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Comparative Biochemistry and Physiology, Part B 144 (2006) 238–244
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Expression of thick filament proteins during ontogenesis of the mussel *Mytilus trossulus* (Mollusca: Bivalvia)

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Received 17 July 2005; received in revised form 6 March 2006; accepted 7 March 2006
Available online 19 April 2006

Abstract

The appearance of thick filament proteins organized into supramolecular complexes was studied by SDS-PAGE and Western-blot analysis at different developmental stages of the mussel *Mytilus trossulus*. Paramyosin appeared at the egg stage, while twitchin and myorod appeared at the blastula stage (12 h after fertilization). In addition, RT-PCR analysis showed that the twitchin genes were expressed starting from the blastula stage. Thus, the proteins forming thick filaments of the contractile apparatus of mussel muscles are expressed long before the formation of the first well-organized muscle system of the veliger larvae (55 h). Further, the ratios actin/myosin heavy chain (MHC) and paramyosin/MHC at the veliger stage (96 h) distinctly differed from those in the adult mussel.

Keywords: Larva; Mollusc; Mussel; Myogenesis; Myorod; Myosin; Paramyosin; Twitchin; Thick filament

1. Introduction

Muscle assemblage is a complex and finely regulated process requiring coordinated synthesis of contractile proteins. Molluscan smooth muscles have a unique protein composition and an unusual structure of their thick filaments, distinct for their extraordinary large size. This unusual structural property of thick filaments correlates with a remarkable functional property of these muscles. They can be locked in the contracted state and will maintain tension for long periods with very little expenditure of energy. This state is called catch, and muscles capable of it are called catch muscles (Ruegg, 1971).

Paramyosin and myosin are the most abundant invertebrate thick filament proteins (Szent-Gyorgyi et al., 1971; Elfvin et al., 1976). Paramyosin forms a paracrystalline core of these filaments, while the motor protein myosin is arranged on the core surface (Elliott, 1974). In addition, two other proteins, twitchin (Vibert et al., 1993) and myorod (Shelud'ko et al., 1999) are co-localized with thick filaments in molluscan catch muscles. Twitchin is a 530-kDa molluscan muscle protein of the same immunoglobulin family as the giant protein titin. A close relationship between the phosphorylation state of twitchin in the smooth muscle and the presence or absence of its catch state was demonstrated by Siegman et al. (1997,1998). It has been proposed that twitchin regulates the muscle catch state in a phosphorylation-dependent via interaction with myosin (Butler et al., 1998) or actin (Shelud'ko et al., 2004). Unlike twitchin, myorod has not been found in the striated or obliquely striated molluscan muscles, therefore it is a specific molecular marker of the phenotype of molluscan smooth muscle cells (Shelud'ko et al., 1999). The myorod content in smooth muscles amounts to only 5–6% of the total protein content. Myorod appears as a product of alternative splicing of the myosin heavy chain RNA (Yamada et al., 2000), but its function is unknown.

The question, when and how such a complex protein supramolecular structure as molluscan thick filament forms has not yet been answered. Molluscs develop various sets of muscles during their life history, with different muscles in larvae and in adults. Mussel embryos and early larvae (blastula and trochophore) move only owing to their ciliary activity. The first larval muscles appear

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1096-4959/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpb.2006.03.002

at the early trochophore stage (Wanninger et al., 1999; Wanninger and Haszprunar, 2002a,b). The veliger was reported to have a specific well-organized musculature (Malakhov and Medvedeva, 1985). In bivalves this musculature consists of a complicated assembly of five pairs of larval retractor muscles and a distinct prototroch muscle ring (Cragg and Crisp, 1991; Wanninger and Haszprunar, 2002b). However, both systems are transient larval structures resorbed during metamorphosis (Malakhov and Medvedeva, 1985). The anterior and posterior adductor muscles that are already present and functional at late veliger stages (at the pediveliger stage, 10–14 days post fertilization, p.f.) form the main adult shell muscle system.

The present study was undertaken to examine the onset and timing of expression of the major myogenic proteins during *Mytilus trossulus* ontogenesis to obtain a better understanding of the sequence of these events.

2. Materials and methods

2.1. Animals

Adult specimens of *M. trossulus* (Mollusca: Bivalvia) were collected in Vostok Bay of the Sea of Japan (June of 2003–2004) and kept in aquaria with aerated running seawater. For experiments, sexually mature mussels were placed each in an individual cap, and spawning was induced by a thermal shock (10 to 20 °C). Mature eggs were inseminated with a sperm suspension and embryos were cultivated in tanks with UV-irradiated seawater at 17–18 °C. Samples were collected at the stages of unfertilized egg, blastula (12 h p.f.), trochophore (17 and 24 h p.f.) and veliger (60 and 96 h p.f.). After 56-h cultivation, the larvae were daily fed on the microalgae *Isochrysis galbana* (100 000 cells/mL). The gross morphology of adult molluscs, unfertilized eggs and the larvae at different developmental stages is presented in Fig. 1.

2.2. Proteins

Unfertilized eggs or larvae (2500–3000 eggs or larvae/mL, 10 mL) were collected by centrifugation (5 min at 2000 g) and homogenized in 1.5 mL of an ice-cold low ionic buffer solution containing 75 mM NaCl, 0.5 mÌ EGTA, 2 mM $MgCl_2$, 3 mM NaN_3, 0.5 mM phenylmethyl sulfonyl fluoride (PMSF), 0.5 mM dithiothreitol (DTT), and 20 mM sodium phosphate, (pH 6.8), with 0.5% Triton Õ-100 at 4 °C. The homogenate was centrifuged for 20 min at 5000 g. The pellet was resuspended in the same solution, but without Triton X-100 and recentrifuged as above. Then the pellet was extracted for 30 min at 4 °C in a high ionic strength solution (0.5 M KCl, 2 mM $MgCl_2$, 0.5 mM DTT, 0.3 mM PMSF, 2.5 mM NaN_3, and 50 mM sodium phosphate, pH 7.5) with subsequent centrifugation at 12 000 g for 20 min.

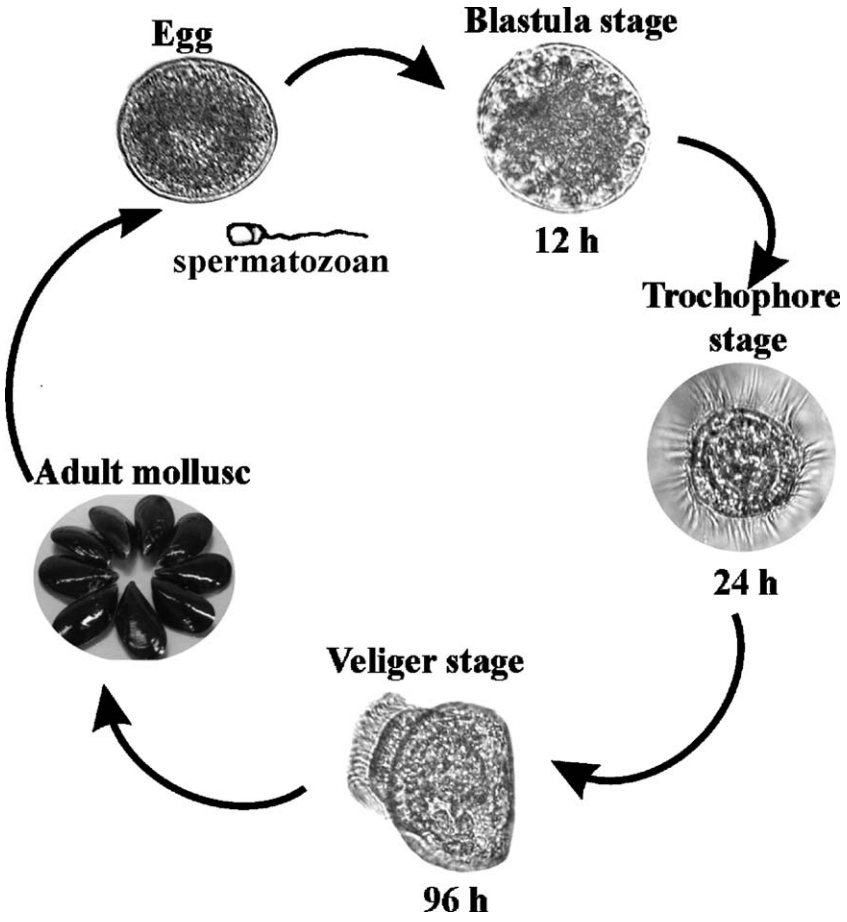

Fig. 1. Some stages of *Mytilus trossulus* ontogenesis (times given refer to hours after fertilization).

Total proteins of the natural actomyosin (AM) extracts were precipitated by 5% trichloroacetic acid; the pellet was dissolved in 8.5 M urea containing 5 mM EDTA and an equal volume of SDS-sample solution (Shelud'ko et al., 1999) was added. The AM extracts from muscles and total tissues of the adult mussel were prepared using the same procedure.

We used a standard treatment of a muscle homogenate for isolating contractile proteins: washing of a muscle homogenate with buffer solutions of low ionic strength (myofibril isolation) and following extraction of the pellet with high ionic strength solutions (natural AM isolation). Thus, we analyzed at different stages of mussel ontogenesis only those contractile proteins which were included in the supramolecular complexes, such as myofibrillar-like structures or dot-like aggregates (Vanderloop et al., 1996). A preparation from the adductor muscles obtained in that way from the adult mollusc *Mytilus edulis* ("natural actomyosin") contains all proteins of the contractile apparatus (Yamada et al., 1997).

Paramyosin (Szent-Gyorgyi et al., 1971), twitchin, myosin, myorod and tropomyosin (Shelud'ko et al., 2004) were isolated from the adductor muscle of the mussel *Crenomytilus grayanus*. Rabbit actin was isolated according to Pardee and Spudich (1982).

2.3. SDS-PAGE

The AM extracts were analyzed by 8% SDS polyacrylamide slab gel electrophoresis, as described by Laemmli (1970) with modifications (Shelud'ko et al., 1999). Under these conditions, the main contractile proteins of various invertebrate species are quite distinct and can be identified (Shelud'ko et al., 2001, 2002, 2004). SDS-PAGE slabs were scanned and the quantitative data were evaluated using the computer-aided image analysis. Staining intensity ratios were converted to: 1) paramyosin /myosin heavy chain (PM/MHC) molecular ratios according to Levine et al. (1976) from the slopes in the dye-binding experiments; 2) actin/MHC (A/MHC) molecular ratios according to Treager and Squire (1973).

2.4. Preparation of polyclonal antibodies

Polyclonal antibodies to paramyosin and twitchin were generated in rabbits using *C. grayanus* paramyosin and twitchin as antigens. Each antigen (0.5 mL, 0.5 mg/mL) was emulsified with Freund's complete adjuvant. The second injection with Freund's incomplete adjuvant was done not earlier than 3 weeks after the first injection. About 3 weeks after the second injection, a blood sample was obtained by rabbit's ear bleeding. The serum was separated from plasma clots and antibodies were purified using standard procedures. Polyclonal rabbit anti-myorod antibodies were kindly provided by Dr. V. Kumeiko (Institute of Marine Biology, Vladivostok, Russia). Specificity of all antibodies was tested by the radial immunodiffusion assay with purified *C. grayanus* myosin, paramyosin, myorod, twitchin, tropomyosin, and rabbit actin. Ouchterlony's immunodiffusion tests were

Fig. 2. A radial immunodiffusion assay: cross-reactivity of the twitchin antibodies with different proteins: 1. twitchin; 2. paramyosin; 3. myorod; 4. tropomyosin; 5. rabbit actin; 6. myosin.

Fig. 3. A schematic representation of *Mytilus* twitchin mRNA (Funabara et al., 2003). The primers were selected from the sequence, adjacent to the twitchin serine–threonine kinase domain and including the sequence of this domain. *Black*, immunoglobulin-like motifs; *white*, fibronectin type III motifs; *S_Tk*, serine–threonine kinase domain.

carried out in 1% agarose gel containing 0.15 M NaCl and 10 mM PBS, pH 7.2. The anti-paramyosin antibody showed cross-reactivity only with paramyosin, while the anti-myorod antibody interacted with both myorod and myosin (Plotnikov et al., 2003). The anti-twitchin antibody showed cross-reactivity with twitchin alone (Fig. 2).

2.5. Western-blot analysis

Separated proteins were blotted onto PVDF membranes (MP Biomedicals, USA) for immunodetection (Towbin et al., 1979). Transferred proteins were visualized with Ponceau Red (Serva, USA). Unspecific binding regions were blocked with 5% non-fat dry milk in a washing buffer solution at room temperature (0.02 M Tris, 0.15 M NaCl, and 0.05% Tween-20, pH 7.6) for 1 h, and the membranes were then incubated with primary antibodies (diluted in washing buffer solution with 0.1% milk at 1:100) for 1 h followed by alkaline phosphatase-conjugated-goat anti-rabbit immu- noglobulins as secondary antibodies. After three 10-min washes, the blots were developed in a buffer (100 mM NaCl, 5 mM $MgCl_2$, and 100 mM Tris–HCl, pH 9.0) containing 5-bromo-4-chloro-3-indolyl phosphate (100 μg/mL) with nitroblue tetrazolium (200 μg/mL) as a substrate.

2.6. RT-PCR of twitchin

Total RNA from the mussel smooth adductor muscle, unfertilized eggs and embryos and larvae at different developmental stages were digested with DNase (Amresco, Solon, Ohio, USA) and purified with BlueSorb (Clonogen, Russia). Complementary DNAs were synthesized using RNA (1–2 μg) by RNA PCR Kit (Silex M, Russia). Reactions were performed in 25-μL aliquots of the reaction mixture which contained RT buffer: 0.2 mM each of the 4 dNTPs, 0.2 μM of oligo-$(dT)_{15}$ primer, 200 U of M-MLV-polymerase at 37 °C for 1 h and 10 μL samples of reverse transcription products were amplified by PCR. The sequence of the mussel twitchin mRNA was determined by Funabara et al., 2003 (DDBJ/GenBank TM/EBI Data Bank with accession no. AB062881, www.ncbi.nlm.nih.gov/PubMed). The primers were selected from the sequence, adjacent to the twitchin kinase domain and including the sequence of this domain (Fig. 3, arrows). The forward (10591–10640) and reverse (11353–1372) primers (5′-CATGGTACCGACAACTGGTC-3′ and 5′-TTTT-CACAGGTTGGGTAGCG-3′) were designed to flank a 780-bp fragment of twitchin mRNA serine–threonine kinase domain. We performed a PCR analysis in an UNO Thermoblock thermal cycler (Biometra, Germany) programmed for an initial denaturation step of 50 s at 95 °C followed by 35 cycles of 50 s at 50 °C and polymerization at 72 °C for 60 s using a Taq DNA polymerase (Silex M, Russia).

3. Results

The composition of major contractile proteins at different stages of *M. trossulus* development was analyzed by SDS-electrophoresis. Fig. 4 shows electrophoretic patterns of AM extracts from adult mussels, unfertilized eggs and larvae. The bands with electrophoretic mobilities corresponding to those of myosin heavy chain, actin and paramyosin were identified. The

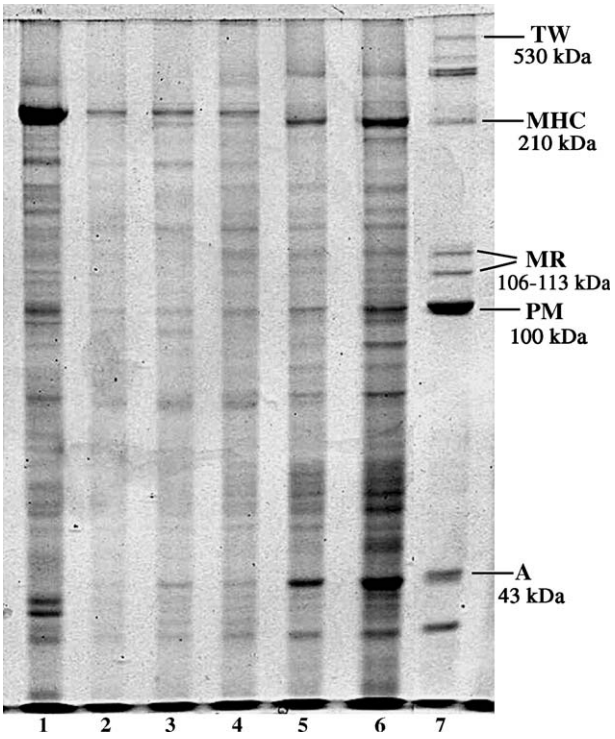

Fig. 4. SDS PAGE profile of actomyosin (AM) extracts isolated from the mussel *M. trossulus* at different stages of ontogenesis. AM extract of the unfertilized eggs (1), different embryonic and larval stages: the blastula stage (2), the trochophore stages 17 h (3) and 24 h (4) p.f., early veliger stage at 60 h p.f. (5), late veliger stage at 96 h p.f. (6), and adult mussel (7). The positions of marker proteins (a sample of the contractile apparatus proteins isolated from the anterior byssus retractor muscle of the mussel as the muscle protein standard) are indicated as: TW, twitchin (530 kDa); MHC, myosin heavy chain (210 kDa); MR, myorod (106 and 113 kDa); PM, paramyosin (100 kDa); A, actin (43 kDa).

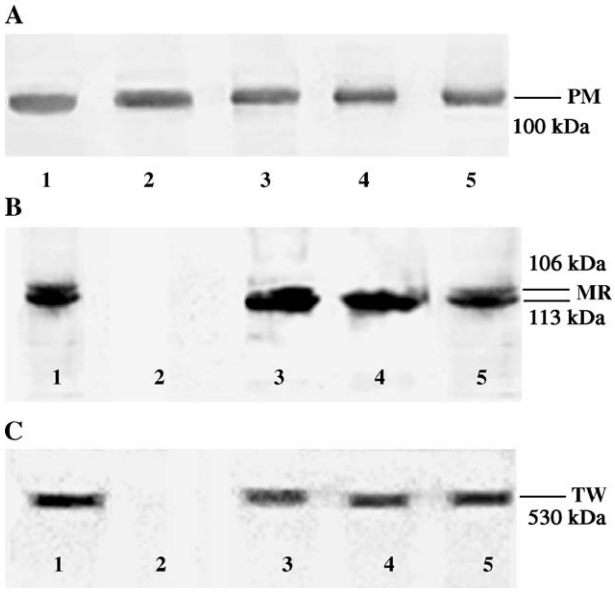

Fig. 5. Western-blot analysis of the mussel selective AM extracts from different stages of *M. trossulus* ontogenesis. AM extracts from adult mussels (1), the unfertilized eggs (2), different embryonic and larval stages: the blastula stage (3); the trochophore stage (4), and an early veliger stage (5) were separated by 8% SDS-PAGE transferred to PDVF membrane and detected with paramyosin (A), myorod (B) and twitchin (C) antibodies. PM, paramyosin (100 kDa); MR, myorod (106 and 113 kDa); TW, twitchin (530 kDa).

paramyosin band was detected at all developmental stages including unfertilized eggs. The MHC band appeared at the trochophore stage (17 h p.f.) (Fig. 4, lane 3), although it was rather weak. Starting from the early veliger stage (60 h p.f.), myosin became a major protein, second to actin. Actin was detected starting from the trochophore stage (17 h p.f.) onwards. At the late veliger stage (96 h p.f.), actin was the leading protein. Note, that an unknown protein with electrophoretic mobility close to that of MHC dominated among the egg proteins. We carried out a series of additional experiments on an interaction of this protein with actin. Measurements of optical density of a mixture of actin and this protein did not detect a formation of protein complexes (data not shown). In other words, the unknown protein is not MHC, because it did not interact with actin. It appears possible that this protein is a lipoprotein, which can be a nutrition component for embryos, since its content decreased during the development process.

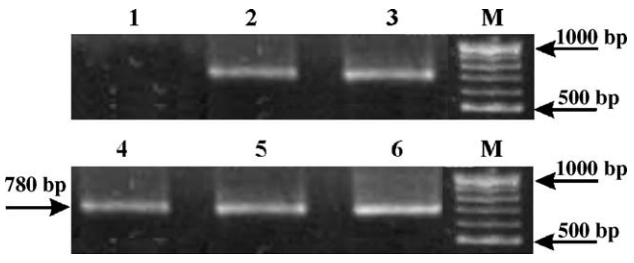

Fig. 6. RT-PCR detection of the messenger RNA of the twitchin gene in several stages of *M. trossulus* development. The unfertilized eggs (1), the blastula stage (2), the trochophore stage (3), an early veliger stage (4), a late veliger stage (5), adult mussel (6). M, synthetic marker. Primers were designed to flank a 780-bp twitchin fragment.

Table 1
Estimates of weight ratios of the paramyosin/myosin heavy chain (PM/MHC) and the actin/MHC in actomyosin (AM) extracts from the veliger and adult mussel *M. trossulus*

Stages of ontogenesis	PM/MHC	A/MHC
Veliger (60 h)	0.37±0.06	1.25±0.03
Veliger (96 h)	0.59±0.1	1.26±0.13
Adult mollusc	5.52±0.67	1.8±0.08
	5.76±0.75[a]	1.33±0.1[b]

$p<0.05$.
Values are presented as mean±SEM obtained in three separate experiments with 3 replicate each.
[a] Levine et al., 1976 (the anterior byssus retractor muscle of *Mytilus edulis*).
[b] Margulis et al. (1979) (smooth muscles of *Mya japonica*).

In order to identify thick filament proteins, we used the Western-blot analysis with polyclonal antibodies (Fig. 5). The results confirmed that paramyosin appeared first at the egg stage (Fig. 5A). Myorod and twitchin were expressed in the mussel larvae starting from the blastula stage (12 h p.f.) (Fig. 5B,C) onwards. In addition, RT-PCR analysis also showed that twitchin was expressed starting from the blastula stage (Fig. 6). Interestingly, twitchin was not found at the egg stage (Fig. 6, lane 1). As a control, we detected an actin PCR product at all developmental stages (data not shown).

Quantitative relations between the major contractile proteins are presented in Table 1. These data show that the PM/MHC weight ratios at the early and late veliger stages were significantly lower than those in the adult molluscan tissues. In addition, the A/MHC weight ratio at all veliger stages was also lower than that for adult molluscs. Note that the ratios of A/MHC and PM/MHC at the veliger stage in the mussel (96 h p.f.) were similar to those of striated muscles in adult scallops (Shelud'ko and Preminger, 1989) but not to those of smooth muscles in adult mussels (Levine et al., 1976).

4. Discussion

4.1. Paramyosin is the first thick filament protein detected during M. trossulus ontogenesis

As shown earlier, paramyosin is present in both embryonic and adult muscles of invertebrates (Honda and Epstein, 1990; Odintsova et al., 2000; Arredondo et al., 2001; Liu et al., 2003; Plotnikov et al., 2003). We found that paramyosin is the first thick filament protein to be detected in *M. trossulus* unfertilized eggs. This fact is consistent with the results of Vinós et al. (1991), who reported that *Drosophila melanogaster* paramyosin was present in relatively high amounts in the cytoskeletal pellet of mature oocytes. Antibody analysis of *D. melanogaster* embryos demonstrated that paramyosin accumulated as a cytoplasmic protein during early embryogenesis before assembling into thick filaments (Liu et al., 2003). It is known that *Caenorhabditis elegans* paramyosin is involved in the initial steps of thick filament formation of body-wall muscles, although its synthesis is independent of myosin synthesis (White et al., 2003). At the same time, as it was shown by those authors, a properly assembled paramyosin-containing thick filament core is essential for

efficient synthesis of muscle myosin. It appears possible that paramyosin, constituting the basis of thick filaments, acts as a 'protein-ruler' to form the contractile apparatus of smooth molluscan muscles.

4.2. Twitchin at early stages of embryogenesis

On the other hand, the giant protein titin, a twitchin homologue (Funabara et al., 2003), is one of the first proteins to appear during myogenesis of vertebrate striated muscles (Hill and Weber, 1986; Fürst et al., 1989). Our RT-PCR data, as well as Western-blot data, suggest that twitchin was expressed in mussel larvae starting from the blastula stage. Hence, newly synthesized twitchin was located only in the supramolecular complexes. The question remains whether this complex is a functional structure or a storage form, like titin from the vertebrate skeletal muscles. During vertebrate embryogenesis, titin is initially observed as dot-like aggregates, while during muscle maturation this high molecular weight protein assumes a cross-striated pattern (Vanderloop et al., 1996). RNAs of proteins related to titin family are also found at the early embryonic stages: RNAs of *D. melanogaster* titin and kettin were detected in the embryos 7 h p.f. until myoblast fusion (Zhang et al., 2000), whereas the corresponding proteins appeared 9–12 h p.f. Perhaps, titin acts as a 'protein-ruler' to regulate an exact myosin filament assembly (Linke, 2000; Tskhovrebova and Trinick, 2002) and is involved in myoblast fusion owing to multiple sites of interaction with other proteins (Fürst et al., 1989; van der Ven et al., 2000). Molluscan twitchin also contains binding sites with muscle proteins, such as myosin (Siegman et al., 1998; Yamada et al., 2001), actin (Shelud'ko et al., 2004), paramyosin and myorod (our unpublished data). It is possible that twitchin not only regulates the catch state (Siegman et al., 1997, 1998; Funabara et al., 2005), but judging from its early appearance in the molluscan ontogenesis, may play along with paramyosin, some role in the assemblage of contractile apparatus, as it is the case with titin (Linke, 2000).

4.3. The ratios of PM/MHC and A/MHC

The paramyosin content varies markedly in different muscles. The degree of muscle structural regularity decreases with an increasing content of paramyosin (Szent-Gyorgyi et al., 1971; Margulis et al., 1979). Paramyosin is most abundant in catch muscles (Winkelman, 1976). The anterior byssus retractor muscle of *M. edulis*, the well-known catch muscle, has one of the highest PM/MHC molecular ratios, where as the highly ordered striated muscles of arthropods have the lowest ratio: 2.88±0.72 (weight ratio of 5.76) and 0.13±0.02 (weight ratio of 0.26), respectively (Levine et al., 1976). Moreover, the scallop striated muscles contain large amounts of myosin (Shelud'ko and Preminger, 1989) and thus show a low A/MHC weight ratio (0.62), while higher ratios (1.25–2.5) are characteristic of smooth muscles (Treager and Squire, 1973). As shown by Margulis et al. (1979), a *Mya japonica* muscle with the A/MHC weight ratio of 1.33 appears to be smooth, *Mercenaria* sp. and *Crassostrea gigas* muscles with the ratios of 0.92–0.81 are obliquely striated, and *Pecten yessoensis* muscles with the ratio of 0.68 are cross-striated. Thus, the PM/MHC and the A/MHC ratios we found at different larval stages of *M. trossulus*, including the veliger stage, are not typical of the smooth muscle in adult molluscs.

Hence, we can conclude that the proteins forming thick filaments of the mussel muscle contractile apparatus are expressed in a definite sequence at the earliest stages of the myofibril assembly, long before the formation of the first well-organized muscle system of the veliger larvae. Perhaps, the contractile apparatus capable to the catch state is formed at the later stages of mussel ontogenesis (at the pediveliger stage, 10–14 days p.f. or later).

Acknowledgements

The authors are very grateful to our reviewers for valuable comments and suggestions. Special thanks to Dr. S.G. Vasetsky for his help in the manuscript editing. We are indebted to Dr. Sergey V. Plotnikov for his kind advice on immunological techniques. This study was supported by the Russian Foundation for Basic Research (grants 05-04-49895 and 06-04-96039), the Far Eastern Branch of Russian Academy of Sciences (grant 06-II-CO-06-025), and the Program for Molecular and Cellular Biology of the Russian Academy of Sciences.

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