Developmental and Comparative Immunology 50 (2015) 87–97

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Characterization and identification of calmodulin and calmodulin binding proteins in hemocyte of the black tiger shrimp (*Penaeus monodon*)

Panjana Sengprasert [a], Piti Amparyup [b,c], Anchalee Tassanakajorn [b], Ratree Wongpanya [a,*]

[a] *Department of Biochemistry, Faculty of Science, Kasetsart University, 50 Pahonyothin, Bangkok 10900, Thailand*
[b] *Center of Excellence for Molecular Biology and Genomics of Shrimp, Department of Biochemistry, Faculty of Science, Chulalongkorn University, 254 Phayathai Road, Bangkok 10330, Thailand*
[c] *National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), 113 Paholyothin Road, Klong 1, Klong Luang, Pathumthani 12120, Thailand*

ARTICLE INFO

Article history:
Received 29 October 2014
Revised 4 February 2015
Accepted 5 February 2015
Available online 10 February 2015

Keywords:
Calmodulin
Penaeus monodon
Shrimp
Immune response
Signaling protein

ABSTRACT

Calmodulin (CaM), a ubiquitous intracellular calcium (Ca^{2+}) sensor in all eukaryotic cells, is one of the well-known signaling proteins. Previously, CaM gene has shown a high transcriptional level in hemocyte of the pathogen infected shrimp, suggesting that shrimp CaM does not only regulate Ca^{2+} metabolism, but is also involved in immune response cascade. In the present study, the CaM gene of shrimp *Penaeus monodon* was identified and the recombinant *P. monodon* CaM (r*Pm*CaM) was produced and biochemically characterized. The identification of CaM-binding proteins was also performed. The *Pm*CaM cDNA consisted of an open reading frame of 447 bp encoding for 149 amino acid residues with a calculated mass of 16,810 Da and an isoelectric point of 4.09. Tissue distribution showed that the *Pm*CaM transcript was expressed in all examined tissues. The results of gel mobility shift assay, circular dichroism spectroscopy and fluorescence spectroscopy all confirmed that the conformational changes of the r*Pm*CaM were observed after the calcium binding. According to the gene silencing of *Pm*CaM transcript levels, the shrimp's susceptibility to pathogenic *Vibrio harveyi* infection increased in comparison with that of the control groups. Protein pull-down assay and LC–MS/MS analysis were performed to identify r*Pm*CaM-binding proteins involved in shrimp immune responses and transglutaminase, elongation factor 1-alpha, elongation factor 2 and actin were found. However, by computational analysis, only the first three proteins contained CaM-binding domain. These findings suggested that *Pm*CaM may play an important role in regulation of shrimp immune system.

1. Introduction

The black tiger shrimp, *Penaeus monodon*, is an important marine species that is widely cultivated especially in tropical countries. The shrimp productions increase every year in Asia and Latin America (Bachère, 2000). However, shrimp aquaculture has faced significant problems with disease outbreaks resulting in the production collapses and economic losses (Flegel, 2006). Like other invertebrates, shrimp defense mechanism is relied mainly on the innate immunity which consists of physical barrier, humoral and particularly cellular responses. In the humoral barrier, many antimicrobial peptides, pathogen recognition receptors (PRRs), prophenoloxidase (proPO) and clotting proteins are produced to eradicate pathogens (Aguirre-Guzman et al., 2009; Zhao et al., 2009). For the cellular response, hemocyte plays a crucial role in phagocytosis, encapsulation and nodule formation (Aguirre-Guzman et al., 2009; Li and Xiang, 2012). However, these immune systems function in a concerted fashion for an effective response. Although the shrimp immune response has been extensively studied, co-operative working of each cascade via signaling pathway is still unclear. Therefore, the elucidation of proteins involved in the immune signaling system is required.

Calcium (Ca^{2+}) ions play crucial roles in cell signaling, helping the activation of many proteins for their various functions such as enzymes, transcription factors and transducers in cytosol. The signal transduction is induced by altering the intracellular concentration of Ca^{2+} ions which depends on various stresses such as external bioenergy, changes in membrane polarization, drug uptake and pathogen infection (Ji et al., 2011; Kiang et al., 2002; Pyrko et al., 2007; Vetter and Leclerc, 2003). Ca^{2+} ions can regulate proteins in

* Corresponding author. Department of Biochemistry, Faculty of Science, Kasetsart University, 50 Pahonyothin, Bangkok 10900, Thailand. Tel.: +66 2 562 5555 ext 2012; fax: +66 2 561 4627.
E-mail address: ratree.w@ku.ac.th (R. Wongpanya).

http://dx.doi.org/10.1016/j.dci.2015.02.003
0145-305X/

the signal transduction by binding directly to the proteins or binding to a Ca^{2+} binding protein that subsequently binds to a target protein (Aslam et al., 2012; Clough et al., 2002; Xu et al., 2012). Calmodulin (CaM) is one of the several proteins reported as a calcium binding protein.

CaM, a ubiquitous intracellular calcium (Ca^{2+}) sensor in all eukaryotic cells, is one of the well-known signaling proteins. The amino acid sequence of CaM is highly conserved through the evolution. This protein can bind and regulate proteins in various cascades. Structural analysis of CaM from various organisms has been characterized (Chattopadhyaya et al., 1992; Symersky et al., 2003). It was found that the CaM contains N- and C-terminal lobes linking with a flexible central linker. Each lobe is composed of two EF hand motifs with two Ca^{2+} binding sites (Babu et al., 1988; Ban et al., 1994; Chou et al., 2001). The binding of Ca^{2+} ions causes the conformational change of CaM by bringing two helices of each lobe to more perpendicular conformation and exposing hydrophobic residues on their surface. Therefore, CaM is able to interact with specific target proteins and regulate their functions (Meador et al., 1992; Wu et al., 2012).

In marine invertebrate, not only CaM does regulate Ca^{2+} metabolism of intracellular processes, but also plays a crucial role in extracellular processes such as biomineralization in mussel leading to shell formation (Zeng et al., 2012) and the regulation of molting in crayfish, *Procambarus clarkii* (Gao et al., 2009). In shrimp, CaM was firstly isolated from *Crangon crangon* and its biological and physiological properties were similar to other invertebrate CaMs (Michael et al., 1992). Recently, it was revealed that CaM gene was highly expressed in *Vibrio harveyi* infected shrimp hemocyte (Wongpanya et al., 2007). Additionally, the expression levels of CaM in shrimp hemocytes of *Litopenaeus vannamei* and *P. monodon* were altered after the pathogen infection (Ji et al., 2011; Somboonwiwat et al., 2010). This indicated that CaM might relate in shrimp pathogen defense mechanism. Although, CaM-mediated processes have been extensively studied, the information of CaM involved in shrimp pathogen defense mechanism is still limited. In this present study, *P. monodon* CaM gene was identified and the effect of CaM gene knockdown was elucidated. Moreover, a recombinant *P. monodon* CaM (r*Pm*CaM) was produced and biochemically characterized and CaM-binding proteins were also identified.

2. Material and methods

2.1. Sample preparation

Healthy shrimps (*P. monodon*) were obtained from a commercial farm in Chantaburi, Thailand. The shrimps (20 g) were reared in a water system with a salinity of 20 ppt at 25 °C for 3 days before experiments. Hemolymph was first withdrawn from the ventral sinus at the first abdominal segment using 10% (w/v) sodium citrate as an anticoagulant. The sample was then centrifuged for 20 min at 10,000 rpm, 4 °C. Hemocyte pellet was collected for RNA extraction. Subsequently, various tissues including gills, hepatopancreas, hearts, lymphoid organs and intestine were dissected and their RNA extractions were carried out.

2.2. Total RNA extraction and sequence analysis

Total RNA was extracted from hemocyte using RNeasy mini Kit (Qiagen). The total RNA was then treated with RNase-free-DNase I (Promega) to eliminate contaminant DNA. First-strand cDNAs were synthesized using oligo (dT) primers and the ImProm-II™ Reverse Transcriptase System kit (Promega), according to the manufacturer's instruction. The cDNA was subsequently used as a template for open reading frame (ORF) amplification. Specific primers were designed based on EST database from Chulalongkorn University. The PCR product was determined by agarose gel electrophoresis and purified by HiYield Gel/PCR DNA Fragment Extraction Kit (RBC Bioscience). The purified PCR product was cloned into pGEM®-T Easy vector (Promega), generating pGEM-*Pm*CaM. The sequence of CaM was confirmed using T7 and SP6 primers (Macrogen). The *Pm*CaM gene was analyzed using BLASTX program with GenBank database. Multiple sequence alignments of various organism CaMs were performed using the ClastalW2 analysis program (http://www.ebi.ac.uk/Tools/clustalw2). Domain architecture was predicted by PROSITE Scan (https://www.ebi.ac.uk/Tools/pfa/ps_scan/).

2.3. Tissue-specific expression

The mRNA expression level of *Pm*CaM in various tissues of healthy shrimp was determined using RT-PCR. Total RNA was extracted from hemocyte, gills, hepatopancreas, hearts, lymphoid organs and intestines as described previously. The specific primers, *Pm*CaM-F and *Pm*CaM-R, were used to amplify *Pm*CaM. An elongation factor 1-α (EF1α) amplified by EF1α-F and EF1α-R primers (Table 1) was used as an internal control. PCR thermocycling conditions were as follows 95 °C for 5 min; 30 cycles of 95 °C for 1 min, 55 °C for 30 s and 72 °C for 1 min, and a final heating at 72 °C for 10 min. The RT-PCR product was analyzed by 1.2% (w/v) agarose gel electrophoresis.

2.4. Synthesis of double-stranded RNA

Double stranded RNA (dsRNA) corresponding to the *Pm*CaM sequence was generated by *in vitro* transcription as described by Charoensapsri et al. (2009). Briefly, DNA template for *Pm*CaM dsRNA preparation was amplified by PCR using gene-specific primers, *Pm*CaM-F and *Pm*CaM-R (Table 1). The PCR product was then used as a template for the synthesis of dsRNA using primers consisting of the same primer sequences flanked by T7 promoter sequence at 5′ end. Sense and anti-sense DNA templates were generated separately using two pairs of primer, *Pm*CaMT7-F and *Pm*CaM-R and *Pm*CaM-F and *Pm*CaMT7-R, respectively (Table 1). For an exogenous gene, green fluorescent protein (GFP) gene amplification was carried out using pEGFP-1 vector as a template with specific primers, GFPT7-F and GFP-R (Table 1) for the sense strand template and GFP-F and GFPT7-R for the anti-sense strand template (Table 1). A proper amount (0.5 μg) of each template was used in an *in vitro* transcription using the T7 RiboMAX™ Express Large Scale RNA Production Systems (Promega), according to the manufacturer's instruction. Equal amounts of sense and anti-sense single stranded RNA were annealed to produce dsRNA. To get rid of the remaining DNA template in the solution, the mixture was treated with RNase-free DNase I. The quality and amount of dsRNAs were determined by 1.2% (w/

Table 1
Primer sequences used for RT-PCR analysis.

Primer	Sequence (5′–3′)
*Pm*CaMexp-F	5′-CATGCCATGGGCCATCATCATCATCATATGGCGGATCAGC TGACCGAAG-3′
*Pm*CaMexp-R	5′-ATAAGAATGCGGCCGCTCACTTCGAGGTCATCATC-3′
*Pm*CaM-F	5′-ACAGTCATGAGGTCCTTGGG-3′
*Pm*CaM-R	5′-TCTCTCCGAGGTTGGTCATC-3′
*Pm*CaMT7-F	5′-GGATCCTAATACGACTCACTATAGGGACAGTCATGAGGTCCTT GGG-3′
*Pm*CaMT7-R	5′-GGATCCTAATACGACTCACTATAGGGTCTCTCCGAGGTTGGTC ATC-3′
GFP-F	5′- ATGGTGAGCAAGGGCGAGGA-3′
GFP-R	5′- TTACTTGTACAGCTCGTCCA-3′
GFPT7-F	5′-TAATACGACTCACTATAGGGATGGTGAGCAAGGGCGAGGA-3′
GFPT7-R	5′-TAATACGACTCACTATAGGGTTACTTGTACAGCTCGTCCA-3′
EF1α-F	5′-GGTGCTGGACAAGCTGAAGGC-3′
EF1α-R	5′-CGTTCCGGT GATCATGTTCTTGAT-3′