Fish & Shellfish Immunology 67 (2017) 518–522

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Short communication

# Characterization and function of a novel calmodulin-like protein from crayfish *Procambarus clarkii*

Baojian Zhu*, Yingying Yu, Jin Gao, Yuanyuan Feng, Lin Tang, Yuxuan Sun, Liangli Yang

*College of Life Sciences, Anhui Agricultural University, Hefei 230036, China*

ARTICLE INFO

*Article history:*
Received 8 March 2017
Received in revised form
24 May 2017
Accepted 3 June 2017

*Keywords:*
*Procambarus clarkii*
Calmodulin-like
Expression
Calcium-binding

ABSTRACT

Calmodulin plays an important role in calcium-dependent signal transduction pathways. In this experiment, a novel calmodulin-like gene (*Pc-CaM-L*) was identified in the crayfish *Procambarus clarkii*; it encodes a polypeptide of 145 amino acids. Quantitative real-time PCR analysis revealed that *Pc-CaM-L* was expressed in all examined tissues, including hepatopancreas, hemocytes, heart, gill, intestine and muscle; the highest *Pc-CaM-L* expression level was detected in the hepatopancreas. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blot analysis demonstrated that a recombinant Pc-CaM-L protein was successfully expressed in *Escherichia coli*. The calcium-binding activity of the purified Pc-CaM-L protein was confirmed by gel mobility shift assay. The expression of *Pc-CaM-L* was significantly upregulated in gut, gill and hemocytes after lipopolysaccharide or polyinosinic:polycytidylic acid induction. These results suggest that *Pc-CaM-L* plays a role in the immune response of *P. clarkii*.



## 1. Introduction

As an important intracellular signal, calcium plays key roles in cell proliferation, apoptosis and metabolism via multiple signal transduction pathways [1–4]. Calmodulin is a $Ca^{2+}$-dependent protein with a molecular weight of about 17 kDa that is ubiquitously expressed in eukaryotic cells of plants and animals [5–7]. It contains EF-hand calcium-binding motifs and regulates many cellular processes, including inflammation, stress response, $Ca^{2+}$ homeostasis and apoptosis, through interaction with targets such as enzymes and cytoskeletal proteins [8–11].

A few calmodulin proteins have been identified in aquatic invertebrates, such as shrimp species *Crangon crangon* [12], *Litopenaeus vannamei* [13] and *Penaeus monodon* [14], the crab *Eriocheir sinensis* [15], oysters *Pinctada fucata* and *Crassostrea gigas* [16,17], abalones *Haliotis discus* and *H. diversicolor* [18,19], the crayfish *Procambarus clarkii* [20] and mussels *Hyriopsis cumingii* and *H. schlegelii* [21,22]. Calmodulins have been described as multifunctional proteins that participate in many physiological processes in crustaceans. For example, *E. sinensis* calmodulin was significantly induced by bacterial challenge, and saline or pH stress obviously altered the expression of calmodulin in various tissues [15]. Similarly, the expression levels of calmodulin significantly changed after *Vibrio parahaemolyticus* or White Spot Syndrome Virus challenge of *L. vannamei* [13]. It was reported that expression of *P. clarkii* calmodulin increased during premolt and postmolt stages, and the upregulation was hormonally mediated [20]. In *P. fucata,* the calmodulin was involved in shell formation [23]. In addition, calmodulin also participats in calcium metabolism in aquatic animals [24].

The crayfish *Procambarus clarkii* is a commercially important decapod aquaculture species in China, but knowledge of this species at the molecular level is limited because of a lack of genomic resources [25]. Although three calcium-binding protein variants have been identified from *P. clarkii* [20,26], other calcium binding proteins in this organism and their functions remain to be discovered. In the present study, we describe the cloning, expression and biological function of a novel calmodulin-like protein from *P. clarkii*.

## 2. Materials and methods

### 2.1. Animals

*P. clarkii* (about 8 g each) were sampled from a market in April and kept in tanks containing continuously flowing water for 1 week before experiments. These crayfish were fed small invertebrates

* Corresponding author.
*E-mail address:* zhubaojian@ahau.edu.cn (B. Zhu).

http://dx.doi.org/10.1016/j.fsi.2017.06.005
1050-4648/

and kept in a 12 h-light and 12 h-dark photoperiod at room temperature. The crayfish were euthanized using carbon dioxide following procedures in agreement with the principles for the care and use of animals [27]. The hepatopancreas, muscle, heart, intestine, hemocytes and gill were collected and immediately frozen in liquid nitrogen until use.

### 2.2. Identification of Pc-CaM-L gene

Total RNA was extracted from hepatopancreas using Trizol™ Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Agrose electrophoresis (1%) and a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, USA) were used to check the integrity of RNA. First strand cDNA was generated using a cDNA Synthesis Kit (TransGen Biotech Co. Ltd., Beijing). Primers F1 and R1 (Table 1) were designed to clone the open reading frame of the *Pc-CaM-L* cDNA sequence which was obtained from the hepatopancreas transcriptome sequenced in our laboratory. PCR was carried out following the protocol: an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s. PCR products were purified and sequenced by Invitrogen, Shanghai. Clustal X software [28] was used for multiple sequence alignments of Pc-CaM-L based on the amino acid sequence and a phylogenetic tree was constructed using MEGA software version 5 with the maximum-likelihood method and a bootstrap test of 1000 replications [29].

### 2.3. Protein expression and purification

PCR products were digested with restriction enzymes (*EcoR*-I and *Xho*-I), then ligated into expression pGEX-4T-1 (Novagen, USA). The resulting recombinant plasmid pGEX-4T-1- CaM-L was confirmed by DNA sequencing and transformed into *Escherichia coli* BL21 (DE3) (Novagen) for protein expression. Isopropyl-β-D-thiogalactopyranoside (IPTG) was used to induce protein expression for 4 h at 37 °C. The *E. coli* cells were harvested by centrifugation at 8000 × g for 15 min at 4 °C and then lysed by sonication. The recombinant fusion protein was analyzed by 12% Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Glutathione Sepharose 4B chromatography was used to purify the recombinant protein. Quantification of total protein was performed using the bicinchoninic acid (BCA) method [30].

### 2.4. Western blotting

Proteins were separated by 12% SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane (Sigma, USA) using an electrophoretic transfer system (Bio-Rad). Membranes were blocked with 5% non-fat milk diluted in PBST (phosphate-buffered saline containing 0.1% Tween-20) for 2 h at 25 °C. After washing with PBST, membranes were incubated with primary antibodies (diluted 1:1000 with PBST) for 2 h at 25 °C. Subsequently the membranes were incubated with secondary antibody (diluted 1:1000 with PBST) for 2 h at 25 °C. Detection was performed with a horseradish peroxidase-3,3-diaminobenzidine (HRP-DAB) detection kit (Qiagen, Germany).

**Table 1**
Primers used for PCR in this study.

| Primer no. | Primer sequence (5′ -3′) |
|---|---|
| F1 | CCGGAATTCATGCCACGGCAGTTGACC |
| R1 | CCGCTCGAGCTATTTAGGGCCGGACATCAT |
| F2 | TCAAATATCCGAGGCCAAAG |
| R2 | CTCTTCAGGGGACTCGACAG |
| 18s SF | CTGTGATGCCCTTAGATGTT |
| 18s SR | GCGAGGGGTAGAACATCCAA |

Note: restriction enzyme sites are underlined.

### 2.5. Electrophoretic mobility shift assay

To investigate the calcium-binding activity of Pc-CaM-L protein, gel mobility shift assays were performed according to a reported method [31]. Purified Pc-CaM-L protein (5 μg) was separated on 12% nondenaturing polyacrylamide gel electrophoresis in the presence of 20 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM $CaCl_2$, or without added $CaCl_2$. The electrophoretic mobility of bovine serum albumin (BSA) was used as a control. The gels were stained with Coomassie Brilliant Blue R-250.

### 2.6. Expression pattern analysis of Pc-CaM-L

Muscle, heart, intestine, gill, hemocytes and hepatopancreas were dissected from six *P. clarkii* crayfish and used to determine the tissue distribution of *Pc-CaM-L* expression. To investigate the expression of *Pc-CaM-L* in response to lipopolysaccharide (LPS) or polyinosinic:polycytidylic acid (poly I: C) challenge, crayfishes were randomly divided into three groups of six individuals per group. In the first two groups, LPS (10 μL, 1 mg/mL diluted in sterilized PBS buffer) or poly I: C (10 μL, 1 mg/mL diluted in sterilized PBS buffer) was, respectively, injected into each crayfish. In the third group, crayfish injected with sterilized PBS were used as the negative control. Intestine, gill and hemocytes were collected from *P. clarkii* 12, 24, 48, 72 and 96 h after injection. Total RNA was extracted using Trizol and the first strand cDNA was synthesized using PrimeScript™ RT Master Mix (Takara). Gene specific primers F2 and R2 (Table 1) were designed using Primer 5.0 software based on known sequences and real-time PCR was performed to determine the expression levels of *Pc-CaM-L*. The real-time PCR reaction was carried out with a SYBR Premix Ex Taq™ Kit (Takara) in an iCycler iQ™ thermocycler (Bio-Rad) using the following procedure: initial denaturation at 95 °C for 3 min, followed by 40 cycles of amplification (95 °C for 10 s, 56 °C for 25 s and 72 °C for 20 s). Continuous fluorescence acquisition at 65–95 °C with an increasement of 0.5 °C per 10 s was used for melting curve analysis. The PCR efficiency of both reference and target genes was verified as approximately equal before real-time PCR. The relative expression level of *Pc-CaM-L* was determined by the $2^{-\triangle\triangle CT}$ method [32] and the 18S rRNA gene (GenBank accession no. AF436001) (Table 1) was used as the internal reference. All data were analyzed by Student's *t*-test or one-way ANOVA; the differences were considered statistically significant when $P < 0.05$.

## 3. Results

### 3.1. Sequence analysis of Pc-CaM-L

A cDNA fragment of 458 bp (GenBank accession no. CAI15835) encoding Pc-CaM-L was obtained by PCR. This open reading frame encodes 145 amino acid residues containing two EF-hand domains (amino acids 80–108 and 116–144) (Fig. S1). The molecular weight and theoretical isoelectric point of the protein are 16.2 kDa and 4.71, respectively. Protein sequence alignment indicated that Pc-CaM-L has a similarity of 68% with the reported calmodulin from *P. clarkii* (GenBank accession no. AGW23861) (Fig. S2A). The three-dimensional structure of Pc-CaM-L was different from that of *P. clarkii* calmodulin (Fig. S2B). These results suggest that Pc-CaM-L is a novel calmodulin like protein from *P. clarkii*. Comparison of Pc-CaM-L with calmodulin proteins from vertebrates and invertebrates