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Calmodulin is a stress and immune response gene in Chinese mitten crab *Eriocheir sinensis*

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ABSTRACT

Calmodulin (CaM) is a multifunctional calcium sensor protein that participates in various cellular processes under normal, stress and pathological conditions. In crabs, however, the involvement of CaM in response to environmental stress and immune challenges has not been revealed yet. In the present study, a *CaM* cDNA (*EsCaM*) was identified from Chinese mitten crab *Eriocheir sinensis* and its mRNA expression patterns in response to ambient (salinity and pH) stress and immune challenges was examined. *EsCaM* encodes a 149-amino-acid protein with a calculated molecular mass of 16.8 kDa and an isoelectric point of 4.09. In unstimulated healthy *E. sinensis*, *EsCaM* mRNA transcript was detected in all tested tissues with predominant expression in hepatopancreas and the lowest expression in haemocytes. Ambient salinity (15‰ and 30‰ salinities) and pH (pH 6 and 8.5) stress significantly altered *EsCaM* mRNA expression in gill, hepatopancreas, haemocytes, intestine and muscle in Chinese mitten crab. In addition, *EsCaM* gene expression was significantly and rapidly induced as early as 2 h after LPS and Poly(I:C) immune stimulations in haemocytes *in vitro*. Furthermore, *EsCaM* expression was significantly up-regulated in *E. sinensis* haemocytes, gill, hepatopancreas, intestine and muscle in response to *Edwardsiella tarda* and *Vibrio anguillarum* challenges. Collectively, our findings suggest that *EsCaM* is an important stress and immune response gene in Chinese mitten crab.

1. Introduction

Chinese mitten crab, belonging to the genus of *Eriocheir* and the family Grapsidae, is an important economic crustacean species with increasing market demand in China. The intensive farming over the last few decades, however, has been accompanied with the outbreak of infectious diseases caused by viruses, bacteria and other pathogens, and has resulted in significant economic losses in Chinese mitten crab aquaculture industry [1–4]. In addition to diseases, the survival of Chinese mitten crab is also often affected by its external environmental stress such as salinity and pH changes. Finding the genes responding to the environmental stress and immune challenges in Chinese mitten crab thus will be helpful in understanding the mechanisms for *Eriocheir sinensis* coping with environmental stress and diseases.

Calmodulin (CaM) is a ubiquitous, highly conserved, eukaryotic protein that binds to and regulates a number of diverse target proteins involved in various immune functions such as programmed cell death, autophagy, inflammation and the immune response in vertebrates [5,6]. CaM has been identified as a tightly bound subunit of inducible nitric oxide synthase (iNOS) in murine macrophages [7] and is required for iNOS enzyme activation [8]. In human beings, CaM has been found to be involved in HIV-triggered and Fas-dependent T-cell apoptosis [9]. Previous study also demonstrated that Ca^{2+}/CaM-dependent protein kinase kinase 2 regulated macrophage-mediated inflammatory responses [10]. In addition, CaM is involved in CaM kinase II gamma initiated cAMP/PKA pathway that induces ERK 1/2 phosphorylation to promote caspase-3 mediated apoptosis of the *Aeromonas hydrophila*-infected catfish head kidney macrophage [11]. In shrimp *Penaeus monodon*, *CaM* has been found to be one of three highly responsive genes (*CaM*, *tubulin* and *asialoglycoprotein* receptor) in response to shrimp pathogens, WSSV and the luminescence bacteria *Vibrio harveyi* [12]. Ji et al. also reported that *CaM* might play an important role in shrimp's defense against pathogenic infection [13]. In addition to the involvement in immunity, *CaM* is also an important

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stress response gene. It has been demonstrated that CaM is involved in goldfish osmoregulation [14] probably through regulation of osmoregulation-related hormone gene expression. In pearl oyster *Pinctada fucata*, seawater acidification can also affect *CaM* gene expression pattern [15]. Moreover, it has been reported that salinity stress can result in an increased expression of *CaM* in the gills of blue mussels [16].

In aquatic invertebrates, *CaM* cDNAs have been identified and characterized from pearl oyster [17,18], mussel [19] and shrimp [13,20]. In crabs, however, the relevance of *CaM* in response to environmental stress and immune challenges has not been revealed yet. In an attempt to examine the potential roles of *CaM* in response to environmental stress and immune challenges in *E. sinensis*, we identified a *CaM* cDNA (termed *EsCaM*) and analyzed its expression patterns by quantitative real-time PCR. We found that *EsCaM* gene expression was significantly altered in response to the environmental (salinity and pH) stress and immune challenges as well. This study highlighted the importance of CaM-mediated signaling pathways in stress and immune response in the Chinese mitten crabs.

2. Materials and methods

2.1. Animals and maintenance

Chinese mitten crabs *E. sinensis* were obtained from a local farm in Qilihai, Tianjin, China and transported to the laboratory. Crabs were cultured with aerated tap water at 19 ± 1 °C for 2 weeks before experiments to acclimate laboratory conditions. Animals were kept on a natural daylight cycle and fed with commercial feed. Only healthy crabs without any pathological signs were used in the experiments.

2.2. RNA preparation and cDNA synthesis

Total RNA from different tissues of *E. sinensis* was isolated by the TRIzol reagent (Invitrogen) and the integrity of RNA was examined by electrophoresis on a 1.2% formaldehyde-denatured agarose gel with ethidium bromide staining on a GelDoc™ XR system (Bio-Rad). Total RNA was quantified by measuring OD_{260} with a NanoDrop 2000 UV/Vis spectrophotometer (Thermo Fisher Scientific) and deoxyribonuclease treated (Invitrogen, amplification grade) to remove genomic DNA contamination according to the manufacturer's protocol. Aliquots of total RNA (2 μg) were then used to synthesize single-strand cDNA with PrimeScript™ RT kit (TaKaRa) following the manufacturer's instructions.

2.3. Isolation of EsCaM cDNA

By searching the transcriptome sequences of combined *E. sinensis* eyestalk, Y-organ and hepatopancreas tissues cDNA library [21], one clone encoding a *CaM* homolog, termed *EsCaM*, was identified. To confirm the correction of *EsCaM* sequence, a two-round of nested PCR was carried out using the cDNAs synthesized from hepatopancreas tissue of *E. sinensis* as template. The first round of PCR was performed with a forward primer F1 (5′-CCTGAGACAGTGGTTCGG-3′) and a reverse gene specific primer R1 (5′-CGATGCTGGACACACCTG-3′), which was followed by a nested amplification with primers F2 (5′-GTCTGTCCCTTGGCTTGT-3′) and R2 (5′-CGCTTCAGAGCCAGATAG-3′) corresponding to the 5′-untranslated and 3′-untranslated regions of *EsCaM* mRNA, respectively, in a MyCycler™ gradient thermocycler (Bio-Rad). PCR products were separated on a 1.5% agarose gel in Tris-acetate EDTA buffer. Amplicons with expected size (664 bp) were excised, purified and cloned into pMD18-T vector. Three independent clones were selected and sequenced with both strands, which showed 100% sequence identity.

2.4. DNA and protein sequence analyses

Nucleotide sequences were searched against GenBank database at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast) using BlastX algorithm to identify their coding proteins. The deduced amino acid sequence was analyzed with the Expert Protein Analysis System (http://www.expasy.org/) to identify the conserved protein domain. Multiple sequence alignments were performed using ClustalW multiple alignment program (http://www.ebi.ac.uk/clustalw/) [22].

2.5. Tissue distribution of EsCaM mRNA transcript in E. sinensis

The tissue distribution pattern of *EsCaM* mRNA transcript in healthy *E. sinensis* was examined by quantitative real-time PCR. Tissues including haemocytes, brain, gill, muscle, stomach, heart, hepatopancreas, ganglion and intestine from 6 individual healthy crabs (average 100 ± 10 g) were dissected and collected and each kind of tissue was equally pooled to minimize individual variability. Aliquots (2 μg) of total RNA from each type of tissue was transcribed into cDNAs as described previously.

2.6. Stress experiment designing

For salinity stress experiment, salinity was prepared by dilution seawater with tap water. One hundred Chinese mitten crabs (mean weight 20 ± 3 g) were divided into three groups: control (tap water), and salinities of 15‰ and 30‰. For pH stress experiment, tap water (control) was adjusted to the pH values of 6.0 and 8.5, respectively, by the addition of sterile 1 M HCl/NaOH. One hundred crabs (average 20 ± 3 g) were divided into three groups: control (tap water), and pH values of 6.0 and 8.5, and kept at 19 °C. Five crabs were randomly collected from each group at 0, 4, 12, 24, 48 and 72 h post treatment. Tissues including haemocytes, gill, hepatopancreas, intestine and muscle were dissected at each time point and total RNA was isolated. The *EsCaM* gene expression under salinity and pH stress conditions is relative to its expression in the control group (tap water), which is determined by quantitative real-time PCR.

2.7. Haemocytes preparation and cell culture

Chinese mitten crab haemocytes were prepared as described previously [23]. Briefly, haemocytes from individual healthy crab (average 100 ± 10 g) were collected with an anti-coagulant treated 1 ml syringe (25 gauge), and immediately diluted with equal volume of anti-coagulant buffer (in mM) [450 NaCl, 100 glucose, 30 sodium citrate, 26 citrate acid, 10 EDTA; pH 7.2]. Haemocytes from 40 individual animals were pooled and centrifuged at 800 g for 10 min at 4 °C. The cells were washed two times with L15 culture medium (Invitrogen) supplemented with 0.6 g/L glutamine and 1% penicillin-streptomycin liquid (Invitrogen). Haemocytes were then cultured overnight at 21 °C in a 24-well plate (2.5×10^6 cell/well) with culture medium supplemented with 10% FBS (Invitrogen) and 1% antibiotics before *in vitro* immune challenge experiments.

2.8. EsCaM mRNA expression in LPS and Poly(I:C) challenged Chinese mitten crab haemocytes

Overnight cultured Chinese mitten crab haemocytes were stimulated with 30 μg/ml Poly(I:C) (Sigma–Aldrich, catalog No. P1530) or LPS from *Escherichia. coli* serotype O55:B5 (Sigma–Aldrich,