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Molecular characterization and expression profiles of myosin essential light chain gene in the Pacific oyster *Crassostrea gigas*

Hong Yu, Huijuan Li, Qi Li

Key Laboratory of Mariculture (Ocean University of China), Ministry of Education, Qingdao 266003, China

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ABSTRACT

In molluscs, muscle contraction is triggered by a direct binding of Ca^{2+} to myosin. The myosin essential light chain (MELC) provides the Ca^{2+} binding site and is of importance for motor function and regulation. In this study, the complete cDNA sequence of MELC gene of the Pacific oyster, *Crassostrea gigas*, was obtained, and the expression profiles were performed in different tissues and different embryo-larval development stages. The results showed that the full length of *C. gigas* MELC (CgMELC) cDNA was 659 bp, containing a 5′-untranslated region of 73 bp, a 3′-untranslated region of 112 bp. The open reading frame encoded a 157 amino acid peptide. The protein sequence of CgMELC contained a conserved EF-hand calcium binding motif, and showed a high sequence identity (68.4–100%) with other bivalves. Quantitative analysis of CgMELC mRNA in tissues indicated that CgMELC was expressed at the highest level in the striated adductor muscle, followed by the smooth adductor muscle and mantle. During the development of embryos and larvae, quantitative analysis and whole-mount in situ hybridization revealed that CgMELC was expressed starting from the blastula stage and abundantly expressed in trochophore and D-shaped larvae, indicating that CgMELC might also be involved in regulation of larval muscle system development. Our data provided valuable information for further researches on the function of MELC and regulation of muscle contraction in oysters.

1. Introduction

Oysters are one of the most important aquaculture species in the world, with the highest annual production of any marine organism (http://www.fao.org). The Pacific oyster (*Crassostrea gigas*) is the most widely cultivated marine bivalve, having been introduced from Asia to many counties worldwide including the US, Canada, Australia, Chile, and so on (Zhu et al., 2016). As sessile marine animals of estuarine and intertidal regions, the adductor muscle is a major feature of oysters. The asymmetric valves are connected by a single adductor muscle, which controls the opening and closing of the two valves. In the oyster *C. gigas*, the adductor muscle consists of a translucent and an opaque portion (Poulet et al., 2003). The translucent portion, also named as the striated phasic adductor, is involved in quick closure of the valves, whereas the opaque portion, also named as the smooth tonic adductor (catch muscle), is responsible for prolonged periods in the “catch” state with little energy expenditure (Poulet et al., 2003; Tsutsui et al., 2007). Although the translucent portion is identified as striated muscle in oysters, it is an obliquely striated muscle but not a cross-striated muscle (Tsutsui et al., 2007). The adductor muscle of oysters is not only an edible part, but also a useful indicator of the health status. The strength of the adductor muscle, required to keep the valves closed, can be used as a diagnosis of juvenile oyster health (Poulet et al., 2003).

A distinguished feature of molluscan muscle regulation system is that contraction is triggered by a direct binding of Ca^{2+} to myosin, which consists of two essential light chains (MELC), two regulatory light chains (MRLC) and two heavy chains (MHC) (Szent-Györgyi et al., 1999). In molluscs, scallops and mussels myosins have been studied extensively (Ashiba and Szent-Györgyi, 1985; Katoh et al., 2002; Odintsova et al., 2006; Dyachuk and Odintsova, 2009; Zhang et al., 2010). The sequences of the three myosin subunits (MHC, MRLC, and MELC) have been determined by cloning and protein chemistry methods (Collins et al., 1986; Goodwin et al., 1987; Perreault-Micale et al., 1996; Andersen et al., 2009). The function has also been revealed through point mutation and chimera construction (Kwon et al., 1990; Jancso and Szent-Györgyi, 1994; Fromherz and Szent-Györgyi, 1995; Zhang et al., 2010). The essential and regulatory light chains are regulatory subunits, of which the MELC contains the Ca^{2+} binding site stabilized by the MRLC. The MRLC also inhibits ATPase activity, tension generation and motility in the absence of Ca^{2+} (Szent-Györgyi et al., 1999).

Myosin essential light chain is a member of the EF hand

Corresponding author.
E-mail address: qili66@ouc.edu.cn (Q. Li).

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superfamily, which is positioned in the neck region of myosin and of importance for motor function and regulation (Fromherz and Szent-Györgyi, 1995). The Ca^{2+} binding site was located within EF hand domains of the MELC in molluscs, which triggered contraction of molluscan muscle (Szent-Györgyi et al., 1999). In addition, Katoh et al. (2002) revealed that essential light chain modulated phosphorylation-dependent regulation of smooth muscle myosin. Although MELC is considered to be very important for muscle contraction in molluscs, information about the MELC gene in molluscs is mainly concentrated in several scallops (Collins et al., 1986; Fromherz and Szent-Györgyi, 1995; Perreault-Micale et al., 1996), and few studies on the MELC gene has been reported in oysters.

In the present study, we cloned the full length cDNA of the MELC gene from *C. gigas* and determined its expression profiles in different tissues and different embryo-larval development stages using RT-qPCR and whole-mount in situ hybridization methods. The results would provide a foundation for studying the molecular mechanisms of oysters muscle contraction and enable us to better understand the function of myosin in molluscs.

2. Materials and methods

2.1. Samples collection

Adult *C. gigas* were collected from an oyster farm in Rushan in Shandong province, China, and dissected to obtained different tissues including gills, female and male gonads, hemolymph, mantle, striated and smooth adductor muscles, digestive gland, and labial palp. Samples were frozen in liquid nitrogen and stored at − 80 °C until processed. Laval culture of *C. gigas* was performed as previously described (Wang et al., 2012). Embryo-larval samples were collected during the following stages: fertilized eggs, two-cell stage, four-cell stage, blastula stage, gastrula stage, trochophore, D-shaped larvae, umbo larvae, and eyed larvae. Samples were preserved in RNAstore solution (Dongsheng Biotech, China) at − 20 °C before extraction of RNA. For whole-mount in situ hybridization, samples were fixed in 4% paraformaldehyde for 12 h at 4 °C, transferred to methanol, and stored at − 20 °C until use. D-shaped larvae, umbo larvae, and eyed larvae, were anesthetized with 7.5% $MgCl_2$ prior to fixation.

2.2. RNA extraction and cDNA synthesis

Total RNA of each sample was extracted with TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions. RNA quantity, purity and integrity were verified using NanoDrop 2000 (Thermo Scientific) and 1.0% agarose gel electrophoresis. For expression profiles, total RNA extracted from different tissues and different development stages was reverse transcripted into cDNA by PrimeScriptTM Reverse Transcription Kit (Takara).

2.3. Cloning the full-length cDNA of MELC

The internal fragment of MELC gene was amplified by PCR based on the bioinformatics prediction of the gene model of LOC105317061 using the cDNA from oyster adductors as the template. Specific primers (MELC-F and MLCe-R3) designed by the Primer Premier 5.0 (http://www.premierbiosoft.com/primerdesign/) were used for the PCR. The PCR reactions were performed as follows: 95 °C for 3 min; 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, 35 cycles; 72 °C for 5 min. The PCR product was purified and sequenced using an ABI 3730 Genetic Analyzer (Applied Biosystems).

Based on the obtained internal fragment sequence, the gene specific primers (Mlce-3′-1 and Mlce-5′-1) for 3′ and 5′ RACE reactions were designed. The 3′ and 5′ cDNA ends of MELC gene were cloned using the RACE method with the SMARTer® RACE 5′/3′ Kit (Clontech). The 3′ and 5′ RACE PCR reactions were performed with Tks Gflex™ DNA Polymerase (Takara) according to the following conditions: 98 °C for 1 min; 98 °C for 10 s, 60 °C for 15 s, 68 °C for 30 s, 30 cycles. Specific primer sequences used in the amplification of MELC are listed in Table 1. The purified PCR products were cloned into the pEASY-Blunt vector (Transgen Biotech, China) and sequenced in both directions (Sangon Biotech, China).

Table 1
Specific primers used in this study.

Primer name	Sequence (5′-3′)	Usage
MELC-F	GGGACGGTCGTGACGCAG	RT-qPCR & PCR
MELC-R	TCGGTTCCGCCATTCTTTCT	RT-qPCR
Mlce-3′-1	′-1TCG[illegible]ACATTCGCCrmMlc5-3	

2.4. Sequence analysis of MELC

The internal fragment, 3′ and 5′ end sequences were assembled using SeqMan (DNAStar) to obtain the full length cDNA of MELC. The open reading frame was predicted by the ORF finder of NCBI (https://www.ncbi.nlm.nih.gov/orffinder/). The conserved domains were predicted with the CD-Search tool of NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The deduced amino acid sequence was analyzed with the Expert Protein Analysis System (http://www.expasy.org/). The secondary structure of the MELC protein was predicted with the PredictProtein program (https://ppopen.informatik.tu-muenchen.de/). The signal peptide and three-dimensional structure of the deduced MELC protein were predicted with the online SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) and Swiss Model (https://swissmodel.expasy.org/interactive).

The protein sequence alignment was performed using the COBALT tool of NCBI (https://www.ncbi.nlm.nih.gov/tools/cobalt/re_cobalt.cgi) and edited with ESPript 3.0 (Robert and Gouet, 2014). A phylogenetic tree was constructed using the neighbor-joining (NJ) method by MEGA 7.0 (Kumar et al., 2016) based on MELC amino acid sequences of 14 molluscs. The sequence of *C. gigas* MELC was obtained in this study and the other molluscan MELC sequences were downloaded from GenBank.

2.5. Expression analysis of MELC by real-time quantitative PCR

Real-time qPCR was used to quantify changes in expression within different tissues and nine developmental stages from fertilized eggs to eyed larvae. Primer sequences (MELC-F and MELC-R) designed for RT-qPCR were showed in Table 1. The RT-qPCR was performed on a LightCycler® 480 real-time PCR system (Roche) using SYBR® Premix Ex Taq™ II kit (Takara) according to the manufacturer's protocols. Elongation factor 1-α (EF1-α) and ribosomal protein S18 (RS18) were used as reference genes in the adult and larva samples, respectively (Du et al., 2013). The RT-qPCR reaction conditions were as follows: 95 °C for 30s; 95 °C for 5 s, 60 °C for 20 s, 72 °C for 20 s, 40 cycles. Melting curve analysis of amplification products was conducted at the end of each PCR reaction to confirm the specificity of each primer pair. The relative expression levels of target genes were calculated using the $2^{-\Delta\Delta Ct}$ method. All data were given in terms of relative mRNA expression level as means ± SD (n = 6). The significant differences between the means were analyzed using the IBM SPSS Statistics 23 by one-way ANOVA followed by a multiple comparison. Differences were considered statistically significant at $P < 0.05$.

2.6. Whole-mount in situ hybridization

Digoxigenin-labelled RNA sense and anti-sense probes were

synthesized from the clone of a cDNA fragment of CgMELC (700 bp) amplified with the primers MELC-F and MLCe-R3 using a DIG-RNA labeling Kit (Roche). Whole-mount in situ hybridization was conducted using the protocol described by Thisse and Thisse (2008) with some modifications. Fixed embryos or larvae were rehydrated stepwise into PBST, and then rinsed for 5 min with 50% PBST + 50% hybridization buffer (50% formamide, 5 × SSC, 50 μg/ml heparin, 500 μg/ml tRNA, 0.1% Tween-20, 9.2 mM citric acid), followed by three washes (100% hybridization buffer) for 10 min each. Prehybridization was done for 4 h at 65 °C in hybridization buffer. Sense or anti-sense probe was added (30 ng/ml) and hybridized to target overnight at 65 °C. Unbound probes were washed away by a series of low-salt washes and samples were incubated in blocking solution for 4 h at room temperature. Antibody incubation was performed in a fresh solution of alkaline phosphatase conjugated anti-digoxigenin antibody (diluted 1:5000 in blocking solution) (Roche) overnight at 4 °C. Samples were then washed with MABT (0.1% Tween-20, 150 mM sodium chloride, 100 mM maleic acid, pH 7.5) for 3 × 30 min, and with alkaline Tris buffer for 3 × 5 min. Samples were incubated with 2% NBT/BCIP solution for 2 h in darkness at room temperature. Color development was stopped by 3 × 5 min washes in PBST. Samples were mounted in 70% glycerol for photography, and pictures were taken with digital camera (Olympus DP73) on the fluorescence light microscope (Olympus BX53).

3. Results

3.1. Sequence analysis of MELC

In the present study, the MELC gene was first cloned from *C. gigas*. The complete sequence of *C. gigas* MELC (CgMELC) cDNA was 659 bp, containing a 5′-untranslated region (5′-UTR) of 73 bp, a 3′-untranslated region (3′-UTR) of 112 bp, and 474 bp open reading frame encoding a 157-amino acid protein with an ATG start codon and TAA stop codon (Fig. 1). The 3′-UTR contained one non-canonical polyadenylation signal (TGTAAAA) locating at the 16 bp distance before the poly (A) tail. The full MELC gene sequence of *C. gigas* was submitted to GenBank (accession no. KY575961). The calculated molecular mass of CgMELC protein was 17.99 kDa and the predicted isoelectric point was 4.45. A conserved EF-hand calcium binding motif was detected in the central to C-terminal of the sequence (Fig. 1), suggesting that the CgMELC was implicated in Ca^{2+} regulation. No signal peptide was detected in the CgMELC protein based on SignalP 4.1 analysis with the D-score of 0.105 (cutoff score of 0.450), indicating that CgMELC protein is a non-secreted protein. The tertiary structure of the CgMELC protein was based on template 1kk7.1.C (Fig. 2), which shared 74.68% identity with

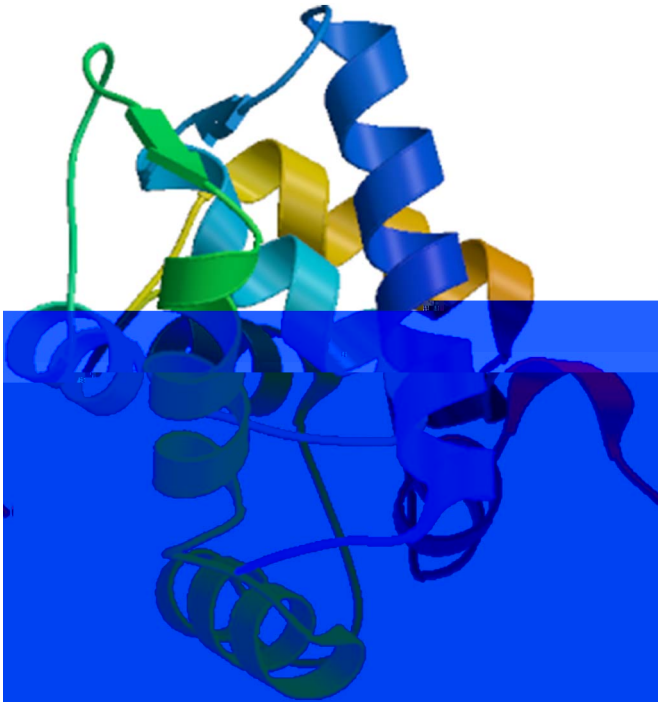

Fig. 2. The predicted three-dimensional structure of the CgMELC protein.

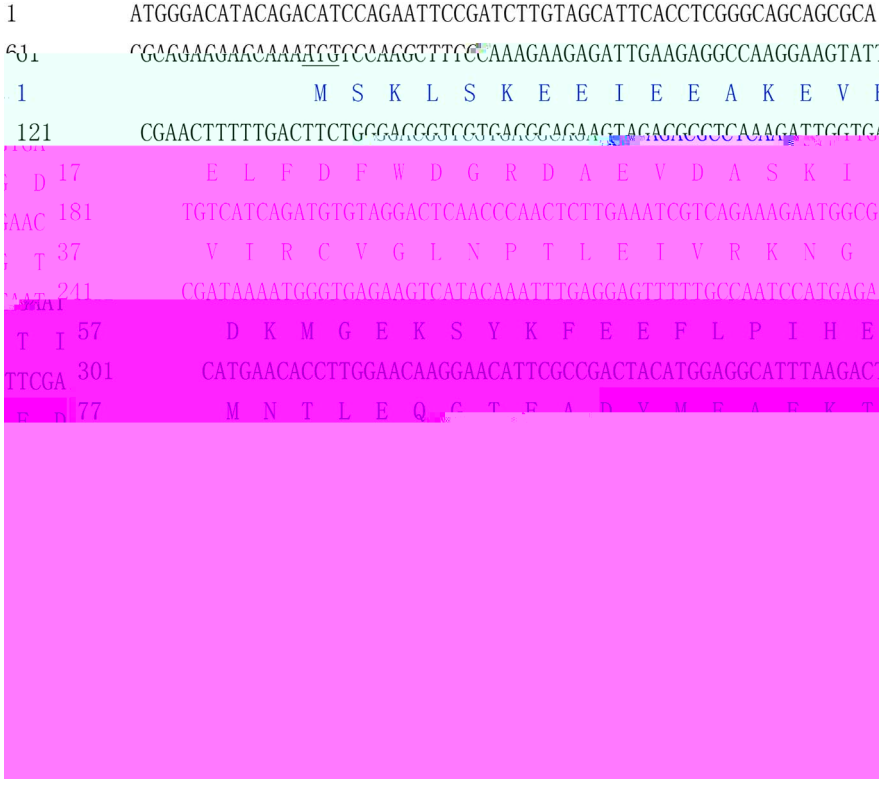

Fig. 1. The cDNA and encoded amino acid sequence of CgMELC. The start (ATG) and stop (TAA) codons are underlined. The polyadenylation signal (TGTAAAA) is marked in red. The conserved EF-hand calcium binding motif is highlighted in grey. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

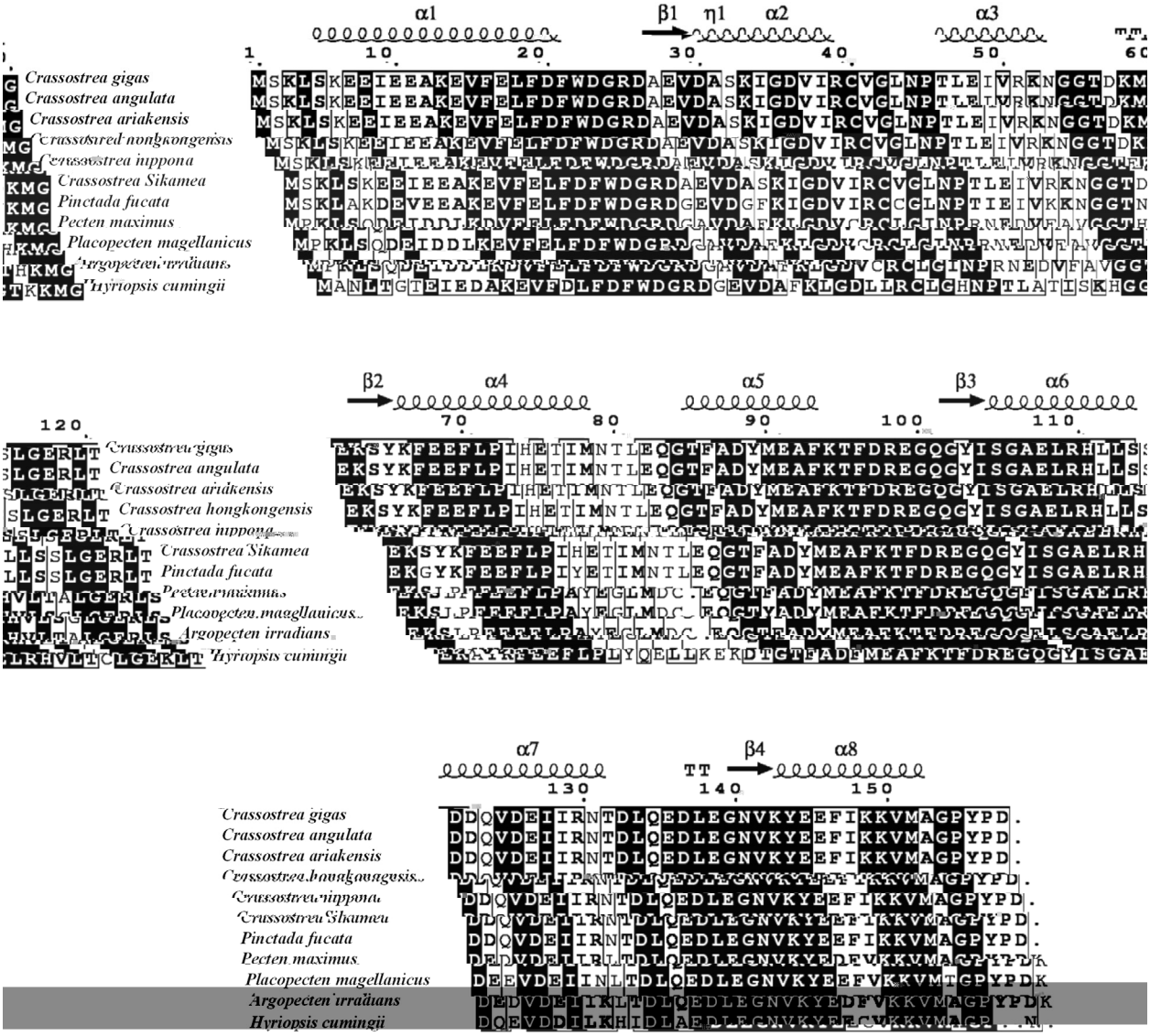

Fig. 3.

CgMELC protein. The CgMELC protein was estimated to comprise eight α-helixes, suggesting that CgMELC protein might consist of four EF hand-like domains (Fig. 3).

3.2. Homology and phylogenetic analysis of CgMELC

The amino acid sequence of CgMELC was aligned with other bivalve MELC amino acid sequences available on GenBank (*Crassostrea angulata* KY575962, *C. ariakensis* KY575963, *C. hongkongensis* KY575964, *C. nippona* KY575965, *C. sikamea* KY575966, *Pinctada fucata* AAZ79490, *Placopecten magellanicus* AAB02928, *Pecten maximus* AAD52843, *Argopecten irradians* AAA27714, *Hyriopsis cumingii* AFR69201). The result revealed that MELC exhibited a relatively high level of sequence conservation (65.8–100%) within the Bivalvia (Fig. 3). A high sequence similarity was detected (99.4–100%) was observed among the genus *Crassostrea*. Furthermore, a high sequence similarity (94.3–99.4%) was detected in the three scallops of Pectinidae. The phylogenetic analysis showed that MELC of *Crassostrea* species was clustered together, and was closely related to the *P. fucata* MELC ortholog (Fig. 4). The gastropod *Haliotis discus discus* (ABO26638) clustered with Bivalvia, and the gastropods *Aplysia californica* (XP_012944415) and *Biomphalaria glabrata* (XP_013081220) formed an independent branch.

3.3. Expression patterns of CgMELC

The CgMELC expression patterns among different tissues, and different embryo-larval developmental stages were analyzed by RT-qPCR. As shown in Fig. 5A, CgMELC mRNA was expressed in the various tissues expect gonads and digest gland. CgMELC mRNA was expressed at the highest level in the striated adductor muscle ($P < 0.05$), followed by the smooth adductor muscle and mantle. During the development of embryos, CgMELC mRNA was not expressed until blastula stage ($P < 0.05$). CgMELC gene expression increased sharply from trochophore ($P < 0.05$) and peaked in D-shaped larvae, and then decreased in umbo and eyed larvae (Fig. 5B).

3.4. Spatiotemporal expression of CgMELC during the embryo-larvae development

The spatiotemporal expression of CgMELC from fertilized eggs to eyed larvae was investigated using whole-mount in situ hybridization. Consistent with the results of RT-qPCR, CgMELC was expressed starting from blastula stage and continuing thereafter (Fig. 6). Specific staining was detectable at the adductor muscle, velum retractor muscle and foot during the D-shaped, umbo and eyed larval development stages.

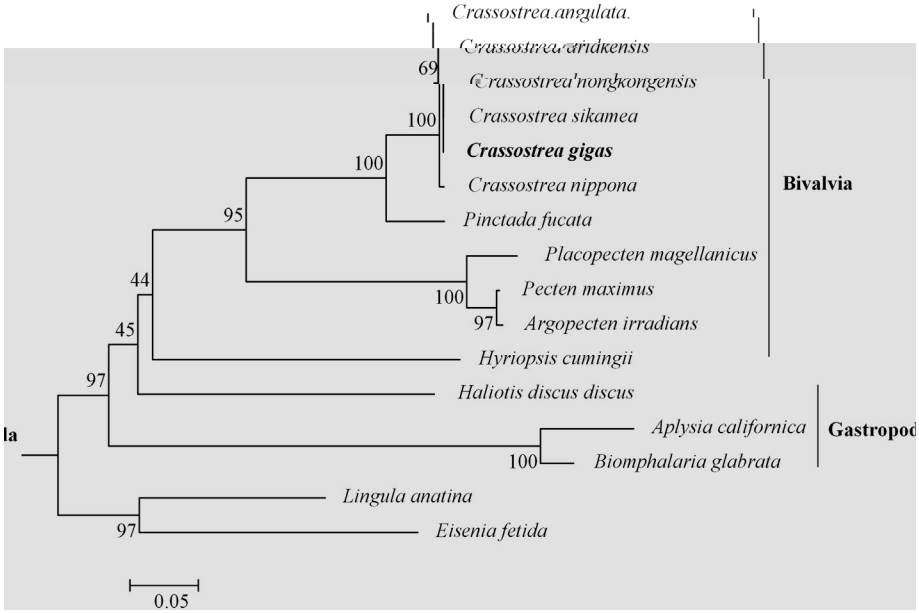

Fig. 4. Neighbor-joining phylogenetic tree based on the amino acid sequences of MELC. Numbers at tree nodes refer to percentage bootstrap values after 1000 replicates.

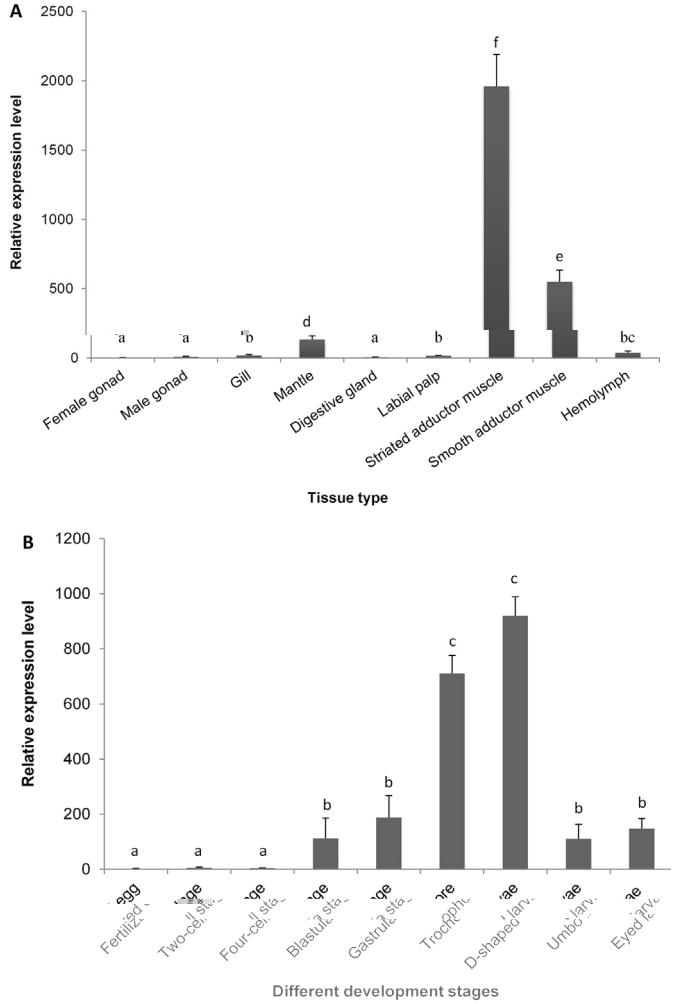

Fig. 5. Expression profiles of CgMELC. Different letters indicated significantly different ($P < 0.05$). A: Expression characterization of CgMELC in various adult tissues. B: The temporal expression of CgMELC during embryo-larval developmental stages.

4. Discussion

MELC genes belong to the EF-hand superfamily and consist of EF hand-like domains. The EF hand is a protein sequence motif that potentially may bind divalent cation. In molluscs, MELC has been confirmed to have Ca^{2+} binding site and involved in regulation of myosin in many species (Kwon et al., 1990; Xie et al., 1994; Fromherz and Szent-Györgyi, 1995; Katoh et al., 2002). In the present study, we identified a 659 bp full-length cDNA sequence in *C. gigas*, which encoded a 157 amino acid sequence containing a conserved EF-hand calcium binding motif, implying the potential Ca^{2+} binding function of CgMELC.

The amino acid sequence of CgMELC showed a high conservativeness (68.4–100%) with other species of the Bivalvia. A sequence comparison of the MELCs among the genus *Crassostrea* yielded only one amino acid difference, which was a conservative replacement. The high sequence similarity suggested the conserved functions of MELC gene among bivalves. Several important conserved amino acid sites unique to molluscan MELCs were observed in the 11 bivalve MELC sequences. Five residues in the Ca^{2+} binding loop (FWDGR), which might be related to the unique ability of molluscan MELCs to bind Ca^{2+} (Fromherz and Szent-Györgyi, 1995), were fully conserved among the 11 bivalves. In addition, the conserved triol group near the end of the α2 helix observed in MELC among the 11 bivalve species (Fig. 3), provided further evidence that this thiol group might be a special feature of molluscan MELC (Barouch et al., 1991). Five amino acids specific to the genus *Crassostrea* were detected at position 27, 32, 41, 51, and 73 (Fig. 3). However, the effect of these mutation was unknown, and more precise researches are still needed. The phylogenetic tree of MELC protein showed that *Crassostrea* had a relatively close genetic relationship with *P. fucata*, in keeping with traditional classification.

The CgMELC was expressed especially highly in the striated adductor muscle, and relatively weakly in the smooth adductor muscle and mantle, but significantly higher than in other non-muscle tissues. In previous studies on scallops, nucleotide and amino acid sequences of MELCs were compared between striated and smooth muscles, and no difference was detected (Perreault-Micale et al., 1996). Yet, few studies on the tissue expression pattern of MELC has been reported in molluscs. Nearly four-folds higher mRNA level of CgMELC was measured in the adult striated adductor compared to the smooth portion. It has been reported that oyster striated muscle myosin moved the actin filaments about five times as fast as smooth muscle myosin (Tsutsui et al., 2007).

Fig. 6. Localization of CgMELC mRNA in various embryo-larvae developmental stages by whole-mount in situ hybridization.

MELC, with the Ca^{2+} binding site, can bind Ca^{2+} and then trigger contraction in molluscan muscles (Fromherz and Szent-Györgyi, 1995). We presumed that the high expression of CgMELC in the striated adductor muscle might contribute to the rapid shortening speed.

During the embryo-larval developmental stages, the CgMELC transcript was expressed in oyster larvae starting from the blastula stage and abundantly expressed in trochophore and D-shaped larvae. In molluscs, the pioneer muscle cells were reported to first appear at the trochophore stage and well-organized musculature was formed at veliger stage (Wanninger et al., 1999; Wanninger and Haszprunar, 2002; Dyachuk and Odintsova, 2009). However, some muscle-related genes (e.g. twitchin, myorod) were expressed in larvae starting from the blastula stage (Dyachuk and Odintsova, 2009). It was proposed that synthesized muscle proteins were located at early developmental stages in the supramolecular complexes and only thereafter they participated in filament assembly (Odintsova et al., 2006; Dyachuk and Odintsova, 2009). In trochophore and D-shaped larvae stages, the larval muscle system sharply developed. During *C. gigas* larvae development, expression of MELC mRNA sharply increased in trochophore and D-shaped larvae stages, indicating that MELC might be involved in regulation of larval muscle system development in oysters. After the well-organized musculature was formed, in umbo and eyed larvae, the

expression of CgMELC decreased.

In summary, the full length of MELC gene of *C. gigas* was identified for the first time, and expression profiles of the MELC transcript in various tissues and different embryo-larval developmental stages were detected by RT-qPCR. Spatial expression patterns of the MELC transcript during the embryo-larvae development were revealed by whole-mount in situ hybridization. It is interesting that the MELC transcript expresses significantly higher in the striated adductor muscle than in the smooth adductor muscle, and abundantly expresses in trochophore and D-shaped larvae stages. These findings will provide useful information on the fundamental knowledge of oyster myosin function.

Acknowledgments

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