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Calponin Isoform Expression in the Japanese Pearl Oyster, *Pinctada fucata*

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Abstract

Calponin is a basic actin-binding protein found widely in invertebrate tissues including catch muscle and therefore may participate in catch contraction. There is limited information about molluscan calponin and molecular characterization to reveal its function in the regulatory system. We previously identified and partially sequenced three calponin isoforms of the Japanese pearl oyster, *Pinctada fucata* (Pifuc-CP-1, Pifuc-CP-2 and Pifuc-CP-3). In this study, the full-length nucleotide sequences of the three isoforms were determined. The primary structures revealed that Pifuc-CP-1 consists of 324 amino acids (aa) with a molecular mass (Mw) of 34.7 kDa and an isoelectric point (pI) of 9.40. Pifuc-CP-2 is 303 aa in length with a Mw of 33.3 kDa and a pI of 9.30, and Pifuc-CP-3 is 398 aa in length with a Mw of 43.8 kDa and a pI of 8.55. Domain architecture prediction showed that the three isoforms have a single calponin homology (CH) domain and multiple calponin (CN) domains. Pifuc-CP-1, Pifuc-CP-2 and Pifuc-CP-3 possess four, three and five CN domains, respectively. Tissue distribution analysis indicated the presence of additional calponin isoforms and these isoforms are distributed widely in muscle and non-muscle tissues. Results of cDNA cloning revealed further four calponin isoforms: Pifuc-CP-4 (402 aa, 42.8 kDa, pI = 9.10), Pifuc-CP-5 (285 aa, 30.7 kDa, pI = 9.45), Pifuc-CP-6 (286 aa, 31.1 kDa, pI = 9.60) and Pifuc-CP-7 (302 aa, 33.3 kDa, pI = 9.10). The domain architecture of these four isoforms also consists of a single CH domain and multiple CN domains. Pifuc-CP-4 possesses six CN domains, whereas Pifuc-CP-5, Pifuc-CP-6 and Pifuc-CP-7 contain three CN domains. Sequence alignment of *P. fucata* calponin isoforms showed that Pifuc-CP-1, Pifuc-CP-2, Pifuc-CP-3 and Pifuc-CP-4 have identical CH domain sequences, whereas Pifuc-CP-5, Pifuc-CP-6 and Pifuc-CP-7 have identical CH domain sequences. The CN repeats were not well conserved. These findings suggest that *P. fucata* calponin isoforms function differently in each tissue.

Keywords

Adductor Muscle, Calponin, Calponin Isoform, Catch Contraction, Pearl Oyster

1. Introduction

Mollusk bivalve adductor muscles are composed of two muscle types: phasic and catch. Phasic muscle is used for the quick closure of shells, whereas catch muscle functions in the sustain closure of shells. The contraction of both muscles is regulated by intracellular Ca^{2+} concentrations [1]. Mollusks employ a thick filament-linked regulatory system where myosin directly binds Ca^{2+}, leading to its activation and subsequent interaction with actin. Following a decrease in the intracellular Ca^{2+} concentration, myosin is inactivated, and its interaction with actin in phasic muscle is abolished. In contrast, once Ca^{2+} concentrations decrease to resting levels, catch muscles enter the high-tension catch state, which is maintained for long periods. Twitchin, a giant myosin-associated protein, tethers together the thin and thick filaments through its phosphorylation sites [2] [3] [4]. The involvement of the thin filament-linked regulatory system in catch contraction remains unresolved.

In contrast to molluscan muscles, vertebrate striated muscles employ a thin filament-linked regulatory system. Troponin (Tn) is the regulator of skeletal muscle contraction. Tn is distributed on thin filaments and inhibits the interaction between actin and myosin. Tn consists of three subunits: troponin C (TnC), troponin I (TnI) and troponin T (TnT). Since Tn is present in mollusk muscles, we have been investigating if there is a thin filament-linked regulatory system of catch contraction.

The Japanese pearl oyster, *Pinctada fucata*, is one of the most important molluscan species in the pearl culture industry. A genome database of *P. fucata* has recently been released and all the major muscle protein genes have been registered [5] [6] [7]. Therefore, we have used *P. fucata* as a model system to elucidate the molluscan muscle regulatory system. We recently performed molecular characterization of TnC and TnI from *P. fucata*, and suggested that Tn may participate in the regulation of the phasic adductor muscle not in catch muscle, because they are predominantly expressed in the phasic muscle [8] [9] [10].

Mammalian smooth muscle exhibits tension maintenance, called latch, which is similar to catch contraction of molluscan smooth muscle [11] [12]. In the latch mechanism, calponin, a basic protein specific to smooth muscle, is involved [13] [14] [15] [16]. Calponin also resides in molluscan muscles [7] [17] [18] [19] [20] [21]. Molluscan calponin has been reported to inhibit actomyosin Mg-ATPase activity [17] [18]. For these reasons, calponin is likely involved in catch contraction in mollusks. However, available information on molluscan calponin is very limited.

We previously revealed that three calponin isoforms are expressed in *P. fucata*

(*Pifuc-CP-1, Pifuc-CP-2 and Pifuc-CP-3*) *by partial sequencing* [7]. In this study, the molecular characterization of *P. fucata* calponin isoforms was performed by conducting 5' rapid amplification of cDNA ends (RACE) to determine the full-length sequences of the three isoforms. In addition, the structural and tissue distribution analysis was performed. Furthermore, we found four more isoforms (*Pifuc-CP-4*, *Pifuc-CP-5*, *Pifuc-CP-6* and *Pifuc-CP-7*) using cDNA cloning.

2. Materials and Methods

2.1. Animal Samples

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

2.2. cDNA Cloning of *Pifuc-CP-1*, *Pifuc-CP-2* and *Pifuc-CP-3*

Total RNA was extracted from the phasic part of the adductor muscle using a conventional method [22]. Partial nucleotide sequences of *Pifuc-CP-1*, *Pifuc-CP-2* and *Pifuc-CP-3*, as determined by 3' RACE, were reported previously [7]. 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-CP-1*, *Pifuc-CP-2* and *Pifuc-CP-3.* For *Pifuc-CP-1*; we used 5'-TCGTATGTCCGAAATGTGAC-3' for synthesizing cDNA, 5'-ACGGCGCCAAAACTCATCCC-3' for the first PCR and 5'-ATT GACTTGCAAACTTATTA-3' for the second PCR. For *Pifuc-CP-2*, we used 5'-ATGTGGCTCCATTAAAAAGAG-3' for synthesizing cDNA, 5'-TCTTCCACC GGCCTAGATCC-3' for the first PCR and 5'-GTAGGAGAAGTTTCTTCGGT-3' for the second PCR. For *Pifuc-CP-3*, we used 5'-ATGTTGGACCATTATAGCTA-3' for synthesizing cDNA, 5'-TTTTCTACTGGTTTCGATCC-3' for the first PCR and 5'-GTAACTGAACTGGACTTGGT-3' for the second PCR. PCR was carried out using SapphireAmp Fast PCR Master Mix (TaKaRa Bio, Shiga, Japan) 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 the DDBJ/EMBL/GenBank (accession numbers LC490357, LC490358 and LC490359, respectively). The motif structures of Pifuc-CP-1, Pifuc-CP-2 and Pifuc-CP-3 were predicted by Pfam (https://pfam.xfam.org/).

2.3. Gene Expression Analysis of *Pifuc-CP-1*, *Pifuc-CP-2* and *Pifuc-CP-3* in Tissues

The gene expression patterns of *Pifuc-CP-1*, *Pifuc-CP-2* and *Pifuc-CP-3* in the catch and phasic muscles, gill, mantle and foot 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 the Universal Probe Library Assay Design Center (Roche Diagnostics, Mannheim, Germany) using the distinct nucleotide sequences between *Pifuc-CP-1*, *Pifuc-CP-2* and *Pifuc-CP-3*. For *Pifuc-CP-1*, the primers used were: 5'-CAAGAAGGTCATGGGGTGAT-3' (forward) and 5'-GACATTCCGGATTGACTTGC-3' (reverse), and the TaqMan probe #80 5'-TCTCCAGG-3'. For *Pifuc-CP-2*, the primers used were: 5'-CAT TGGAGCGGTGAGACATA-3' (forward) and 5'-CAAGGACTGCTTGTCGTAA TCA-3' (reverse), and the TaqMan probe #122 5'-TCAGGGCA-3'. For *Pifuc-CP-3*, the primers used were: 5'-AAGGAAAGAGCTTTATCAACTTGC-3' (forward) and 5'-TCATACCCTTCTGCGATGC-3' (reverse), and the TaqMan probe #164 5'-GCAACCAG-3'. *P. fucata β-actin* (AF378128) was used as an internal standard. For *β-actin*, the primers used were 5'-TCGTTCCTCGGAATG GAA-3' (forward) and 5'-TCGACATCGCATTTGAGAAT-3' (reverse), and the TaqMan probe #151 5'-GCTGGAAT-3'. The PCR reaction was performed using Eagle Taq Master Mix with ROX (Roche Diagnostics).

2.4. Protein Expression Analysis of Calponin in Tissues of *P. fucata*

Protein expression patterns of calponin in tissues of *P. fucata* were analyzed by immunoblotting using the anti-Yesso scallop calponin antiserum prepared in our previous study [23]. Catch and phasic muscles, gill, mantle and foot were homogenized in phosphate-buffered saline and subjected to 10% SDS-PAGE, followed by electro-blotting onto a polyvinylidene difluoride membrane. After blocking, the membrane was hybridized with an anti-Yesso scallop calponin antiserum. Horseradish peroxidase-conjugated goat anti-rabbit IgG was used as the secondary antibody. Detection was carried out with 0.2 mg/ml 3,3'-diaminobenzidine and 0.005% hydrogen peroxide in Tris-buffered saline.

2.5. cDNA Cloning of *P. fucata* Calponin Isoforms

Protein expression analysis revealed the possibility that other isoforms were expressed in *P. fucata* tissues. Thus, we carried out reverse transcriptase (RT)-PCR to obtain cDNA clones encoding calponin isoforms. cDNA was synthesized from total RNA of catch and phasic muscles with the 3'-Full RACE Core Set (TaKaRa Bio). Because *Pifuc-CP-1*, *Pifuc-CP-2* and *Pifuc-CP-3* have identical sequences of 633 nt from the 5'-end, which encodes the N-terminal region of the calponin homology (CH) domain, we postulated that all calponin isoforms share the same exon encoding the N-terminal region of the CH domain. Therefore, first, PCR was carried out to amplify DNA fragments from the common sequence of the 5' region to the poly-A tail. We designed 5'-ACATTTAGTCTGTCTATTTG-3' (CP-full-1F) and 5'-ATAAGGTTCCACTCAGCAGT-3' (CP-full-2F) as forward primers, based on the sequence of the upstream start codon. PCR was performed with CP-full-1F and a 3 Sites Adaptor Primer (reverse) included in the cDNA

synthesis kit above, and then nested PCR was performed with CP-full-2F and the same reverse primer using the first PCR products as templates. Polymerase KOD-Plus-Neo (Toyobo) was used in the first and nested PCR. The products of the nested PCR were used to amplify calponin isoform genes. Primers were designed to cover the open reading frames (ORF) of *Pifuc-CP-1*, *Pifuc-CP-2* and *Pifuc-CP-3*. The forward primer used was 5'-ATGGCTGAGCGTATGAAACC-3'. As *Pifuc-CP-1*, *Pifuc-CP-2* and *Pifuc-CP-3* have distinct sequences in the 3' region at their C-termini, we designed three reverse primers 5'-TCATCCGCCGC GGATATCGG-3' (from *Pifuc-CP-1*), 5'-TCATCCGGTGTACATAATCT-3' (from *Pifuc-CP-2*) and 5'-CTACATATCATTCTCTGCTT-3' (from *Pifuc-CP-3*). PCR was carried out using SapphireAmp Fast PCR Master Mix with the forward primer and each of the reverse primers. 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 the DDBJ/EMBL/GenBank with accession numbers LC490360 (*Pifuc-CP-4*), LC490361 (*Pifuc-CP-5*), LC490362 (*Pifuc-CP-6*) and LC490363 (*Pifuc-CP-7*). The domain architectures of Pifuc-CP-4, Pifuc-CP-5, Pifuc-CP-6 and Pifuc-CP-7 were predicted by Pfam (https://pfam.xfam.org/). Deduced amino acid sequences of all Pifuc-CP isoforms were compared using the ClustalW algorithm.

2.6. Phylogenetic Analysis of Calponin

Phylogenetic analysis was carried out using the primary structures of calponin from various species following sequence alignment using the ClustalW algorithm [24]. The sequences used were: human, *Homo sapiens* (S80560); chicken, *Gallus gallus* (M63559); zebrafish, *Danio rerio* (BC059802); fruit fly, *Drosophila melanogaster* (AF217286); kissing bug, *Triatoma infestans* (EF638975); Mediterranean mussel, *Mytilus galloprovincialis* (AB052656); abalone, *Haliotis diversicolor* (EF542809); blood fluke, *Schistosoma mansoni* (HE601630); Asian tapeworm, *Taenia asiatica* (EF201933); pig roundwarm, *Ascaris suum* (JI170148); and filaria, *Onchocerca volvulus* (U01099).

3. Results

3.1. Molecular Characteristics of *Pifuc-CP-1*, *Pifuc-CP-2* and *Pifuc-CP-3*

We used 5' RACE to determine 799 bp of new sequence including the 5'-untranslated region (UTR) of *Pifuc-CP-1*, 532 bp of new sequence of *Pifuc-CP-2* and *Pifuc-CP-3*. Combined with known sequences, the full nucleotide sequences of *Pifuc-CP-1*, *Pifuc-CP-2* and *Pifuc-CP-3* were determined (**Figures 1-3**). The 5'-UTR, ORF and 3'-UTR of the *Pifuc-CP-1* gene are 214, 975 and 1067 bp in length, respectively (**Figure 1**). The 5'-UTR, ORF and 3'-UTR of the *Pifuc-CP-2* gene are 214, 909, and 627 bp in length, respectively (**Figure 2**), whereas the same regions in the *Pifuc-CP-3* gene are 214, 1197 and 668 bp in

(a)

```
ACATTTAGTCTGTCTATTTGATAAGGTTCCACTCAGCAGTGCGGTGAAGCGCCGGCGCAG   60
ACAGCCAAATTCACTTTTAGGGGATTGCGATAAGATTCTCTTCGGATTTCTCCAACAGGG  120
TCCATTCCGCTTGAAGGGAGGCGAATTTATTCTTAAGTTTTAGACACTTTTATTCTGTGC  180
TTCGTCTTTTTAGTTAGAACACAAATTTTTCAAAATGGCTGAGCGTATGAAACCAATGGG  240
                                      M  A  E  R  M  K  P  M  G     9
GATGGACCGTGCACTTTCGTCAAAGATGGGCGCTAAATATGACCCTGCTGCTGAGGCGGA  300
  M  D  R  A  L  S  S  K  M  G  A  K  Y  D  P  A  A  E  A  E    29
AGTCAGGAACTGGATAAAACAGCTGATCGGCGAGGACATCGGTGACGGCGCCATGAACGT  360
  V  R  N  W  I  K  Q  L  I  G  E  D  I  G  D  G  A  M  N  V    49
GGAGAAAAACCTCAGAGATGGAGTCATTCTTATCAAACTTTTACAAAAACTATACGAAGG  420
  E  K  N  L  R  D  G  V  I  L  I  K  L  L  Q  K  L  Y  E  G    69
GACCTCCAACCTACCCAACAAGGCCAGAACCATGAAACTCTCATACAACACAATGAATGC  480
  T  S  N  L  P  N  K  A  R  T  M  K  L  S  Y  N  T  M  N  A    89
TCCTTTCAAACAGATGGAGAATATAGAAGTTTTCCTTAAAGGTGCAAACGCCTATGGTGT  540
  P  F  K  Q  M  E  N  I  E  V  F  L  K  G  A  N  A  Y  G  V   109
CCCCCTTAACAGTTTGTTCCAGACAGTTGACCTGTATGAAGGCAGAAATATGGCCATGGT  600
  P  L  N  S  L  F  Q  T  V  D  L  Y  E  G  R  N  M  A  M  V   129
CGTAGCCACTATTCTACAGCTGGGTACTGAGGCTCAAAGGAACGGTCATGAGTCGGTTGA  660
  V  A  T  I  L  Q  L  G  T  E  A  Q  R  N  G  H  E  S  V  E   149
AAAGGGCTTAATCTGTGGGGCAAAACCTGTCGAGAAACATGTGGTACATTTTACCGAGGA  720
  K  G  L  I  C  G  A  K  P  V  E  K  H  V  V  H  F  T  E  E   169
ACAAAAGAGGGCAGGTCAAGGCATCATCGGATTACAAGCTGGAACAAACAAATGCGCAAG  780
  Q  K  R  A  G  Q  G  I  I  G  L  Q  A  G  T  N  K  C  A  S   189
TCAAAAAGGAATGAAAATAGGAGGAGCACGCCACATTGCAGACATCAAAGCAGACGACAT  840
  Q  K  G  M  K  I  G  G  A  R  H  I  A  D  I  K  A  D  D  M   209
GAATCGTGAGGGCCAAGGTATACTTTCTGCTCAAGCAGGCACCAATAAATTTGCTTCCCA  900
  N  R  E  G  Q  G  I  L  S  A  Q  A  G  T  N  K  F  A  S  Q   229
GAAGGGCATGTCGATCGGATCAGTCAGGCATATCGCTGATATTAGGGCAGACGACATGTC  960
  K  G  M  S  I  G  S  V  R  H  I  A  D  I  R  A  D  D  M  S   249
TCAAGAAGGTCACGGGGTCATTGGTCTACAAGCCGGAAGTAATAAGTTTGCAAGTCAATC 1020
  Q  E  G  H  G  V  I  G  L  Q  A  G  S  N  K  F  A  S  Q  S   269
GGGGATGAGTTTTGGCGCCGTTCGTCACATTTCGGACATACGAGCTGACGACATGTCACA 1080
  G  M  S  F  G  A  V  R  H  I  S  D  I  R  A  D  D  M  S  Q   289
AGAAGGTCATGGGGTGATTGGTCTCCAGGCCGGAAGTAACAAGGGCGCAAGTCAATCCGG 1140
  E  G  H  G  V  I  G  L  Q  A  G  S  N  K  G  A  S  Q  S  G   309
AATGTCATTCGGAAGTGTTCGCCATATTGCCGATATCCGCGGCGGATGAATACGACCAAA 1200
  M  S  F  G  S  V  R  H  I  A  D  I  R  G  G  *               324
GCAGGCGCCGGAACCATTGGACTGCAGTACGGCAGTAACGAAGGAGCGAGTCAATCAGGC 1260
ATGACTGGAGTCGGCACAACGCGCGGTGTGGATGACATGAAAAATGACGAGCTCGCCGAA 1320
GCTTCCGGGGCGATGGATTGAAGTGATTACAATGTATAGGATGGGTCATTAGGATGAAAA 1380
GTACCAAAATCGAGTTAGCTTTATCCAATGTTTGTCGTGTAATGAAAGACAAATCCGAAA 1440
AAAATAGTTATATGTTTGAAAGATAGTTACATGTACAATAACAACAGAGAAAGAAAAATT 1500
GAATTCAAAGAAATCGTTTCACGAGACGCGTCATTAAACACATGAATAAAAATTTTGAAA 1560
TTATAGAAAAATATTTAAGAAAAACTGCAAGAAAAGTGTTGATGGATATTATTTAGATTC 1620
TTCAAAAACTCTAATTTATCTTTTTTAAAGGTTTTAACCTTCATGATTTTAACACTTCAT 1680
TCCAAAAACTCTTTCATTTCACATCATTTCACTTGTCTTGTTGTGTCAGAATCATCATGT 1740
ACGTGTTTCATGTAAAGCGTTTCTAGATTATTTACTTAGCAGCTTTGTTACGAATATTTC 1800
GTATTTACATTTAAACTTACTGAAAATATCAGCTGTAAAGAAAAATATACAACAGGCGAT 1860
CACAAAAGGAAAACAGAAACAAAGTTTAGATACTAGTAAATGGAAAGTTTTGAGAAGAAA 1920
GGATCAAACCATATTTTTGATTTACAGAAAGACTCATGAGTACGCAGAAATTTGAAATAA 1980
TAAAATGATAAAAATCAGAATATGGCCACCTCAATCTTTATTTAAATCCTTCTTTTAGTG 2040
TGCGAGGGAAGAGACCGAATAGTTGAATACTTGTGATTTACAAATTAAATAAATGTATTT 2100
TCTCTAAGGTTGATATTTCCATCAAGGTTTATCGATCTGCTTTTAGTTAGATTATCATGA 2160
TGTTCTGAATTTCTAACACGGTAGTTTCTATGGTGAATTCTCATCCAGTCCATGTTTTGA 2220
ATAAAACAGCTTTGATTCGTGAAGAAAAAAAAAAAA                          2256
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(b)

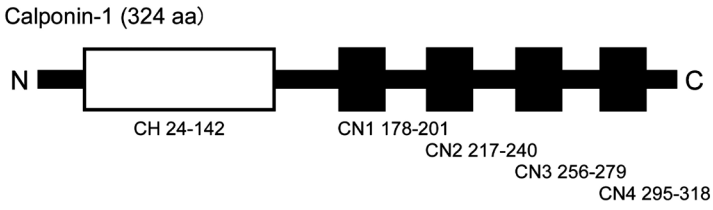

Figure 1. Molecular characteristics of calponin-1 of the Japanese pearl oyster, *Pinctada fucata* (Pifuc-CP-1). (a) Nucleotide and deduced amino acid sequences. Numbers at the right of the sequences represent nucleotide and amino acid residues from the 5'-end and N-terminus, respectively. The region of the calponin homology (CH) domain is shaded. Bold letters represent the calponin (CN) domain sequences. An asterisk represents the termination codon. (b) Pifuc-CP-1 motif structure predicted by Pfam. White and black boxes represent CH and CN domains, respectively. Numbers under the black boxes represent the amino acid residues that constitute each CN domain.

(a)

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ACATTTAGTCTGTCTATTTGATAAGGTTCCACTCAGCAGTGCGGTGAAGCGCCGGCGCAG   60
ACAGCCAAATTCACTTTTAGGGGATTGCGATAAGATTCTCTTCGGATTTCTCCAACAGGG  120
TCCATTCCGCTTGAAGGGAGGCGAATTTATTCTTAAGTTTTAGACACTTTTATTCTGTGC  180
TTCGTCTTTTTAGTTAGAACACAAATTTTTCAAAATGGCTGAGCGTATGAAACCAATGGG  240
                                    M  A  E  R  M  K  P  M  G     9
GATGGACCGTGCACTTTCGTCAAAGATGGGCGCTAAATATGACCCTGCTGCTGAGGCGGA  300
  M  D  R  A  L  S  S  K  M  G  A  K  Y  D  P  A  A  E  A  E    29
AGTCAGGAACTGGATAAAACAGCTGATCGGCGAGGACATCGGTGACGGCGCCATGAACGT  360
  V  R  N  W  I  K  Q  L  I  G  E  D  I  G  D  G  A  M  N  V    49
GGAGAAAAACCTCAGAGATGGAGTCATTCTTATCAAACTTTTACAAAAACTATACGAAGG  420
  E  K  N  L  R  D  G  V  I  L  I  K  L  L  Q  K  L  Y  E  G    69
GACCTCCAACCTACCCAACAAGGCCAGAACCATGAAACTCTCATACAACACAATGAATGC  480
  T  S  N  L  P  N  K  A  R  T  M  K  L  S  Y  N  T  M  N  A    89
TCCTTTCAAACAGATGGAGAATATAGAAGTTTTCCTTAAAGGTGCAAACGCCTATGGTGT  540
  P  F  K  Q  M  E  N  I  E  V  F  L  K  G  A  N  A  Y  G  V   109
CCCCCTTAACAGTTTGTTCCAGACAGTTGACCTGTATGAAGGCAGAAATATGGCCATGGT  600
  P  L  N  S  L  F  Q  T  V  D  L  Y  E  G  R  N  M  A  M  V   129
CGTAGCCACTATTCTACAGCTGGGTACAGAGGCTCAGAGGAACTCTTTTAATGGAGCCAC  660
  V  A  T  I  L  Q  L  G  T  E  A  Q  R  N  S  F  N  G  A  T   149
ATGTGGATCTAGGCCGGTGGAAGAAAACCGAAGAAACTTCTCCTACGAACGACTGAAGTC  720
  C  G  S  R  P  V  E  E  N  R  R  N  F  S  Y  E  R  L  K  S   169
CTCCCATGGAAGTATCGGTTTACAGTCAGGAACCAACAAATTTGACTCCCAGAAGGGCAT  780
  S  H  G  S  I  G  L  Q  S  G  T  N  K  F  D  S  Q  K  G  M   189
GACAGCCATGGGGGCCGTGCGTCACATATCCGACATCCGAGCGGATAAGTTCGACCAGAC  840
  T  A  M  G  A  V  R  H  I  S  D  I  R  A  D  K  F  D  Q  T   209
GTCCGAGGGCTGTATCACCCTGCAGGCCGGTACCAACAAGTTTGCCAGCCAGAAAGGAAT  900
  S  E  G  C  I  T  L  Q  A  G  T  N  K  F  A  S  Q  K  G  M   229
GACGGCCATTGGAGCGGTGAGACATATCTCGGACATCAGGGCAGATGATTACGACAAGCA  960
  T  A  I  G  A  V  R  H  I  S  D  I  R  A  D  D  Y  D  K  Q   249
GTCCTTGTGTGACATCAATCTTCAGTCCGGTACTAACAAATTTGACAGTCAGAAGGGAAT 1020
  S  L  C  D  I  N  L  Q  S  G  T  N  K  F  D  S  Q  K  G  M   269
GCGAGGATTTGGCGCGGTCCGCCATATTTCTGACATCACAGCAGACGACTTGTCACGTGA 1080
  R  G  F  G  A  V  R  H  I  S  D  I  T  A  D  D  L  S  R  E   289
GGCGCGCAGTACGATCTCACCACAGATTATGTACACCGGATGAGACAGTCAGGCGGGAAT 1140
  A  R  S  T  I  S  P  Q  I  M  Y  T  G  *                    302
GAGAGGATTTGGCGCCGCCCGTCACGTTTCAGATATCATAGTACCCGACCTTGCTGAGGA 1200
GATGGCACAAAAGACTGGATATAAACCTGCAAGGCGCGTGGATTCTGGCGCTAATCTGGC 1260
AGAGGCTCAGCCGGAAGAGGAAGCAGAAGATAATTAGAATCAACATCATTTGAACGCTTG 1320
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AAATCTTTAATTTTACGCGATAAGTGACGATTCATGTGTAGGCAGGGACACTAATTAGTA 1440
TTGTATAAACAAAGACACTCCTTTATTTTCTTTTTTTCTGTGATATTAGGAAGTTCCTTT 1500
ATTTTAGTCTCTTCACTTTGAAATATGGTCGCTTATTTATTTTATAAACAAAAACTATCA 1560
ACTATCAATAACATATATATAATTCAGAGAGCTTTGATGAACCCTAGTATATTGTAAATC 1620
AAATATTTTTGGATTATATGTGAAAATGAACTTTTTAAATTCTTCACCTATATGTAATGC 1680
TTGTTTTAACCAATCAATATAGAAATAAACTTAAATACTTTTAAAAAAAAAAAAAAAAAC 1740
CCAAAAAAAA                                                     1750
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(b)

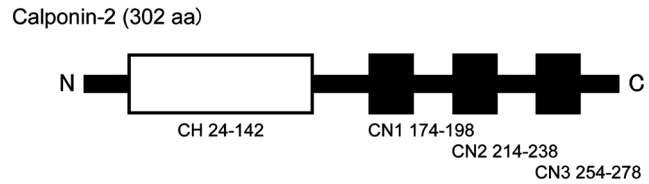

Figure 2. Molecular characteristics of calponin-2 of the Japanese pearl oyster, *Pinctada fucata* (Pifuc-CP-2). See legend of **Figure 1**.

length, respectively (**Figure 3**). Pifuc-CP-1 consists of 324 amino acids (aa) with a molecular mass (Mw) of 34.7 kDa and an isoelectric point (pI) of 9.40. Pifuc-CP-2 is 303 aa in length with a Mw of 33.3 kDa and a pI of 9.30. Pifuc-CP-3 is 398 aa in length with a Mw of 43.8 kDa and a pI of 8.55. Pfam prediction indicates that the three proteins share an identical CH domain but have different numbers of CN domain repeats (**Figures 1-3**). There are five CN domains in Pifuc-CP-1, three in Pifuc-CP-2 and six in Pifuc-CP-3.

3.2. Gene and Protein Expression Analyses of Pifuc-CP-1, Pifuc-CP-2 and Pifuc-CP-3

Gene expression analysis showed that the *Pifuc-CP-1*, *Pifuc-CP-2* and *Pifuc-CP-3*

genes were expressed predominantly in adductor phasic muscle, whereas relatively weaker expression was detected in catch muscle (Figure 4). Gene expression of the three genes was barely detectable in gill, mantle and foot. Immunoblotting analysis of the protein expression profiles in *P. fucata* tissues detected multiple proteins

(a)

```
ACATTTAGTCTGTCTATTTGATAAGGTTCCACTCAGCAGTGCGGTGAAGCGCCGGCGCAG   60
ACAGCCAAATTCACTTTTAGGGGATTGCGATAAGATTCTCTTCGGATTTCTCCAACAGGG  120
TCCATTCCGCTTGAAGGGAGGCGAATTTATTCTTAAGTTTTAGACACTTTTATTCTGTGC  180
TTCGTCTTTTTAGTTAGAACACAATTTTTTCAAAATGGCTGAGCGTATGAAACCAATGGG  240
                                        M  A  E  R  M  K  P  M  G     9
GATGGACCGTGCACTTTCGTCAAAGATGGGCGCTAAATATGACCCTGCTGCTGAGGCGGA  300
  M  D  R  A  L  S  S  K  M  G  A  K  Y  D  P  A  A  E  A  E    29
AGTCAGGAACTGGATAAAACAGCTGATCGGCGAGGACATCGGTGACGGCGCCATGAACGT  360
  V  R  N  W  I  K  Q  L  I  G  E  D  I  G  D  G  A  M  N  V    49
GGAGAAAAACCTCAGAGATGGAGTCATTCTTATCAAACTTTTACAAAAACTATACGAAGG  420
  E  K  N  L  R  D  G  V  I  L  I  K  L  L  Q  K  L  Y  E  G    69
GACCTCCAACCTACCCAACAAGGCCAGAACCATGAAACTCTCATACAACACAATGAATGC  480
  T  S  N  L  P  N  K  A  R  T  M  K  L  S  Y  N  T  M  N  A    89
TCCTTTCAAACAGATGGAGAATATAGAAGTTTTCCTTAAAGGTGCAAACGCCTACGGTGT  540
  P  F  K  Q  M  E  N  I  E  V  F  L  K  G  A  N  A  Y  G  V   109
CCCCCTTAACAGTTTGTTCCAGACAGTTGACCTGTATGAAGGCAGAAATATGGCCATGGT  600
  P  L  N  S  L  F  Q  T  V  D  L  Y  E  G  R  N  M  A  M  V   129
CGTAGCCACTATTCTACAGTTGGGTACTGAGGCCCAGAGAAATAGCTATAATGGTCCAAC  660
  V  A  T  I  L  Q  L  G  T  E  A  Q  R  N  S  Y  N  G  P  T   149
ATGTGGATCGAAACCAGTAGAAAAACACCAAGTCCAGTTCAGTTACGAGCAACTTAAAAA  720
  C  G  S  K  P  V  E  K  H  Q  V  Q  F  S  Y  E  Q  L  K  N   169
TTCCCATGGTGTTATTGGTTTACAGTCGGGAACCAACAAATTTGCATCTCAAAAGGGCAT  780
  S  H  G  V  I  G  L  Q  S  G  T  N  K  F  A  S  Q  K  G  M   189
GCGCGGAGGTTTTGGTGCAGTACGTCACATTTCTGACATCAGGGCAGATGACCTTTCCAG  840
  R  G  G  F  G  A  V  R  H  I  S  D  I  R  A  D  D  L  S  R   209
AGATGGCCAAGATAACATAGGTCTTCAAGCTGGATCAAACAAGTTTGCCAGTCAGAAGGG  900
  D  G  Q  D  N  I  G  L  Q  A  G  S  N  K  F  A  S  Q  K  G   229
CATGACGGGATTCGGCGCTGTCCGCCATATTGCTGACATCAGAGCAGATGATTTTAACAA  960
  M  T  G  F  G  A  V  R  H  I  A  D  I  R  A  D  D  F  N  K   249
GGAGTCGTCGACAGACATCAGTTTACAGTCGGGTACCAACAAATTCGCCTCCCAGAAAGG 1020
  E  S  S  T  D  I  S  L  Q  S  G  T  N  K  F  A  S  Q  K  G   269
AATGCGTGGTGGTTTCGGCGCCGTCCGCCATATTTCGGATATCCGCGCAGATCAGTATTC 1080
  M  R  G  G  F  G  A  V  R  H  I  S  D  I  R  A  D  Q  Y  S   289
AGAAGAAGGAAAGAGCTTTATCAACTTGCAAGCCGGAAGCAACCAGTTTGCATCGCAGAA 1140
  E  E  G  K  S  F  I  N  L  Q  A  G  S  N  Q  F  A  S  Q  K   309
GGGTATGACAGGTTTCGGAGCTGTGCGTCACATTGCTGATATCAGGGCCGATGATTTAGA 1200
  G  M  T  G  F  G  A  V  R  H  I  A  D  I  R  A  D  D  L  D   329
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  R  E  A  A  S  T  V  S  L  Q  Y  G  T  N  Q  F  D  S  Q  A   349
AGGCATGAGAGGTTTTGGCGCGCAACGTCACGTGTCTGATATCAAAGTCAATGATCTTGC 1320
  G  M  R  G  F  G  A  Q  R  H  V  S  D  I  K  V  N  D  L  A   369
TGAGCAGCTCAGAATGCAGCAAATGGGTGTAACACCTCAACAGTACACACAGATCAAGAG 1380
  E  Q  L  R  M  Q  Q  M  G  V  T  P  Q  Q  Y  T  Q  I  K  R   389
GGAAGAAGAAGAAGCAGAGAATGATATGTAGGCCAATTTGGACAACATCTGACTCTATCT 1440
  E  E  E  E  A  E  N  D  M  *                                  398
GTTCACTTTGCTTTTAGAAACATCTGAACTTGAAACAAACGAACAATCAAGACGCTGTAA 1500
GATCAAAGATACGAAACAAAGAAAACCATGGTGATATTTTTTCGCGATTTTCCTAGGAAC 1560
TGAAACATCGAAAAAGAAGAACAAAAAAATCGACGTCGTCTAGTGAAGTTTACGGATATC 1620
CGAGTCCCCCGTTCTCTGCTCACATTTTATCTTCATGCTTTAAAGTAGGGCTCATTCATT 1680
CGGAGAATCAGACTCTTGCTTTCCTCTCTCTGTCTGCGTACGCTGTCGCTAAGCTTTCCC 1740
CGTTCGGACACTACAGACCAAAATTCTTGTATTCATTTTGCTTTCGATACTCCTCGGATT 1800
GTTACCATATCTAAGGGCCCTTATCAAACAAACCGGAACCGGAAATATCATATAAGACTG 1860
AACATAGATGTGTAAATTCTTTACGCTGCATTTAATGGAATATTTTTATTTTCATATTTT 1920
GATATGTAAAAATTGGATCGTAGACTCATGGATAAGTTATTTATTTTATAATCTATGAAT 1980
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TGTTGTGAAATAAACATTTTAAAAAAAAAAAAAAAAAAA                       2079
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(b)

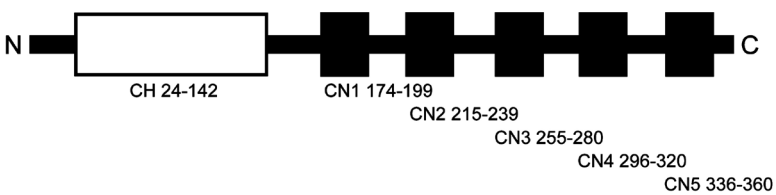

Figure 3. Molecular characteristics of calponin-3 of the Japanese pearl oyster, *Pinctada fucata* (Pi-fuc-CP-3). See legend of Figure 1.

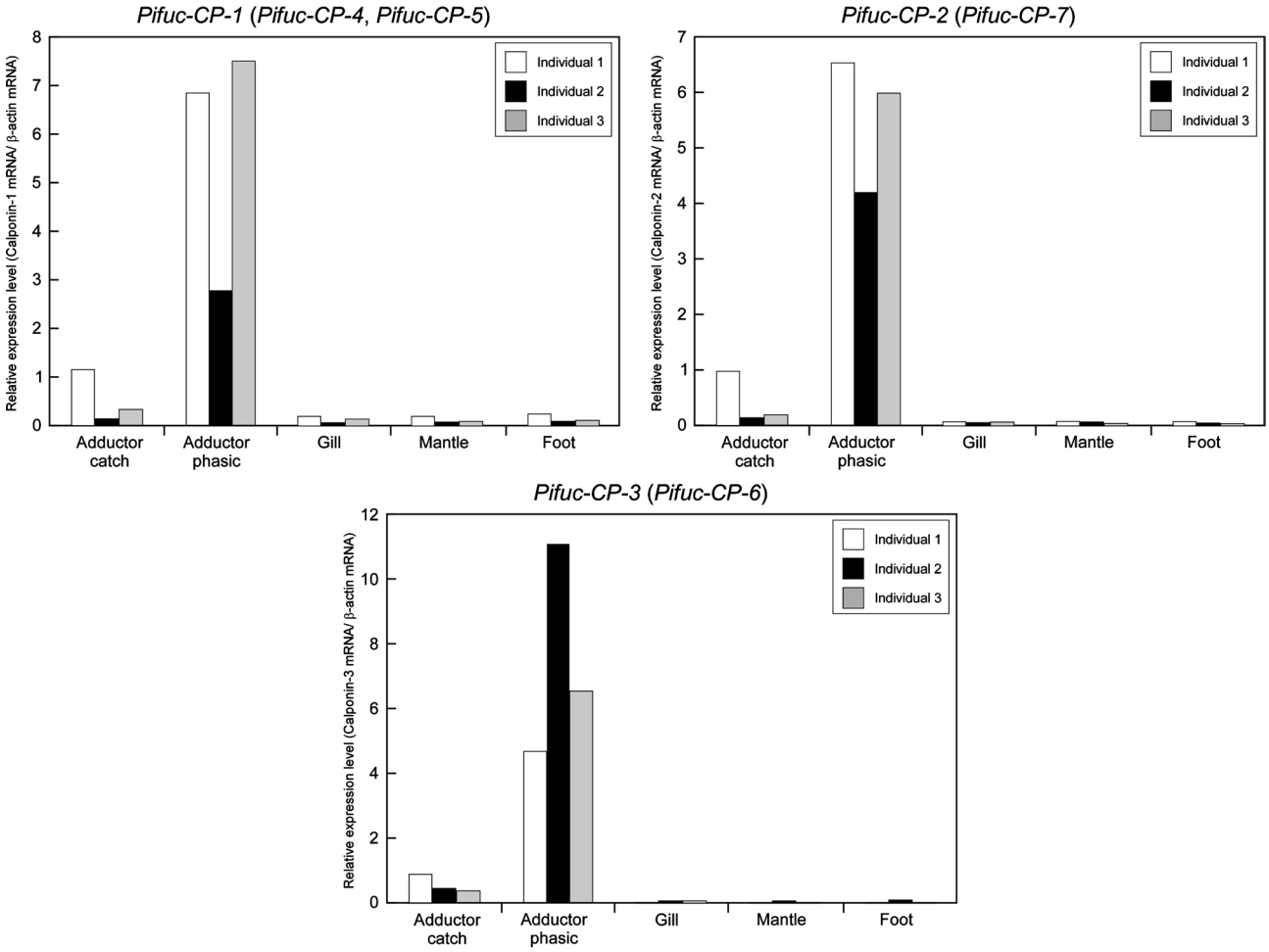

Figure 4. Gene expression patterns of calponin isoforms in *Pinctada fucata* tissues. Quantitative real-time PCR analysis was performed to examine calponin gene expression in *P. fucata* adductor catch muscle, adductor phasic muscle, gill, mantle and foot. The data shown are representative of three independent experiments. The *y*-axis indicates the relative calponin expression levels using *β-actin* as an internal standard. Left panel, *Pifuc-CP-1* including *Pifuc-CP-4* and *Pifuc-CP-5*; middle panel, *Pifuc-CP-2* including *Pifuc-CP-7*; right panel, *Pifuc-CP-3* including *Pifuc-CP-6*.

in all tissues (Figure 5). SDS-PAGE patterns of the mantle and foot tissues indicated that they contain muscle cells because their electrophoretic patterns were similar to those of the catch and phasic muscles, which consist of muscle proteins such as myosin, paramyosin and actin. Therefore, detection of calponin in the mantle and foot tissues was anticipated. Additionally, calponin was detected in the gill, of which SDS-PAGE patterns were dissimilar to those of the other tissues, indicating that Pifuc-CP might be distributed in non-muscular tissues. Multiple bands were detected in all lanes of the immunoblotting analysis, suggesting that there are calponin isoforms besides Pifuc-CP-1, Pifuc-CP-2 and Pifuc-CP-3. We then carried out cDNA cloning to identify other Pifuc-CP isoforms.

3.3. Molecular Characteristics of *Pifuc-CP-4*, *Pifuc-CP-5*, *Pifuc-CP-6* and *Pifuc-CP-7*

cDNA cloning of *P. fucata* calponin isoforms gave four more isoforms, *Pifuc-CP-4*,

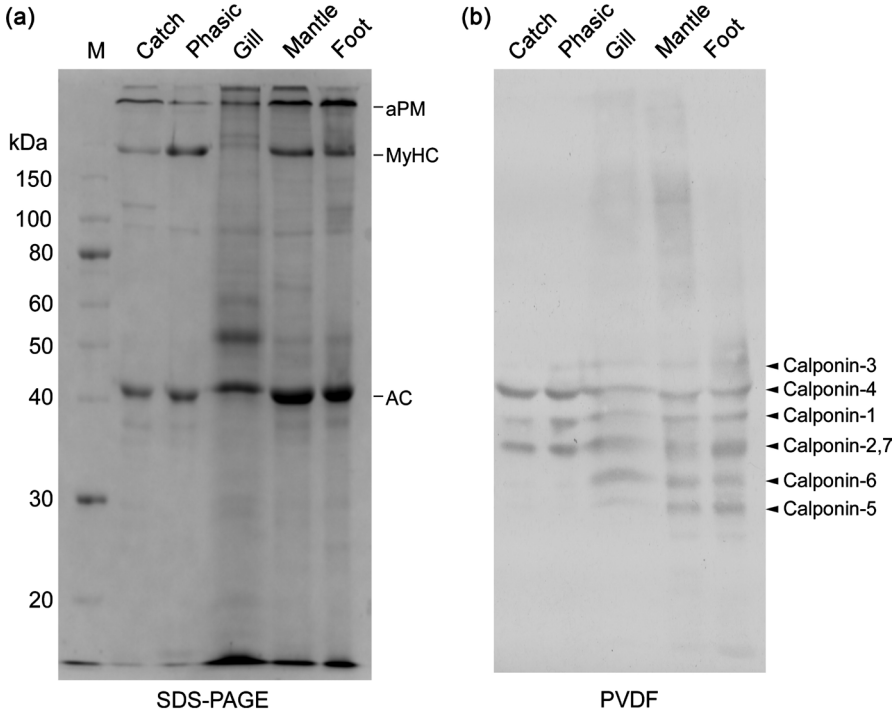

Figure 5. Protein expression patterns of calponin in *Pinctada fucata* tissues. Immunoblotting analysis was performed to examine calponin in *P. fucata* adductor catch muscle, adductor phasic muscle, gill, mantle and foot. (A) SDS-PAGE patterns of each tissue homogenate, showing aggregated paramyosin (aPM), myosin heavy chain (MyHC) and actin (AC). M: molecular weight markers. Positions of molecular weight standards are indicated (left). (B) The polyvinylidene difluoride membrane reacted with the anti-Yesso scallop calponin antiserum. Black arrow-heads indicate calponin bands.

Pifuc-CP-5, Pifuc-CP-6 and Pifuc-CP-7 (**Figures 6-9**). Pifuc-CP-4 is 402 aa in length with a Mw of 42.8 kDa and a pI of 9.10. Pifuc-CP-5 is 285 aa in length with a Mw of 30.7 kDa and a pI of 9.45. Pifuc-CP-6 is 286 aa in length with a Mw of 31.1 kDa and a pI of 9.60. Pifuc-CP-7 is 302 aa in length with a Mw of 33.3 kDa and a pI of 9.10. Predicted structural motifs revealed that all isoforms have one CH domain and multiple repeats of the CN domain. Pifuc-CP-4 has six CN domains, whereas the other three isoforms have three CN domains.

Sequence alignment of the *P. fucata* calponin isoforms was carried out by ClustalW (**Figure 10**). Pifuc-CP-1, -2, -3 and -4 have identical CH domain sequences, whereas Pifuc-CP-5, -6 and -7 have identical CH domain sequences. The multiple repeats of the CN domains are not well conserved.

We tried tissue distribution analysis for the *Pifuc-CP-4*, *Pifuc-CP-5*, *Pifuc-CP-6* and *Pifuc-CP-7* genes, but there was no region specific to respective genes by nucleotide sequences. As the position of the primers and TaqMan probe for *Pifuc-CP-1* was shared by *Pifuc-CP-4* and *Pifuc-CP-5*, the gene expression of *Pifuc-CP-1* shown in **Figure 4** includes that of *Pifuc-CP-4* and *Pifuc-CP-5*. In the same way, the gene expression of *Pifuc-CP-2* includes that of *Pifuc-CP-7*, and the gene expression of *Pifuc-CP-3* includes that of *Pifuc-CP-6*.

Immunoblotting analysis revealed that calponin isoforms are expressed in

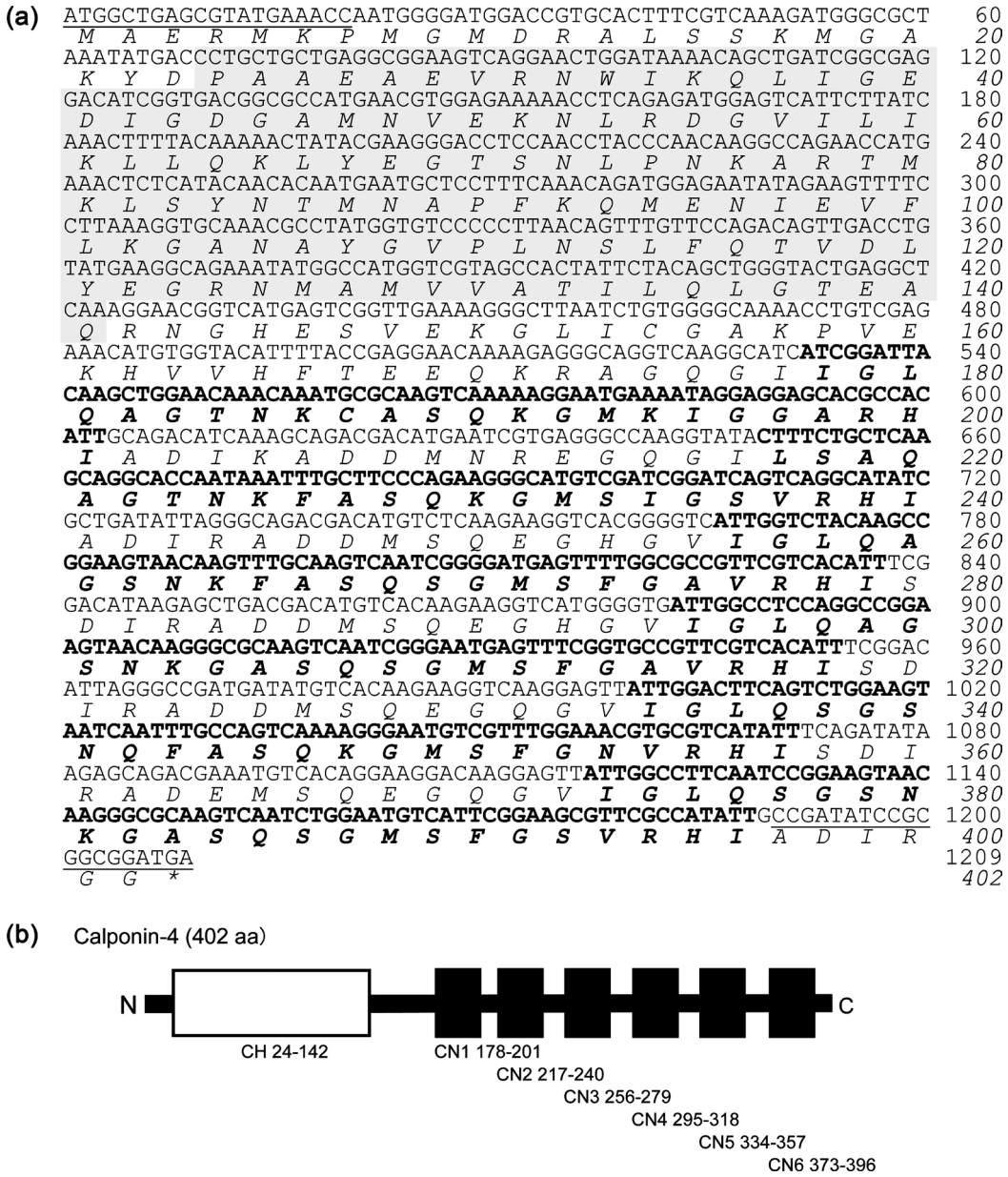

Figure 6. Molecular characteristics of calponin-4 of the Japanese pearl oyster, *Pinctada fucata* (Pifuc-CP-4). See legend of Figure 1. Underlined sequences at the 5'- and 3'-end of the nucleotide sequence represent the sequences of the primers used for RT-PCR.

each *P. fucata* tissue (Figure 5). Based on their calculated Mw from their primary structures, we identified bands corresponding to the respective isoforms. In catch and phasic adductor muscles, Pifuc-CP-4 and Pifuc-CP-2 (or 7) are mainly expressed, whereas Pifuc-CP-1 is weakly expressed. In gill, mantle and foot, all calponin isoforms appear to have similar expression levels.

3.4. Phylogenetic Analysis of Calponin

Phylogenetic tree analysis showed that Pifuc-CP isoforms are grouped into the same clade (Figure 11). Calponin from the Mediterranean mussel *Mytilus*

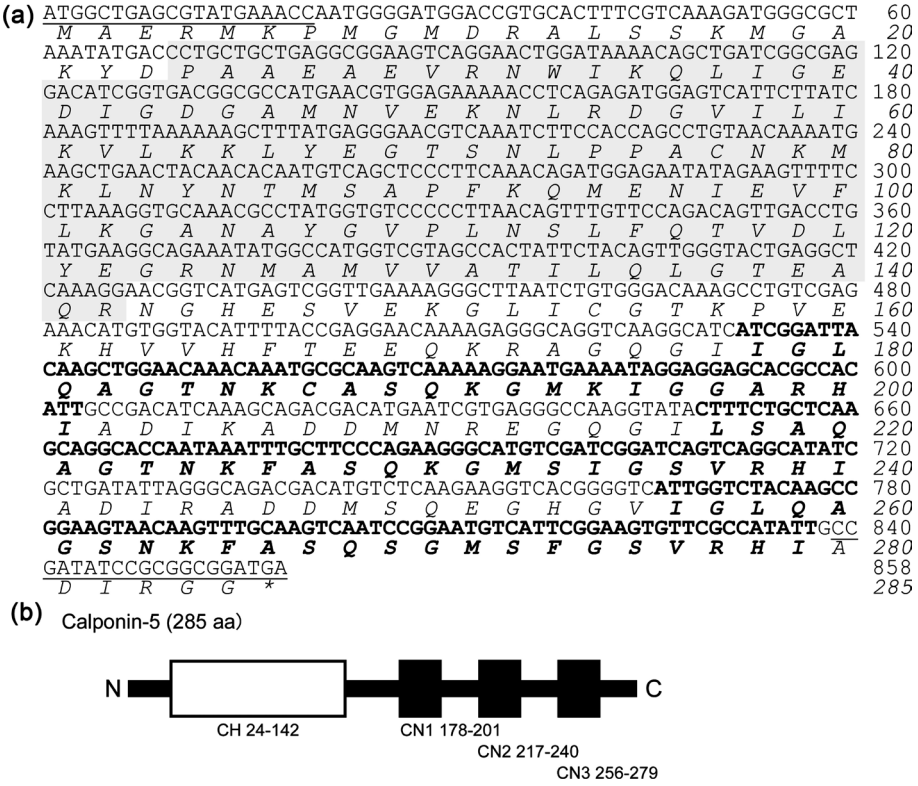

Figure 7. Molecular characteristics of calponin-5 of the Japanese pearl oyster, *Pinctada fucata* (Pifuc-CP-5). See legends of **Figure 1** and **Figure 6**.

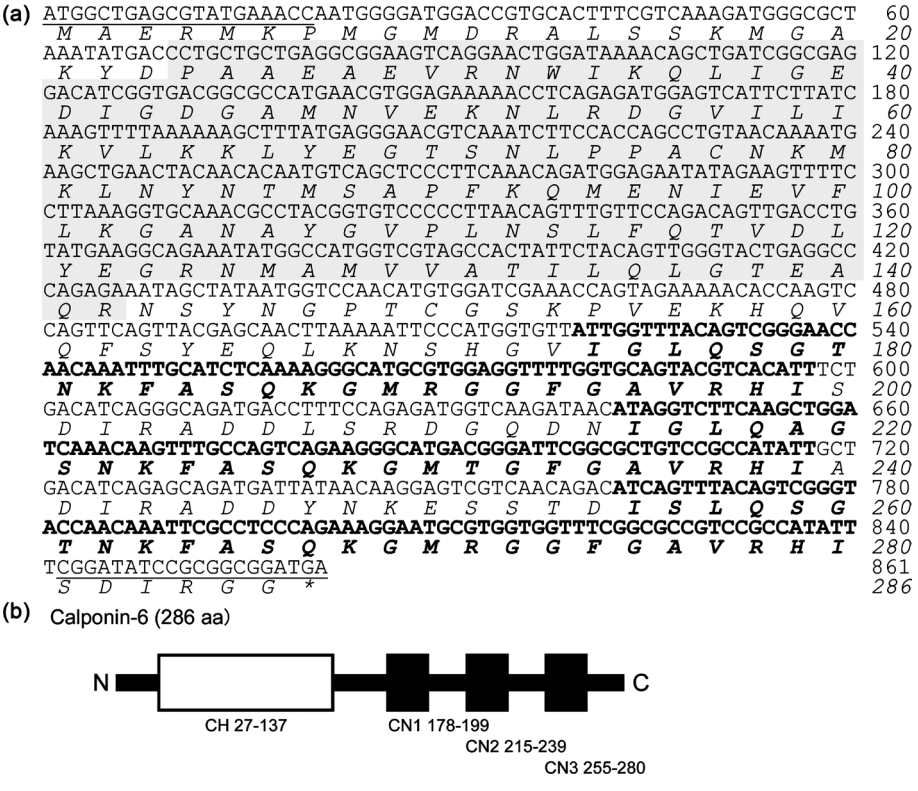

Figure 8. Molecular characteristics of calponin-6 of the Japanese pearl oyster, *Pinctada fucata* (Pifuc-CP-6). See legends of **Figure 1** and **Figure 6**.

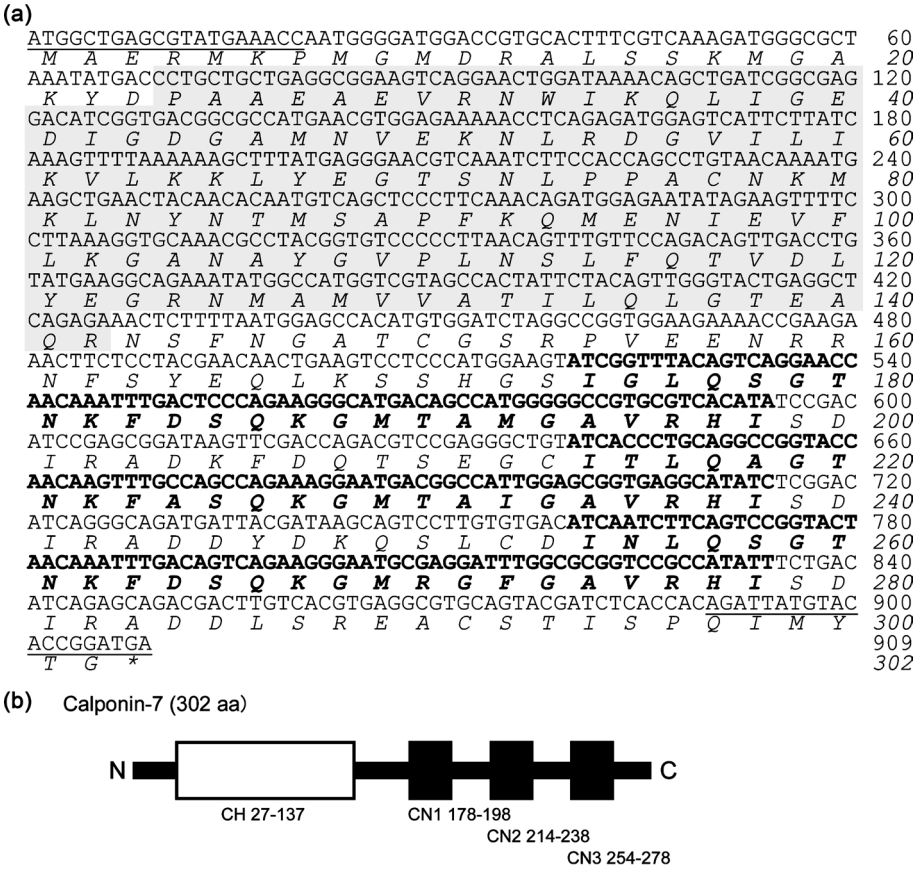

Figure 9. Molecular characteristics of calponin-6 of the Japanese pearl oyster, *Pinctada fucata* (Pifuc-CP-7). See legends of **Figure 1** and **Figure 6**.

galloprovincialis, which is found in catch muscle, were separated into the same clade, implying that bivalve calponin works in the same fashion in muscle contraction [17].

4. Discussion

In this study, we found that seven calponin isoforms (Pifuc-CP-1, Pifuc-CP-2, Pifuc-CP-3, Pifuc-CP-4, Pifuc-CP-5, Pifuc-CP-6 and Pifuc-CP-7) are expressed in the Japanese pearl oyster, *Pinctada fucata*. All isoforms are composed of a single CH domain and multiple repeats of the CN domain, which is in agreement with the domain architecture found in other species. Reported bivalve calponins have five calponin domains [21] [23] [25]. The CH domain is found widely throughout actin-binding proteins such as cytoskeletal and signal transduction proteins [26]. The CH domain is involved in actin binding in some actin-binding proteins. However, in calponin, the CH domain is not involved in actin-binding activity [27]. The CN domain repeats are essential for the actin-binding function of calponins and the strength of actin-binding correlates directly with the number of CN domains [28]. The number of CN domains in *Pinctada* calponin isoforms varies between three and six, and may reflect the different roles these isoforms play in muscle and non-muscle tissues. Molluscan

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Pifuc-CP-1  MAERMKPMGMDRALSSKMGAKYDPAAEAEVRNWIKQLIGEDIGDGAMNVEKNLRDGVILI 60
Pifuc-CP-2  ............................................................ 60
Pifuc-CP-3  ............................................................ 60
Pifuc-CP-4  ............................................................ 60
Pifuc-CP-5  ............................................................ 60
Pifuc-CP-6  ............................................................ 60
Pifuc-CP-7  ............................................................ 60

Pifuc-CP-1  KLLQKLYEGTSNLPNKARTMKLSYNTMNAPFKQMENIEVFLKGANAYGVPLNSLFQTVDL 120
Pifuc-CP-2  ............................................................ 120
Pifuc-CP-3  ............................................................ 120
Pifuc-CP-4  ............................................................ 120
Pifuc-CP-5  .V.K..........PACNK...N....S..................................... 120
Pifuc-CP-6  .V.K..........PACNK...N....S..................................... 120
Pifuc-CP-7  .V.K..........PACNK...N....S..................................... 120

Pifuc-CP-1  YEGRNMAMVVATILQLGTEAQRNGHESVEKGLICGAKPVEKHVVHFTEEQKRAGQGIIGL 180
Pifuc-CP-2  ......................SFN----.AT..SR...ENRRN.SY.RLKSSH.S... 176
Pifuc-CP-3  ......................SYN----.PT..S......Q.Q.SY..LKNSH.V... 176
Pifuc-CP-4  ..........................................................  180
Pifuc-CP-5  ..........................T.......................... 180
Pifuc-CP-6  ......................SYN----.PT..S......Q.Q.SY..LKNSH.V... 176
Pifuc-CP-7  ......................SFN----.AT..SR...ENRRN.SY..LKSSH.S... 176

Pifuc-CP-1  QAGTNKCASQKGMK--IGGARHIADIKADDMNREGQGILSAQAGTNKFASQKGMS-IGSV 237
Pifuc-CP-2  .S....FD.....T-AM.AV...S..R..KFDQTSE.CITL.............TA..A. 235
Pifuc-CP-3  .S....F......RGGF.AV...S..R...LS.D..DNIGL...S.........TGF.A. 236
Pifuc-CP-4  ..............--.....................................-.... 237
Pifuc-CP-5  ..............--.....................................-.... 237
Pifuc-CP-6  .S....F......RGGF.AV...S..R...LS.D..DNIGL...S.........TGF.A. 236
Pifuc-CP-7  .S....FD.....T-AM.AV...S..R..KFDQTSE.CITL.............TA..A. 235

Pifuc-CP-1  RHIADIRADDMSQEGHGVIGLQAGSNKFASQSGMSF--GAVRHISDIRAD---------- 285
Pifuc-CP-2  ...S......YDKQSLCD.N..S.T...D..K..RG-F.........T..---------- 284
Pifuc-CP-3  ..........FNK.SSTD.S..S.T......K..RGGF............QYSEEGKSFI 296
Pifuc-CP-4  ....................................--............---------- 285
Pifuc-CP-5  ................................---------------------------- 269
Pifuc-CP-6  ..........YNK.SSTD.S..S.T......K..RGGF..........GG---------- 286
Pifuc-CP-7  ...S......YDKQSLCD.N..S.T...D..K..RG-F............---------- 284

Pifuc-CP-1  ------------------------------DMSQEGHGVIGLQAGSNKGASQS------- 308
Pifuc-CP-2  ------------------------------.L.R.ARST.SP.IM--------------- 299
Pifuc-CP-3  NLQAGSNQFASQKGMTGFGAVRHIADIRAD.LDR.AASTVS..Y.T.QFD..AGMR---- 352
Pifuc-CP-4  ------------------------------.....................GMSFGAV 315
Pifuc-CP-5  ------------------------------------------------------------
Pifuc-CP-6  ------------------------------------------------------------
Pifuc-CP-7  ------------------------------.L.R.ACST.SP.IMI-------------- 300

Pifuc-CP-1  ------------------------------------------------------------
Pifuc-CP-2  ------------------------------------------------------------
Pifuc-CP-3  ----------------------------------------GFGAQRHVSDIKVNDLA 369
Pifuc-CP-4  RHISDIRADDMSQEGQGVIGLQSGSNQFASQKGMSFGNVRHISDIRADEMSQEGQGVIGL 375
Pifuc-CP-5  ------------------------------------------------------------
Pifuc-CP-6  ------------------------------------------------------------
Pifuc-CP-7  ------------------------------------------------------------

Pifuc-CP-1  -----------GMSFGSVRHIADIRGG-- 324
Pifuc-CP-2  --------------YTG------------ 302
Pifuc-CP-3  EQLRMQQMGVTPQQYTQIKREEEEAENDM 398
Pifuc-CP-4  QSGSNKGASQSGMSFGSVRHIADIRGG-- 402
Pifuc-CP-5  -----------GMSFGSVRHIADIRGG-- 285
Pifuc-CP-6  ----------------------------
Pifuc-CP-7  --------------YTG------------ 303
```

Figure 10. Comparison of calponin isoforms from the Japanese pearl oyster, *Pinctada fucata.* Identical residues to those of Pifuc-CP-1 are indicated by dots. Dashes are inserted to maximize the alignment. Numbers at the right of the sequences represent amino acid residues from the N-terminus. The sequences of the CH domains are shaded.

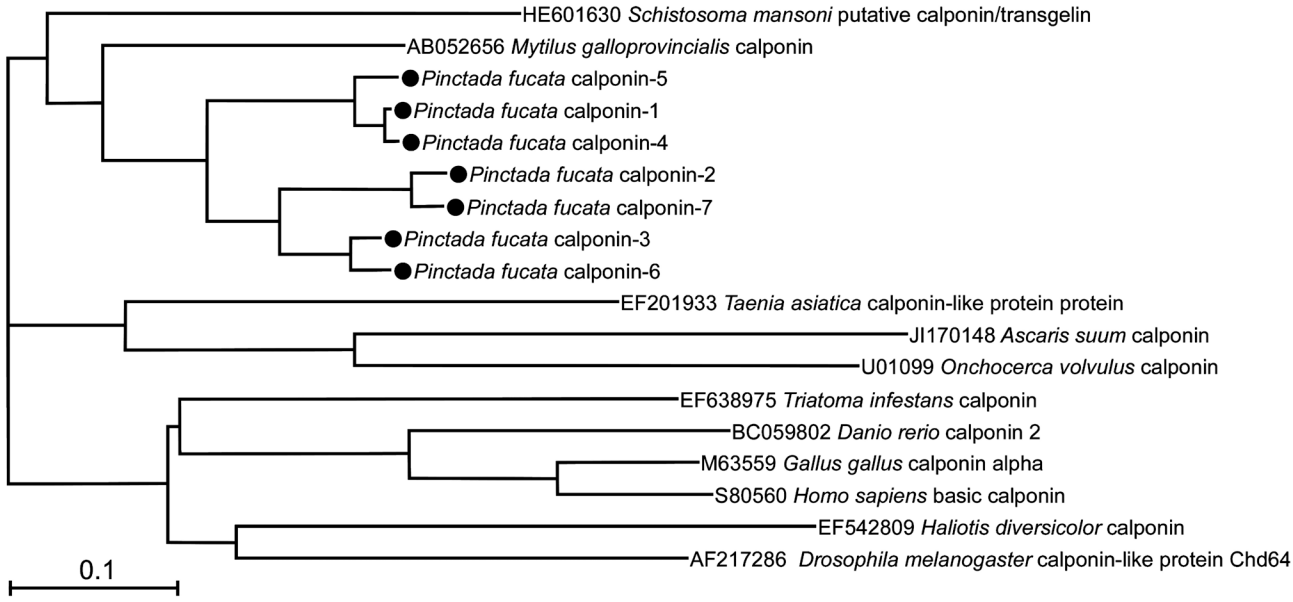

Figure 11. Phylogenetic tree showing the relationship among the calponin amino acid sequences from *Pinctada fucata* and other species. The tree was generated using the ClustalW algorithm. The GenBank accession numbers of each sequence used in the analysis are listed in the Materials and Methods.

calponin inhibits actomyosin Mg-ATPase activity *in vitro* and interacts with F-actin [17] [18] [29]. Therefore, *Pinctada* calponin may interact with F-actin in the same fashion and its affinity for F-actin may depend on the number of CN domains.

Protein expression analysis revealed that *P. fucata* calponin isoforms are expressed in muscle tissues and in non-muscle tissues, gill, mantle and foot (**Figure 5**). These findings are consistent with previous studies on molluscan calponin [21] [23]. In these studies, RT-PCR and protein expression analyses revealed that Yesso scallop calponin is expressed in catch and phasic muscles, gill, mantle and foot. These findings indicate that molluscan calponin is widely distributed in various tissues. The different number of bands detected by immunoblotting for respective tissues examined suggests that calponin isoforms function differently in tissues (**Figure 5**). In vertebrates, three types of calponin isoforms, basic, neutral and acidic, have been identified and have distinct functions [19] [30] [31] [32]. However, only basic calponin is present in mollusks [17] [21] [23] [33]. There is no available data describing the presence of neutral and acidic calponins in mollusks. Basic calponin isoforms may work distinctly in molluscan tissues, and studies on each calponin isoform, e.g., by using recombinant calponins, are required to elucidate their specific functions.

Catch contraction of molluscan smooth muscle is regulated by twitchin, a member of the titin/connectin family, through its phosphorylation and dephosphorylation [1]. *In vitro* studies revealed that twitchin binds simultaneously to myosin and actin in a phosphorylation-sensitive manner. The D2 site that is phosphorylated by cAMP-dependent protein kinase (PKA) is thought to be involved in tension maintenance of catch contraction. The binding site of the

twitchin D2 fragment on actin was found to overlap with the actin region that electrostatically interacts with loop 2 of myosin to initiate the movement of myosin over actin filaments. In addition, loop 2 of myosin binds to the twitchin D2 site. The formation of the complex among myosin, actin and twitchin may contribute to maintaining tension in the catch state. Therefore, the tethering of thick- and thin-filaments by twitchin is likely to be an essential event in catch contraction [2] [3]. Mammalian smooth muscles exhibit latch contraction similar to catch contraction [11]. The molecular mechanism of the tension maintenance of the latch contraction remains unresolved but it has been suggested that calponin participates in the tethering of thick- and thin-filaments, like molluscan twitchin [13]. In the resting stage of mammalian smooth muscle, calponin interacts longitudinally with two actin monomers that involve its low and high affinity binding sites. Upon increasing Ca^{2+} concentration within the stimulated cells, the N-terminus of calponin (most likely residues 1–52), which contains a low affinity calmodulin (CaM)-binding domain, is antagonized by the Ca^{2+}/CaM complex in concert with ATP. This leads to the dissociation of the N-terminal half of calponin from actin filaments. The released calponin fragment bends and interacts with the phosphorylated myosin regulatory light chain, whereas the central fragment of calponin (residues 145–163) remains bound to F-actin. In this scenario, calponin acts to tether thick- and thin-filaments and slows down the detachment rate of activated cross-bridges. This reaction introduces an internal load that triggers maximal contraction [13]. This model reminds us that thick- and thin-filaments are tethered by calponin besides twitchin in molluscan catch muscle. A question for the twitchin model described above is that the amount of twitchin (molar ratio to myosin = 1:15) [34] seems to be too small to tether thick- and thin-filaments to maintain the tension in the catch state. To answer this question, the calponin model might be used to catch contraction together with the twitchin model. Further studies on proteins that interact with molluscan calponin are required to elucidate the calponin function in catch contraction.

Acknowledgements

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Conflicts of Interest

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

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