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Characterization of cDNAs for calmodulin and calmodulin-like protein in the freshwater mussel *Hyriopsis cumingii*: Differential expression in response to environmental Ca^{2+} and calcium binding of recombinant proteins

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ABSTRACT

Calmodulin and calmodulin-like protein are two crucial calcium regulators in bivalves. However, molecular characteristics and expression patterns of these genes in the freshwater mussel are poorly understood. In this study, two cDNAs encoding novel calmodulin and calmodulin-like protein (*Hc*CaM and *Hc*CaLP) were cloned and characterized from the freshwater pearl mussel *Hyriopsis cumingii*. The full-length cDNA of *Hc*CaM was 726 bp, including a 118-bp 5′-untranslated region (UTR), a 447-bp open reading frame (ORF), and a 161-bp 3′-UTR. The 1217-bp *Hc*CaLP cDNA comprised of a 51-bp 5′-UTR, a 447-bp ORF, and a 716-bp 3′-UTR. The potential phosphorylation sites of, Arg^{80} and Phe^{100} in deduced HcCaM were mutated to Thr^{80} and Tyr^{100} in HcCaLP. Tissue-specific expression analysis revealed that *Hc*CaM mRNA was prominently expressed in the gill, mantle center, and foot. In contrast, *Hc*CaLP mRNA was mainly expressed in the mantle edge. The recombinant HcCaM and HcCaLP proteins expressed in *Escherichia coli* showed the typical Ca^{2+} dependent electrophoretic shift characterization as CaM and differed in the calcium binding affinity. The calcium stimulation test that lasted 5 weeks implied that *Hc*CaM and *Hc*CaLP had differential expression patterns in response to various environmental Ca^{2+} concentrations (0.25–1.25 mM). The expression of *Hc*CaM mRNA was up-regulated by low Ca^{2+} concentration (0.25 mM), and the highest expression of HcCaLP mRNA occurred under Ca^{2+} concentration of 1 mM.

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1. Introduction

Bivalve shell consists of calcium carbonate, matrix proteins and other organic matrices, and calcium carbonate accounts for approximately 95% of shell weight. Biomineralization of bivalve shell is regulated by calcium metabolism, including the sequential processes of calcium absorption, accumulation, transportation, and incorporation (Huang et al., 2007; Machado and Lopes-Lima, 2011). Calcium metabolism is controlled by many Ca^{2+} regulators, such as calcitonin (Breimer et al., 1988), troponin C (Ojima et al., 1994), calmodulin (CaM) (Li et al., 2004), calmodulin-like protein (CaLP) (Li et al., 2005), and calconectin (Duplat et al., 2006). CaM is a crucial and ubiquitous calcium sensor protein in eukaryotes and mediates cellular functions by interacting with numerous target proteins, including calcineurin, myosin light chain kinases, and phosphorylase kinase in various metabolic and signaling pathways (Friedberg and Rhoads, 2001; Ikura and Ames, 2006). In marine bivalves, CaM is a regulator of the membrane Ca^{2+}-ATPase system that is responsible for uptake, transportation, and secretion of Ca^{2+} in the gill and mantle (Stommel et al., 1982; Stommel and Stephens, 1985; Li et al., 2004). Calmodulin-like protein (CaLP), another member of the CaM superfamily, has been identified in a wide range of organisms from bacteria to mammals (Nikapitiya et al., 2010). CaLP acts as a multifunctional calcium sensor and regulates calcium metabolism in various processes, such as Ca^{2+} transportation in buffalo sperm (Sidhu and Guraya, 1993), epithelial cell differentiation in humans (Rogers et al., 2001), and shell formation in pearl oyster *Pinctada fucata* (Li et al., 2005). As a component of the organic layer in bivalve shell, CaLP also induces the nucleation of aragonite through binding with the 16-kDa nacre protein, and regulates the growth of calcite in the prismatic layer or participates in the shell regeneration (Yan et al., 2007; Fang et al., 2008). To date, the presence of multiple *CaM* and *CaLP* genes has been observed in marine molluscs (Li et al., 2004; Li et al., 2005; Simpson et al., 2005).

Calcium metabolism pattern is obviously different between freshwater and marine bivalves due to the Ca^{2+} concentration difference in freshwater and seawater (Richardson et al., 1981). Earlier studies reported that increasing calcium availability can enhance calcium

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deposition in shell of freshwater mussels (Wilbur and Jodrey, 1952; Dalesman and Lukowiak, 2010). However, few calcium regulators have been reported from freshwater bivalves, and the molecular mechanism of calcium metabolism in freshwater mussels is not clear (Zeng et al., 2012). In the present study, we isolated and characterized full length cDNAs of two calcium regulators, *Hc*CaM and *Hc*CaLP from freshwater pearl mussel *Hyriopsis cumingii*, which contributes more than 95% pearl yield in the world (Wang et al., 2009). We also tested the differential expression of *Hc*CaM and *Hc*CaLP under different environmental Ca^{2+} concentrations.

2. Materials and methods

2.1. Mussel and sample collection

Mussels from a commercial freshwater pearl mussel farm at Fengqiao, Zhuji (Zhejiang, China) were collected during the fast growth stage in September, 2012. Six mussels including 3 1-year-old mussels and 3 2-year-old mussels were sampled, and 9 samples were isolated from each mussel for tissue expression profiling, including gill (GL), intestine (IN), foot (FT), gonad (GN), adductor muscle (AM), mantle center (MC), mantle edge (ME), posterior of mantle pallial (pMP), and middle of mantle pallial (mMp). The samples were frozen in liquid nitrogen and stored at −80 °C.

2.2. Rapid amplification of cDNA ends (RACE)

Total RNA was extracted from the mantle pallial of 2-year-old mussels using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer protocol, and then quantified by measuring absorbance at 260 nm with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The full-length cDNAs of *Hc*CaM and *Hc*CaLP were synthesized from total RNA using the SMART™ RACE cDNA Amplification Kit (Clontech, PaloAlto, CA, USA) according to the manufacturer's instructions. To obtain the 3′ terminal sequence of the *Hc*CaM and *Hc*CaLP cDNA ends, the forward primer HcCaMF1 was designed based on the conserved regions in the open reading fragment (ORF) of *H. schlegelii Hs*CaM cDNA sequence (GenBank Accession No. FJ194962) and a *H. cumingii* EST sequence (GW692024) (Table 1). 3′-RACE PCR was performed in a 50-μL reaction mixture comprising 1.0 μL of Advantage 2 Polymerase Mix (Clontech, PaloAlto, CA, USA), 5.0 μL of Advantage 2 PCR Buffer, 1.0 μL of *Hc*CaMF2 primer (10 μM), 5 μL of universal primer mix (UPM, 10×), and 2.5 μg of total RNA. PCR products were electrophoresed on a 1.2% agarose gel and stained with ErBr. Bands of approximately 700 bp and 1200 bp were excised from the gel respectively, purified using a DNA Fragment Purification Kit (TaKaRa, Dalian, China), and subsequently cloned into a pMD19-T vector using a TA cloning kit (TaKaRa) and sequenced. Based on the 3′-cDNA end sequences of *Hc*CaM and *Hc*CaLP, gene-specific primers were designed for two rounds of 5′-RACE PCR and listed in Table 1. PCR of 5′-cDNA ends and cloning of the products were carried out as described above for 3′-cDNA ends.

Table 1
Primers and their applications in this study.

Primer name	Sequence 5′ → 3′	Use
HcCaMF1	GGAGGCGTTCAGCCTGTTT	3′ RACE
HcCaMR1	AACAACATTTGCATTACTTATGCCCTTCCAC	1st round 5′ RACE
HcCaMR2	TGCCCTTCCACAGCTCTATCTCCTTATTGT	2nd round 5′ RACE
HcCaLPR1	AACTTCGGTGAAGCGGAAAAGGATTTG	1st round 5′ RACE
HcCaLPR2	GGAATTCKGGGAAATCWATCGTKCCATT	2nd round 5′ RACE
HcCaLPF3	TTTCACTTCCATAACACCTGCT	ORF amplification
HcCaLPR3	AACTTCGGTGAAGCGGAAAAGGATTTG	ORF amplification
RT-HcCaMF	GCAGCAGAACTCAGACACGTGAT	Realtime PCR forward
RT-HcCaMR	CCCTTCCACAGCTCTATCTCCTTAT	Realtime PCR reverse
RT-HcCaLPF	CAAATCCTTTTCCGCTTCACC	Realtime PCR forward
RT-HcCaLPR	CGTTGTTGACATTGCTCCAGAA	Realtime PCR reverse
RC-HcCaMF	ATGGCTGACCAACTGACGGAAGAAC	Domain amplification
RC-HcCaMR	TTATTTACTCGTCATCATCTGCACG	Amplification domain and positive recombinant clone
RC-HcCaLPF	ATGGCAGACCAACTAACAGAAGAAC	Domain amplification
RC-HcCaLPR	TCACTTCGACATCATCATCCTCACG	Amplification of domain and positive recombinant clone
actin-F	CCCTGGAATCGCTGACCGTAT	Realtime PCR forward
actin-R	GCTGGAAGGTGGAGAGAGAAG	Realtime PCR reverse
T7 Promoter	TAATACGACTCACTATA	Recombinant clone amplification

2.3. Sequence analysis

Determination of the gene, ORF, and protein sequence was performed using the Expert Protein Analysis System (ExPASy, http://au.expasy.org). Multiple sequence alignments were analyzed using the Cluster W 1.81 (Thompson et al., 1994). Domain prediction was undertaken using the simple modular architecture research tool (SMART, http://smart.embl-heidelberg.de/). The prediction of phosphorylation sites was carried out by NetPhos 2.0 Server (http://www.cbs.dtu.dk/services/NetPhos/).

2.4. Tissue specific expression of the HcCaM and HcCaLP genes

Expression analysis was performed by real-time quantitative PCR using the iQTM5 Multicolor Real-Time PCR Detection System Cycler (BioRad, Hercules, CA, USA). Total RNA was extracted with TRIzol Reagent (Invitrogen). cDNA from total RNA was synthesized by a PrimeScript® Reverse Transcriptase kit (TaKaRa), using oligo $(dT)_{18}$ as primer. Gene-specific primers, RT-HcCaMF and RT-HcCaMR for PCR were designed based on the 3′ terminal region of ORF and 3′-UTR sequence of *Hc*CaM mRNA respectively, and RT-CaLPF and RT-HcCaLPR on the 3′-UTR sequences of *Hc*CaLP mRNA (Table 1). *β-actin* was used as endogenous reference gene for calibration, and primers actin-F and actin-R were designed according to the *β-actin* cDNA sequence (GenBank Accession No. HM045420) (Table 1).

Real-time quantitative PCR was performed with the SYBR® Premix Ex Taq™ PCR kit (TaKaRa). Amplifications were performed in 25 μL. An aliquot of 1.0 μL of the cDNA synthesized above was used as template. The cycling conditions for *Hc*CaM, *Hc*CaLP and *β-actin* were the same as follows: 1 min at 95 °C, followed by 40 cycles (95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s). Melting curves were also performed with 0.5 °C increments from 50 °C to 95 °C to ensure that a single PCR product was amplified using each pair of primers. The relative mRNA levels were normalized to *β-actin* transcripts using the following formula $N = 2^{(Ct\ \beta\text{-actin} - Ct\ \text{target gene})}$.

2.5. Recombination and protein purification of HcCaM and HcCaLP

The full coding regions of *Hc*CaM and *Hc*CaLP were amplified using the primers of RC-HcCaMF/R and RC-HcCaLPF/R separately (Table 1), and were ligated to the expression vector pEASY-E1 (TransGen Biotech, Beijing, China). The recombinant plasmids, pEASY-E1-HcCaM and pEASY-E1-HcCaLP were transformed into Trans1-T1 phage resistant chemically competent cell of *Escherichia coli* (TransGen Biotech). The forward positive clones were screened by PCR with vector primer T7 promoter primer and recombinant reverse primers (Table 1), and further confirmed by sequencing. The positive recombinant plasmid, pEASY-E1-HcCaM and pEASY-E1-HcCaLP, were isolated by AxyPrep ™ Plasmid Miniprep Kit (Axygen Biosciences, Union City, Ca, USA) and transferred into *E. coli* BL21 (DE3) (TransGen Biotech), incubated in LB medium. The *E. coli* BL21 (DE3) inserted pEASYE1 vector without insert fragment was also cultured in LB medium as negative control. Protein expression was induced for 4 h after addition of 1.0 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37 °C. The

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