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Ca^{2+}-Induced Conformational Change of Troponin C from the Japanese Pearl Oyster, *Pinctada fucata*

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Abstract

Troponin is a thin filament-associated regulator of vertebrate striated muscle contraction. Troponin changes its structure upon Ca^{2+} binding to troponin C, one of the subunits of troponin, allowing myosin to interact with actin. We recently elucidated the molecular characteristics of the Japanese pearl oyster *Pinctada fucata* troponin C (Pifuc-TnC), revealing the possibilities that Pifuc-TnC and vertebrate muscle TnC play dissimilar roles in muscle contraction. Pifuc-TnC has four EF-hand motifs, but, unlike vertebrate TnC, only one (site IV) was predicted to bind Ca^{2+}. To confirm the number of Ca^{2+}-binding sites in Pifuc-TnC and whether Ca^{2+} binding induces a conformational change, we purified the full-length protein and a variant, Pifuc-TnC-E142Q (that has a mutation in the predicted Ca^{2+}-binding site of site IV), following their expression in laboratory *E. coli*. Isothermal titration calorimetry demonstrated Ca^{2+} binding to Pifuc-TnC, whereas Pifuc-TnC-E142Q was unable to bind Ca^{2+}, confirming that site IV is the only Ca^{2+}-binding site in Pifuc-TnC. Pifuc-TnC eluted in a later fraction from a gel filtration column in the presence of Ca^{2+} compared with the condition when Ca^{2+} was absent. In contrast, the elution profiles of Pifuc-TnC-E142Q were equivalent in both the presence and absence of Ca^{2+}, suggesting that Ca^{2+} binding to Pifuc-TnC induces a conformational change that delays its elution from the column. UV-absorption spectral analysis revealed that binding of Ca^{2+} to Pifuc-TnC caused an increase in absorption at a wavelength of approximately 250 nm, possibly because phenylalanine residues had been exposed on the surface of the molecule as a result of a conformational change. Differential scanning calorimetric analyses of Pifuc-TnC showed aggregation in the presence of Ca^{2+} in accordance with an increase of temperature, but no aggregation was seen in the absence of Ca^{2+}. In combination, these findings suggest that Ca^{2+} binding to site IV induces a conformational change in Pifuc-TnC.

Keywords

Ca^{2+}-Binding, Catch Muscle, Conformational Change, EF-Hand, Troponin C

1. Introduction

Troponin (Tn) is the sarcomeric Ca^{2+}-dependent regulator for striated muscle contraction in vertebrates. It is distributed on thin filaments and inhibits the interaction between actin and myosin. Troponin consists of three subunits: troponin C (TnC), troponin I (TnI), and troponin T (TnT). The binding of Ca^{2+} to TnC induces a conformational change in the troponin complex structure that enables myosin to interact with actin [1]-[6].

Both vertebrate and molluscan muscle contraction are regulated by intracellular Ca^{2+} concentrations. However, in contrast to vertebrates, mollusks employ a thick filament-linked regulatory system where myosin binds Ca^{2+} directly, leading to its activation and subsequent interaction with actin. Although Tn is also located in molluscan muscles, it is currently unclear whether it is involved in a similar thin filament-linked regulatory system to that in vertebrates.

Bivalve adductor muscle is composed of two types of muscles, phasic and catch. Phasic muscle is used for quick shell closures and catch muscle for the prolonged closure of shells, a process that utilizes little energy (catch contraction) [7]. Recently, we revealed the molecular characteristics of troponin C from the Japanese pearl oyster *Pinctada fucata* (Pifuc-TnC) [8] [9]. The Pifuc-TnC gene is predominantly expressed in phasic muscle, which leads us to suggest that Pifuc-TnC is involved in phasic muscle contraction. Our prediction is also supported by a recent comprehensive survey of gene expression patterns in scallop adductor muscle using proteomic and transcriptomic analyses that showed that the genes of all the troponin subunits are more highly expressed in phasic muscle compared with catch muscle [10]. However, to evaluate our prediction, elucidation of the function(s) of Tn in molluscan phasic muscle is required.

Pifuc-TnC possesses four EF-hand motifs (site I - IV) and, analogous to other molluscan TnCs, primary structure analyses indicate that only site IV is able to bind to Ca^{2+} [8]. A predicted three-dimensional model of Pifuc-TnC resembled that of chicken fast skeletal TnC. However, there was an additional loop structure in the α-helix connecting its N- and C-terminal lobes, suggesting that Pifuc-TnC might work differently in molluscan muscles compared with TnC in vertebrate muscles. Moreover, it has been reported that scallop TnC, which binds to Ca^{2+} only at site IV, is likely to have distinct functions from vertebrates [11].

Here, we studied Pifuc-TnC and Pifuc-TnC-E142Q, a Pifuc-TnC mutant variant lacking Ca^{2+}-binding ability, and investigated whether the Pifuc-TnC structure changes upon binding of Ca^{2+}.

2. Materials and Methods

2.1. Protein Preparation

We designed Pifuc-TnC-E142Q, a mutant variant lacking the ability to bind Ca^{2+} due to the substitution of a glutamic acid (E142) residue located in the site IV EF-hand motif to glutamine (Q) (**Figure 1**). DNA fragments encoding Pifuc-TnC or Pifuc-TnC-E142Q, 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 pET15b (Novagen Darmstadt, Germany), creating an in-frame N-terminal fusion of six histidine residues. *E. coli* BL21(DE3) transformed with *pET-Pifuc-TnC* or *pET-Pifuc-TnC-E*142*Q* were cultured in auto-induction media at 37˚C for 24 hours [12]. The cultured *E. coli* collected by centrifugation was suspended in a lysis buffer included in a kit EzBactYeast Crusher (ATTO, Tokyo, Japan). The supernatant of the lysate obtained by centrifugation containing Pifuc-TnC or Pifuc-TnC-E142Q was subjected to affinity chromatography with a Bio-Scale Mini Profinity IMAC cartridge (Bio-Rad, Hercules, CA, USA) under native conditions according to the manufacturer's instructions. The purity of the eluted proteins was confirmed using SDS-PAGE and Coomassie blue staining. Protein concentrations were measured by the Bradford method using bovine serum albumin as a standard. Purified protein samples were freeze-dried following dialysis against 10 mM ammonium bicarbonate (pH 8.0).

2.2. Isothermal Titration Calorimetric Analysis

Ca^{2+} binding to Pifuc-TnC or Pifuc-TnC-E142Q was measured by isothermal

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Pifuc-TnC       MGSSHHHHHHSSGLVPRGSHMSEFKVSEKQFADAHQTFNLFDKKGTGQVSTKELEKVFKS  60
Pifuc-TnC-E142Q .....................................................  60

Pifuc-TnC       LALQVDDEKLKEWSDELDEEATGYFTWDQFKILFERKLKTDEDERELREAFRVLDKGNKG 120
Pifuc-TnC-E142Q ............................................................ 120

Pifuc-TnC       TIPVEDLRWILKSLGDDLTDEELDEMIAETDTDGSGTVDYEEFKTLMTSD           170
Pifuc-TnC-E142Q ..........................................Q.........           170
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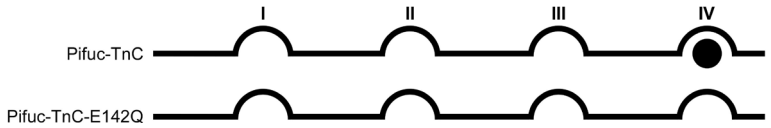
(a)



(b)

Figure 1. Sequences of *Pinctada fucata* troponin C (Pifuc-TnC) and Pifuc-TnC-E142Q. (a) Primary structures of Pifuc-TnC and its mutant variant, Pifuc-TnC-E142Q, which was constructed in this study. Additional sequences, including a histidine tag and a thrombin recognition site are shaded. Each of the four EF-hand motifs is underlined. Dots represent identical residues in Pifuc-TnC and Pifuc-TnC-E142Q; (b) Schematic representation of Pifuc-TnC and Pifuc-TnC-E142Q. The four EF-hand motifs are represented by semicircles. The black circle represents Ca^{2+} binding. Only the site IV EF-hand motifs in Pifuc-TnC is able to bind Ca^{2+} (Pifuc-TnC-E142Q is unable to bind Ca^{2+}).

titration calorimetry (ITC) using a MicroCal iTC200 calorimeter (Malvern Panalytical Ltd., Malvern, UK). Freeze-dried protein samples were resuspended and dialyzed overnight against 10 mM PIPES-KOH (pH 6.8) containing 0.15 M NaCl and 1 mM 2-mercaptoethanol at 4˚C. The protein samples and $CaCl_2$ solution were adjusted to final concentrations of 2.4 mM and 120 mM, respectively, using the external dialysis buffer. Experimental parameters were: total injections, 18 times; cell temperature, 25˚C; reference power, 10 μcal/s; syringe concentration, 120 mM; cell concentration, 2.4 mM; stirring speed, 1000 rpm. Injection parameters were: volume, 2.0 μL; spacing, 200 s. Titration was performed at 25˚C by injecting 2.0 μL of 120 mM $CaCl_2$ into the ITC cell containing 300 μL of 2.4 mM Pifuc-TnC or Pifuc-TnC-E142Q. The data thus obtained were corrected for the heat of dilution and analyzed using MicroCal Analysis Launch software (Malvern Panalytical Ltd.).

2.3. Gel Filtration Analysis

To compare the gel filtration chromatography elution profiles of Pifuc-TnC in the presence and absence of Ca^{2+}, Pifuc-TnC (2 mg) was injected onto a Hi-Prep16/60 Sephacryl S-200 high resolution column (GE Healthcare Ltd., Buckinghamshire, UK) equilibrated with 10 mM PIPES-KOH (pH 6.8) containing 0.15 M NaCl, 1 mM 2-mercaptoethanol, and 1 mM $CaCl_2$ or 0.5 mM EDTA at 4˚C. Elution was performed at the flow rate of 0.5 mL/min and fraction volumes were 3.0 mL. The column effluent was monitored at 280 nm. Equivalent procedures were used to analyze the elution profiles of Pifuc-TnC-E142Q in the presence and absence of Ca^{2+}.

2.4. UV-Absorption Spectral Analysis

Pifuc-TnC was dialyzed against phosphate buffered saline (PBS) and adjusted to a final concentration of 2.0 mg/mL. The absorption spectrum of Pifuc-TnC (2.0 mg/mL) in the presence or absence of 1 mM Ca^{2+} (where appropriate), was measured between 240 nm and 320 nm using an Agilent Cary 60 UV-Vis scanning spectrometer (Agilent Technologies, Santa Clara, CA) at room temperature. The UV-absorption spectra of Pifuc-TnC in the presence and absence of Ca^{2+} were then compared. Equivalent procedures were used to analyze the UV-absorption spectra of Pifuc-TnC-E142Q in the presence and absence of Ca^{2+}.

2.5. Differential Scanning Calorimetric Analysis

The thermostability of Pifuc-TnC in the presence or absence of Ca^{2+} was analyzed by differential scanning calorimetry (DSC). Pifuc-TnC was dialyzed against PBS and adjusted to a final concentration of 2.0 mg/mL. Pifuc-TnC was analyzed by DSC in the presence or absence of 1 mM Ca^{2+} (where appropriate) using a Microcal VP-DSC (Malvern Panalytical Ltd.). Thermal scanning was performed between 10˚C and 80˚C with a scanning rate of 1˚C/min.

3. Results

3.1. Protein Preparation

Histidine-tagged Pifuc-TnC and Pifuc-TnC-E142Q were successfully expressed in laboratory E. coli and purified to near-homogeneity using affinity chromatography as shown in **Figure 2**. The purity was sufficient for use in all experiments carried out in this study. Following freeze-drying, both proteins were able to be resuspended in the buffers required for all analyses.

3.2. Isothermal Calorimetric Analysis

Ca^{2+}binding to Pifuc-TnC was confirmed by ITC analysis (Kd = 16.7 mM), whereas no Ca^{2+} binding was detected for Pifuc-TnC-E142Q. This confirms that only the Pifuc-TnC site IV EF-hand motif is able to bind to Ca^{2+} (**Figure 3**). Each injection of Ca^{2+} into the calorimetry cell containing Pifuc-TnC produced an endothermic heat of reaction that decreased in magnitude with subsequent injections. The results indicated that the binding of Ca^{2+} to Pifuc-TnC was driven by entropy changes.

3.3. Gel Filtration Chromatography

In the presence of Ca^{2+}, Pifuc-TnC eluted in fraction 30 from a HiPrep16/60 Sephacryl S-200 high resolution gel filtration column, whereas it eluted earlier, in fraction 27, in the absence of Ca^{2+} (**Figure 4(a)**). In contrast, the elution profiles of the mutant variant, Pifuc-TnC-E142Q, which is unable to bind Ca^{2+}, were equivalent in both the presence and absence of Ca^{2+} (**Figure 4(b)**). This indicates that Ca^{2+} binding to Pifuc-TnC induces a conformational change that delays its elution from the column.

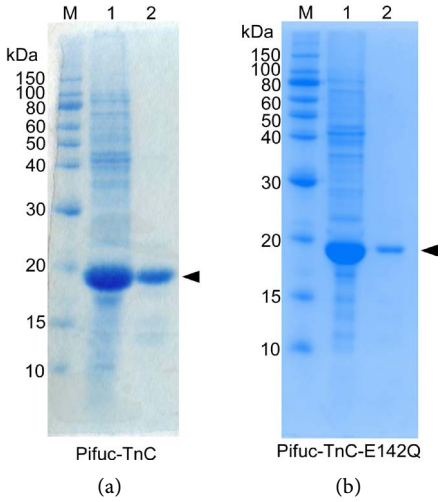
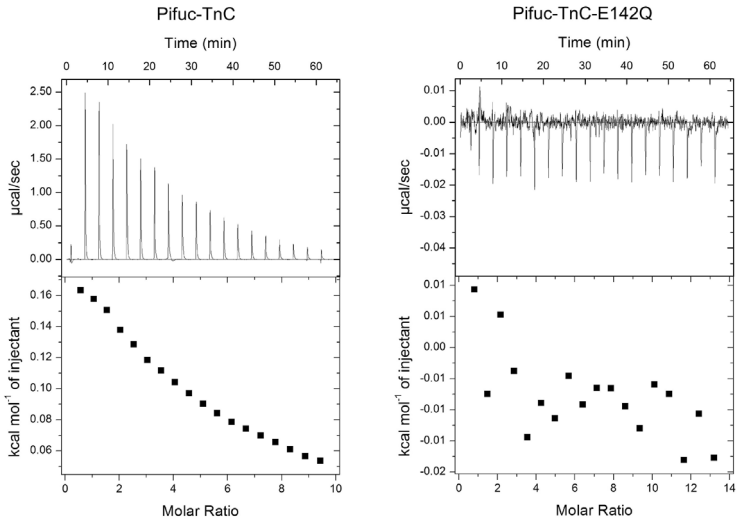
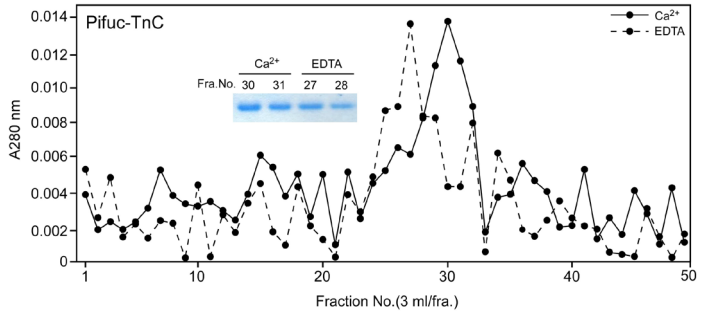


Figure 2. Expression and purification of Pifuc-TnC and Pifuc-TnC-E142Q. (a) Coomassie blue-stained SDS-PAGE gel showing Pifuc-TnC upon expression in laboratory *E. coli* (lane 1) and following affinity purification (lane 2); (b) SDS-PAGE gel showing Pifuc-TnC-E142Q upon expression (lane 1) and following affinity purification (lane 2). M: molecular weight markers. Arrowheads indicate the purified protein bands.

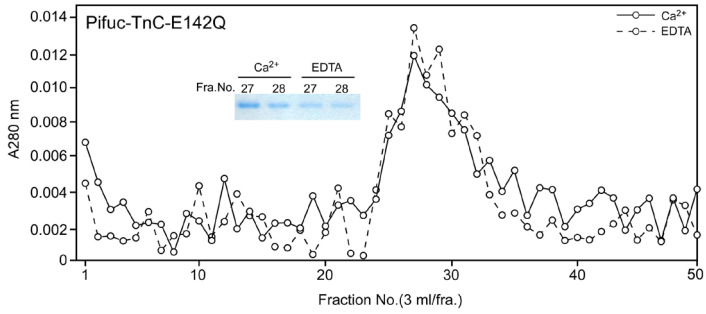


(a) (b)

Figure 3. Isothermal titration calorimetric analysis of Ca^{2+} binding to Pifuc-TnC and Pifuc-TnC-E142C. Titration curve of interactions between Pifuc-TnC and Ca^{2+} (a) and Pifuc-TnC-E142C and Ca^{2+} (b). In each case, the upper panel shows raw energy changes during the titration (time), while the lower panel presents the derived integrated total energy changes as a function of the molar ratio of the interactants. An endothermal reaction was detected upon interactions between Pifuc-TnC and Ca^{2+}.



(a)



(b)

Figure 4. Isothermal titration calorimetric analysis of Ca^{2+} binding to Pifuc-TnC and Pifuc-TnC-E142C. Titration curve of interactions between Pifuc-TnC and Ca^{2+} (a) and Pifuc-TnC-E142C and Ca^{2+} (b). In each case, the upper panel shows raw energy changes during the titration (time), while the lower panel presents the derived integrated total energy changes as a function of the molar ratio of the interactants. An endothermal reaction was detected upon interactions between Pifuc-TnC and Ca^{2+}.

3.4. UV-Absorption Spectra

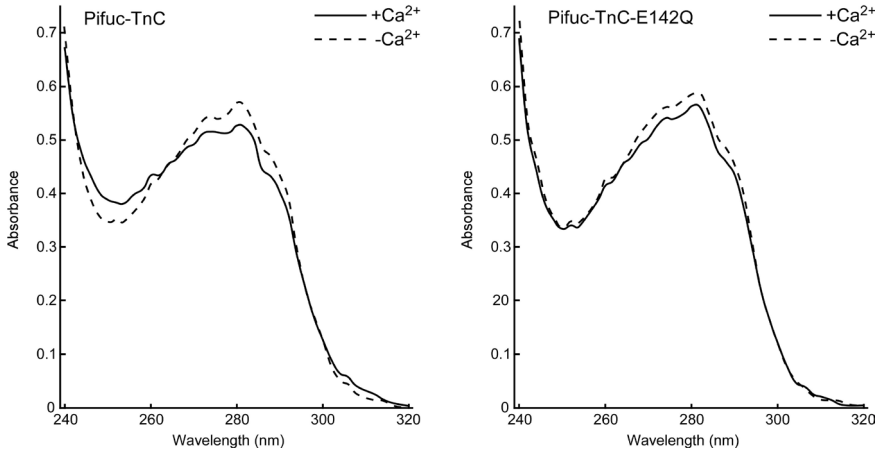
UV-absorption spectral analysis revealed that the binding of Ca^{2+} to Pifuc-TnC caused an increase in absorption at a wavelength of approximately 250 nm (Figure 5). In contrast, no significant difference was observed in UV absorption spectra of the Pifuc-TnC-E142Q mutant variant in the presence and absence of Ca^{2+}.

3.5. Differential Scanning Calorimetry

We also examined if Ca^{2+} binding affects Pifuc-TnC stability by monitoring its thermal unfolding using differential scanning calorimetry (DSC). The DSC thermogram of the Ca^{2+}-unbound Pifuc-TnC did not show a clear thermal transition, which may be due to gradual unfolding as the temperature increases. However, a very different spectrum was observed for the Ca^{2+}-bound Pifuc-TnC: a possible structural transition was observed at 50˚C followed by a number of smaller peaks that are probably the result of aggregation at higher temperatures (Figure 6). The difference between the Ca^{2+}-bound and -unbound forms is likely to be a result of changes in protein folding, owing to a conformational change upon Ca^{2+} binding. Unfortunately, no thermodynamic parameters were obtained from the DSC data.

4. Discussion

Here, we successfully constructed, expressed, and purified recombinant Pifuc-TnC and its mutant variant Pifuc-TnC-E142Q (Figure 1 and Figure 2). ITC analyses confirmed that Pifuc-TnC, as predicted from its primary structure, is able to bind to Ca^{2+} only at its site IV EF-hand motif (Figure 3), analogous to other molluscan TnCs. Gel-filtration chromatographic studies of Pifuc-TnC in the presence and absence of Ca^{2+} ions strongly suggested that Ca^{2+} binding



(a) (b)

Figure 5. UV-absorption spectral analysis. (a) UV-absorption spectrum of Pifuc-TnC in the presence (solid line) and absence (dotted line) of Ca^{2+}; (b) UV-absorption spectrum of Pifuc-TnC-E142Q in the presence (solid line) and absence (dotted line) of Ca^{2+}.

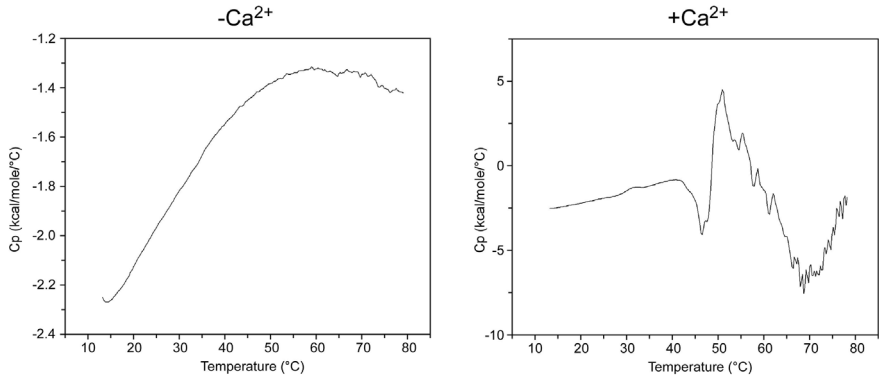


Figure 6. Differential scanning calorimetric analysis. DSC thermograms of Pifuc-TnC in the absence (a) and presence (b) of Ca^{2+}.

induces a conformational change, an observation that was supported by UV-absorption spectra and DSC analyses (**Figures 4-6**). It is possible that this increase in absorption at 250 nm of the UV-absorption spectra was caused by the exposure of phenylalanine residue at the surface of Pifuc-TnC as a result of a conformational change induced upon Ca^{2+} binding. These findings agree with reports that revealed that troponin C from Akazara scallop adductor muscle also has a single Ca^{2+} binding site at site IV, and, like Pifuc-TnC, alters its steric structure upon Ca^{2+} binding [11] [13] [14].

In our previous study, we predicted a three-dimensional model of Pifuc-TnC using SWISS-MODEL. The predicted structure of Pifuc-TnC was very similar to TnCs from chicken fast skeletal muscle and American lobster [8]. The only notable difference was the presence of a short loop (four amino acids) within the α-helix connecting the N- and C-lobes, suggesting functional differences between molluscan and vertebrate TnCs. In contrast to molluscan TnCs, Ca^{2+} binding of vertebrate skeletal TnC involves all four EF-hand motifs. Ca^{2+} binding to sites I and II in the N-terminal lobe of skeletal TnC triggers actin-myosin activation, whereas sites III and IV in the C-terminal lobe are thought to contribute to the stabilization of the molecular structure. In contrast, Ca^{2+} binding to site IV of scallop TnC confers Ca^{2+} sensitivity to actomyosin, which suggests that the conformational change induced by Ca^{2+} binding of molluscan TnC may control the interaction between actin and myosin filaments [15] [16].

Pifuc-TnC is predominantly distributed in phasic adductor muscle [8]. Our findings in this study support previous reports that suggest molluscan TnC plays a role in the regulation of phasic muscle contraction. In mollusks, it is thought that muscle contraction begins following the direct binding of Ca^{2+} to myosin, which leads to its activation and subsequent interaction with actin. The detachment of Ca^{2+} from myosin reverses the process and relaxes the muscle [17]. Troponin is distributed on thin filaments and it is currently unclear how thin-filament related proteins contribute to the regulation of muscle contraction in mollusks. However, it is highly likely that the Ca^{2+}-induced conformational

change of TnC is involved in the regulation of molluscan muscle contraction.

Troponin is a complex of three regulatory proteins, TnC, TnI and TnT. Previous studies have revealed that Ca^{2+}-bound scallop TnC binds to a different site in TnI in the troponin complex compared with vertebrate TnCs [13]. Further studies on the molecular interaction of Ca^{2+}-bound TnC with the other subunits of the troponin complex are required to clarify the function of troponin in molluscan muscle contraction.

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Conflicts of Interest

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

References

[1] Leavis, P.C., Gergely, J. and Szent-Gyorgyi, A.G. (1984) Thin Filament Proteins and Thin Filament-Linked Regulation of Vertebrate Muscle Contraction. *Critical Reviews in Biochemistry*, **16**, 235-305. https://doi.org/10.3109/10409238409108717

[2] Ohtsuki, I., Maruyama, K. and Ebashi, S. (1986) Regulatory and Cytoskeletal Proteins of Vertebrate Skeletal Muscle. *Advances in Protein Chemistry*, **38**, 1-67. https://doi.org/10.1016/S0065-3233(08)60525-2

[3] Zot, A.S. and Potter, J.D. (1987) Structural Aspects of Troponin-Tropomyosin Regulation of Skeletal Muscle Contraction. *Annual Review of Biophysics and Biophysical Chemistry*, **16**, 535-559. https://doi.org/10.1146/annurev.bb.16.060187.002535

[4] Grabarek, Z., Tao, T. and Gergely, J. (1992) Molecular Mechanism of Troponin-C Function. *Journal of Muscle Research and Cell Motility*, **13**, 383-393. https://doi.org/10.1007/BF01738034

[5] Farah, C.S. and Reinach, F.C. (1995) The Troponin Complex and Regulation of Muscle Contraction. *The FASEB Journal*, **9**, 755-767. https://doi.org/10.1096/fasebj.9.9.7601340

[6] Tobacman, L.S. (1996) Thin Filament-Mediated Regulation of Cardiac Contraction. *Annual Review of Physiology*, **58**, 447-481. https://doi.org/10.1146/annurev.ph.58.030196.002311

[7] Funabara, D., Kanoh, S., Siegman, M.J., Butler, T.M., Hartshorne, D.J. and Watabe, S. (2005) Twitchin as A Regulator of Catch Contraction in Molluscan Smooth Muscle. *Journal of Muscle Research and Cell Motility*, **26**, 455-460. https://doi.org/10.1007/s10974-005-9029-2

[8] Funabara, D., Urakawa, Y. and Kanoh, S. (2018) Molecular Cloning and Tissue Distribution of Troponin C from the Japanese Pearl Oyster, *Pinctada fucata*. *American Journal of Molecular Biology*, **8**, 166-177. https://doi.org/10.4236/ajmb.2018.83014

[9] Funabara, D., Watanabe, D., Satoh, N. and Kanoh, S. (2013) Genome-Wide Survey of Genes Encoding Muscle Proteins in the Pearl Oyster, *Pinctada fucata*. *Zoological*

Science, **30**, 817-825. https://doi.org/10.2108/zsj.30.817

[10] Sun, X., Liu, Z., Wu, B., Zhou, L., Wang, Q., Wu, W. and Yang, A. (2018) Differences between Fast and Slow Muscles in Scallops Revealed through Proteomics and Transcriptomics. *BMC Genomics*, **19**, 377. https://doi.org/10.1186/s12864-018-4770-2

[11] Ojima, T. and Nishita, K. (1992) Akazara Scallop Troponin C: Ca^{2+}-Induced Conformational Change and Interaction with Rabbit Troponin Subunits. *Archives of Biochemistry and Biophysics*, **299**, 344-349. https://doi.org/10.1016/0003-9861(92)90285-5

[12] Studier, F.W. (2005) Protein Production by Auto-Induction in High-Density Shaking Cultures. *Protein Expression and Purification*, **41**, 207-234. https://doi.org/10.1016/j.pep.2005.01.016

[13] Yumoto, F., Tanaka, H., Nagata, K., Miyauchi, Y., Miyakawa, T., Ojima, T. and Tanokura, M. (2008) Spectroscopic and ITC Study of the Conformational Change upon C^{a2+}-Binding in TnC C-Lobe and TnI Peptide Complex from Akazara Scallop Striated Muscle. *Biochemical and Biophysical Research Communications*, **369**, 109-114. https://doi.org/10.1016/j.bbrc.2007.11.124

[14] Ojima, T., Koizumi, N., Ueyama, K., Inoue, A. and Nishita, K. (2000) Functional Role of Ca^{2+}-Binding Site IV of Scallop Troponin C. *The Journal of Biochemistry*, **128**, 803-809. https://doi.org/10.1093/oxfordjournals.jbchem.a022818

[15] Doi, T., Satoh, A., Tanaka, H., Inoue, A., Yumoto, F., Tanokura, M., Ohtsuki, I., Nishita, K. and Ojima, T. (2005) Functional Importance of Ca^{2+}-Deficient N-Terminal Lobe of Molluscan Troponin C in Troponin Regulation. *Archives of Biochemistry and Biophysics*, **436**, 83-90. https://doi.org/10.1016/j.abb.2005.01.004

[16] Ojima, T. and Nishita, K. (1986) Troponin from Akazara Scallop Striated Adductor Muscles. *The Journal of Biological Chemistry*, **261**, 16749-16754.

[17] Funabara, D., Watabe, S. and Kanoh, S. (2015) Phosphorylation Properties of Twitchin from Yesso Scallop Catch and Striated Muscles. *Fisheries Science*, **81**, 541-550. https://doi.org/10.1007/s12562-015-0866-7