represent regions of exons. Sequences used for antibody production are shaded.

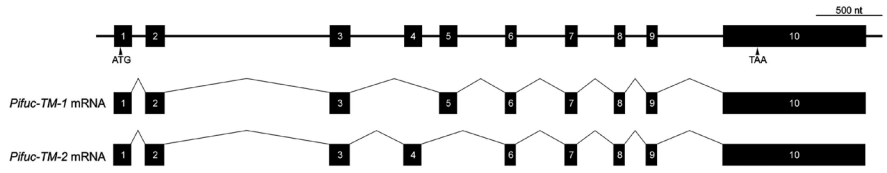

Figure 3. Gene structure of *Pinctada fucata* tropomyosin. Black boxes indicate exons, and intervening lines represent introns. The ATG start codon and TAA stop codons are indicated. Exons 1 - 3 and 5 - 10 are used to transcribe *Pifuc-TM*-1 mRNA, and exons 1 - 4 and 6 - 10 for *Pifuc-TM*-2 mRNA.

3.3. Gene and Protein Expression Analyses of Pifuc-TM-1 and Pifuc-TM-2

Gene expression analysis showed that the *Pifuc-TM*-1 gene was predominantly expressed in adductor phasic muscle, while relatively weaker expression was detected in catch muscle; the *Pifuc-TM*-2 gene was expressed equally in phasic and catch muscle (**Figure 4**). Both genes were barely detectable in gill and mantle. These findings remind us that the two isotype proteins Pifuc-TM-1 and Pifuc-TM-2 were differentially expressed in the two muscle types. Immunoblotting using antibodies that specifically recognize Pifuc-TM-1 and Pifuc-TM-2 was carried out to analyze the protein expression profiles in *P. fucata* tissues (**Figure 5**). The results revealed that Pifuc-TM-1 was present in adductor catch and phasic muscles, whereas Pifuc-TM-2 was present only in catch muscle. Neither Pifuc-TM-1 nor Pifuc-TM-2 was detected in gill or mantle. Paramyosin, which has a similar structure to TM in that it consists of an α-helix that spans the entire molecule, was nonspecifically detected by the anti-Pifuc-TM-1 and anti-Pifuc-TM-2 antibodies (**Figure 5(B)** and **Figure 5(C)**). Taken together, the data suggested that adductor phasic muscle contained mainly Pifuc-TM-1 while adductor catch muscle contained equally amounts of both Pifuc-TM-1 and Pifuc-TM-2. To confirm this result, we performed DSC analysis using TM purified from adductor phasic (phasic-TM) and catch muscle (catch-TM), and bacterially expressed Pifuc-TM-1 and Pifuc-TM-2.

3.4. DSC Analysis of Pifuc-TM-1, Pifuc-TM-2, Phasic-TM and Catch-TM

All DSC thermograms of Pifuc-TM-1, Pifuc-TM-2, phasic-TM and catch-TM had two peaks (labeled as T_m1 and T_m2 in the order of temperature from low to high) (**Figure 6**). The T_m1 values were 46.49˚C for Pifuc-TM-1, 47.20˚C for Pifuc-TM-2, 46.65˚C for phasic-TM and 46.83˚C for catch-TM. The T_m2 values were 54.34˚C for Pifuc-TM-1, 53.71˚C for Pifuc-TM-2, 51.34˚C for phasic-TM and 53.75˚C for catch-TM. Thus, all of the TM molecules had similar T_m values. However, Pifuc-TM-1 and Pifuc-TM-2 had thermograms that differed based on the distinct sequences between them. A comparison of the heights of the T_m2 peaks implied that catch-TM is composed of both Pifuc-TM-1 and Pifuc-TM-2.

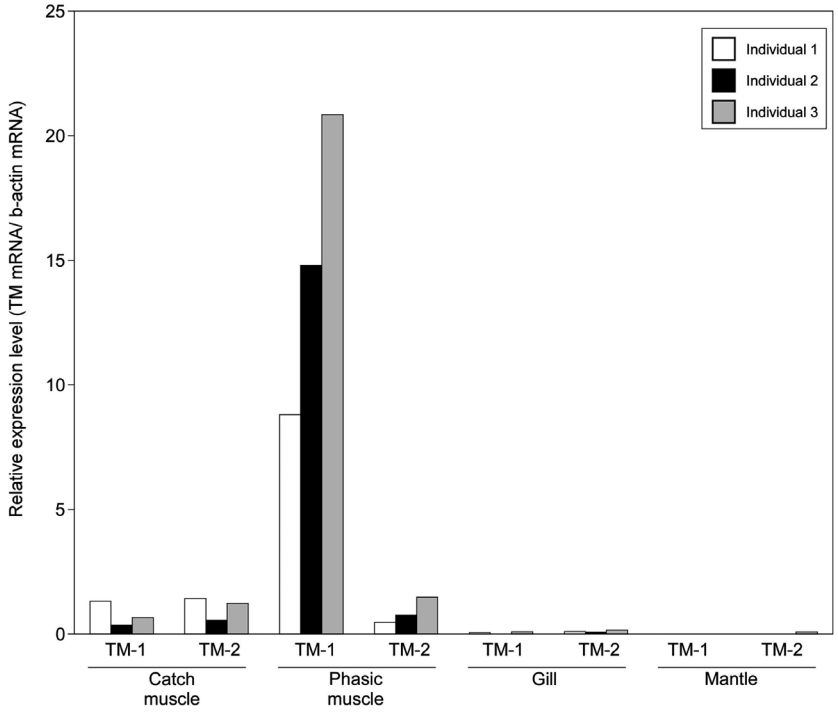

Figure 4. Gene expression patterns of *Pifuc-TM*-1 and *Pifuc-TM*-2 in *Pinctada fucata* tissues. Quantitative real-time PCR analysis was performed to examine *Pifuc-TM*-1 and *Pifuc-TM*-2 gene expression in *P. fucata* adductor catch muscle, adductor phasic muscle, gill and mantle. The data shown are from three independent experiments. The y-axis indicates relative gene expression levels using β-actin as an internal standard.

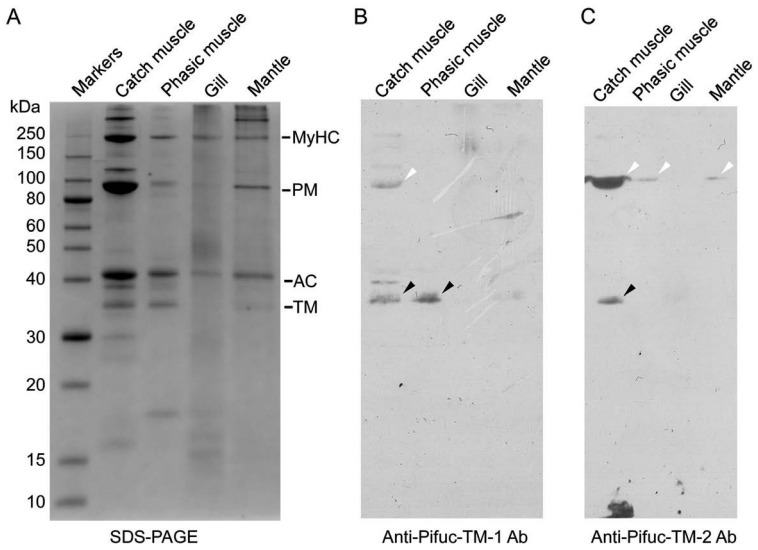

Figure 5. Protein expression patterns of Pifuc-TM-1 and Pifuc-TM-2 in *Pinctada fucata* tissues. Immunoblotting analysis was performed to examine Pifuc-TM-1 and Pifuc-TM-2 in *P. fucata* adductor catch muscle, adductor phasic muscle, gill and mantle. (A) SDS-PAGE patterns of each tissue homogenate, showing myosin heavy chain (MyHC), paramyosin (PM), actin (AC), and tropomyosin (TM). The polyvinylidene difluoride membrane reacted with anti-Pifuc-TM-1 antibodies (B) and anti-Pifuc-TM-2 antibodies (C). Black arrow heads indicate TM bands. White arrow heads indicate the nonspecific detection of PM.

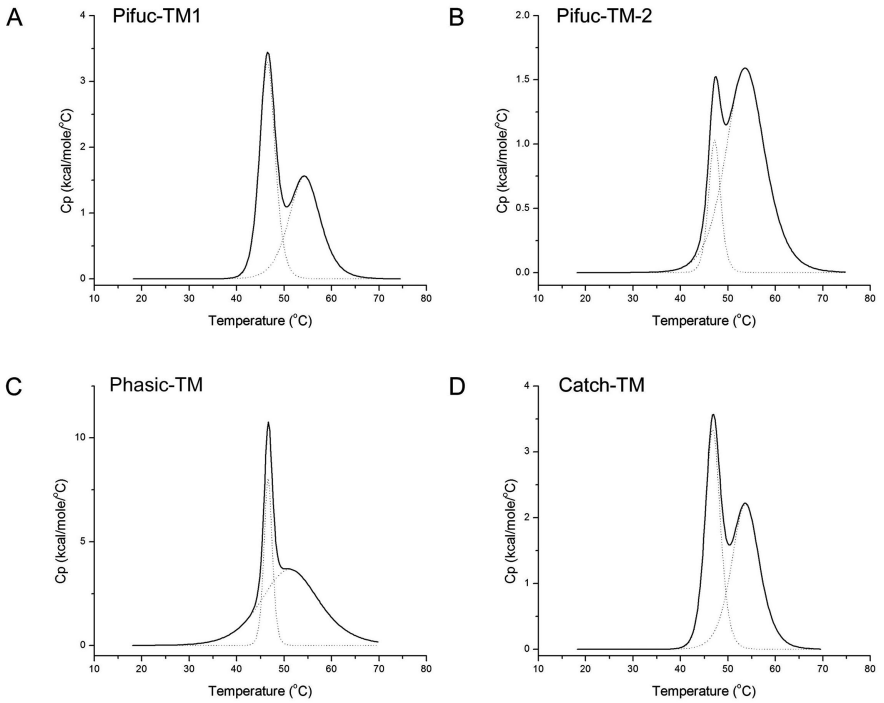

Figure 6. Differential scanning calorimetric (DSC) analysis. DSC thermograms of bacterially expressed Pifuc-TM-1 (A) and Pifuc-TM-2 (B), and phasic-TM (C) and catch-TM (D) purified from *Pinctada fucata* tissues. Cp = measured heat capacity.

4. Discussions

We determined that, like other muscle TMs, the full sequences of Pifuc-TM-1 and Pifuc-TM-2 consist of 284 amino acid residues (**Figure 1** and **Figure 2**). The distinct sequence segment (residues 126 - 160) of Pifuc-TM-1 and Pifuc-TM-2 includes a region considered to be involved in interaction with troponin T (TnT) [18], implying that the affinity of TnT to Pifuc-TM possibly depends on isoform types. As shown in **Figure 4** and **Figure 5**, Pifuc-TM-2 was mainly expressed only in phasic muscle. It was reported in a scallop striated muscle study that the movement of TM was induced by the Ca^{2+} concentration, as it is in vertebrates [6]. Pifuc-TM-2 might be involved in phasic muscle regulation in collaboration with Tn, which is expressed mainly in phasic muscle [5] [19].

TM molecules are joined together by a head-to-tail interaction of eight to nine residues at the N and C-termini. A sequence of MDAIKKKM at the N-terminus is well conserved across vertebrates and invertebrates, including both Pifuc-TM-1 and Pifuc-TM-2 (**Figure 2**). Conversely, at the C-terminus, vertebrate TM has a sequence of ALNDMTSI, whereas molluscan TM, including Pifuc-TM-1 and Pifuc-TM-2, has a sequence of TFAEL(I) AGY. This difference at the C-terminal sequence possibly confers differences in TM assembly and muscle regulation. It was reported that TM in different bivalves inhibited the Mg-ATPase activity of rabbit reconstituted actomyosin, and that the relative viscosities in the absence of KCl were 3.5 - 6 times higher than those of vertebrate TMs [11]. Taken together, these data suggest that Pifuc-TM-1 and Pifuc-TM-2 might have distinct

functions from the isotypes of vertebrate TM.

It is well known that many TM variants are produced by alternative RNA processing that is tissue specific [20]. In this study, we revealed that Pifuc-TM-1 and Pifuc-TM-2 are synthesized from a single gene and that their expression patterns are different in adductor phasic and catch tissue, suggesting that the adductor muscle selectively employs tropomyosin isoforms for different muscle functions. A study of scallop adductor muscle that parallels the results of this study showed that different myosin heavy chain isoforms produced by alternative RNA processing from a single gene were used selectively in striated and catch muscles [21]. Our previous reports showed that isoforms of twitchin and calponin are selectively used in scallop striated and catch muscles [22] [23]. Different properties that distinguish phasic and catch muscles in bivalves might be derived from isoforms of muscle proteins that are produced by alternative RNA processing. Although molluscan muscle TM has been widely studied, there are few reports of genomic analysis. In this study, a BLAST search against the *P. fucata* genome database yielded only one scaffold including the *TM* gene, indicating that there is one *TM* gene in the *P. fucata* genome. It was reported that there might be multiple TM genes in the genome of the mussel *Mytilus galloprovincialis* [7]. Numbers of TM genes might depend on the mollusc species.

We recently showed that TnC, a subunit of Tn, might participate in the regulation of phasic muscle by virtue of its molecular characterization [5] [19]. In this study, DSC analysis clearly revealed that the types of TM isolated from phasic and catch muscles are different (**Figure 6**), raising the possibility that Tn might interact with Pifuc-TM-1. However, how TnC collaborates with other subunits of Tn to regulate molluscan muscle contraction is unclear. Molluscan TnI and TnT are considered to have different characteristics from those of vertebrates [24] [25] [26] [27]. To better understand the molecular mechanism of molluscan muscle contraction regulation, we need to elucidate the functions of the Tn subunits, and the interaction between Tn and TM.

Acknowledgements

This study was supported by JSPS KAKENHI Grant Number JP16K07872. We thank Michelle Kahmeyer-Gabbe, PhD, from Edanz Group (http://www.edanz.com/ac) for editing a draft of this manuscript.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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