Fish & Shellfish Immunology 50 (2016) 175–190

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

A single CRD C-type lectin from *Eriocheir sinensis* (*EsLecB*) with microbial-binding, antibacterial prophenoloxidase activation and hem-encapsulation activities

Zi-Yan Fang [1], Dan Li [1], Xue-Jie Li, Xing Zhang, You-Ting Zhu, Wei-Wei Li**, Qun Wang*

Laboratory of Invertebrate Immunological Defense & Reproductive Biology, School of Life Science, East China Normal University, Shanghai, China

ARTICLE INFO

Article history:
Received 12 August 2015
Received in revised form
13 January 2016
Accepted 24 January 2016
Available online 27 January 2016

Keywords:
C-type lectin
Pattern recognition receptor
Eriocheir sinensis
Antibacterial activity

ABSTRACT

C-type lectins (CTLs) exist widely in crustaceans. To date, thirteen CTLs have been reported in crustaceans, and play significant roles in pathogen recognition, encapsulation of hemocytes and antimicrobial activity in the innate immune response. Based on the initial expressed sequence tags (EST) of a hepatopancreatic cDNA library, a novel CTL, designated as *EsLecB*, with a 470 bp open reading frame encodes a polypeptide of 156 amino acids, including a signal peptide of 19 amino acid residues and one carbohydrate-recognition domain of 131 aa residues, was cloned from the crustacean *Eriocheir sinensis*. By qRT-PCR analysis, *EsLecB* was detected in all tested tissues, and showed highest expression in hemocytes, hepatopancreas and heart. The expression of *EsLecB* was up-regulated following injections of PAMPs or bacteria. The recombinant protein (rEsLecB) expressed in *Escherichia coli* had a calcium-independent but carbohydrate-dependent microbial-binding and microbial-agglutinating, microorganism growth inhibitory and hem-encapsulation activities. Moreover, the rEsLecB could stimulate the activation of prophenoloxidase in vitro. These results indicated that *EsLecB*, as an antibacterial pattern recognition receptor is involved in innate immunity, and may act as an upstream detector of the prophenoloxidase activating system, which can detect pathogen invasion in *E. sinensis*.

1. Introduction

The immune system is usually divided into two branches: innate or natural immunity, and adaptive or acquired immunity. As invertebrates, crustaceans do not have a typical adaptive immunity, which comprises various immunocytes and induced immunological memory [1]. Crustaceans rely solely on a series of germ-line encoded pattern recognition receptors (PRRs) that mediate immune recognition and defense against various pathogens [2–4]. PRRs are expressed constitutively in the host and perceive the invaders, regardless of their life-cycle stage [5]. PRRs recognize the conserved molecules exposed on the surface of microorganisms, known as pathogen-associated molecular patterns (PAMPs), which include lipopolysaccharides (LPSs), lipoproteins, flagellins and peptidoglycans (PGs), that are essential for the survival of the microorganism [5–7]. Immune recognition is the first line of innate immunity defense against invaders, in this step, PRRs have an important role [4]. Different PRRs react with specific PAMPs, and show distinct expression patterns, activate a downstream cascade of specific signaling pathways, and lead to distinct anti-pathogen responses, such as the prophenoloxidase (proPO) activated system, phagocytosis, encapsulation and antimicrobial activity [8–11].

In invertebrates, at least eight groups of distinct PRRs were identified, including C-type lectins (CTLs), Toll-like receptors (TLRs), Dscam, multidomain scavenger receptors (SCRs), galactoside-bindinglectins (galectins) [13], peptidoglycan-recognition proteins (PGRPs) [14], thioester-containing proteins (TEPs), Gram-negative binding proteins (GNBPs) and fibrinogen-like domain immunolectins [11,12,15]. Among these PRRs, CTLs are recognized as an important group in crustaceans, and their classification, characterization and functions have been reported extensively [7,16,17]. CTLs are a large family of Ca^{2+}-dependent carbohydrate binding proteins that are distinguished from the other types of lectins by containing at least one carbohydrate-recognition domain (CRD) of about 110–130 amino acids [10,18].

* Corresponding author.
** Corresponding author.
E-mail addresses: wwli@bio.ecnu.edu.cn (W.-W. Li), qwang@bio.ecnu.edu.cn (Q. Wang).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.fsi.2016.01.031
1050-4648/

The CRD has four conserved cysteine residues that form a characteristic double-loop to maintain the spatial structure [7]. The inner loop, also called the long loop region, lies in the overall loop and participates in the calcium-mediated carbohydrate interaction. The Ca^{2+}-binding site 2 motif is structurally conserved and is involved in carbohydrate binding in CRDs, such as EPN/WND or QPD/WND, which are thought to specify binding to mannose or galactose, respectively [19,20]. Variations of these motifs are always associated with stabilizing the structure, such as EPD, EPK, EPS, EPQ, QPN, QPG, QPT, QPS, YPG, YPT, WID, WSD, WHD, FSD, LSD and mutated motifs, such as WHD, FND, and MND occupy the corresponding sites [10,21]. CTLs containing this specific domain play important roles in innate immunity, where they serve as PRRs to discriminate self and non-self by recognizing PAMPs of pathogens by binding to carbohydrates on the surface of microorganisms. CTLs are involved in many immune responses, such as agglutinating activities, opsonization and hemocytes encapsulation, activation of the respiratory burst [22], induction of prophenoloxidase activating system, microorganisms binding activity, and the antibacterial response [8–11].

CTLs are conserved in vertebrates, but are diverse in invertebrates. For example, *Drosophila melanogaster* has 32 genes encoding C-type lectin-like domains (CTLDs), and at least 183 genes have been found in *Caenorhabditis elegans* [23]. The mechanism by which *Drosophila* defends itself against intrusion by pathogens supplies a model to study innate immunity and host–pathogen interactions [24]. Two *Drosophila* CTLs, DL2 and DL3, might act as PRRs to mediate hemocyte encapsulation and melanization by recruiting hemocytes to the lectin-coated surface directly [25]. In red swamp crayfish (*Procambarus clarkii*), *PcLec2* is an upstream detector, and the recombinantly expressed protein, rPcLec2, could stimulate the activation of prophenoloxidase in vitro and in vivo, which demonstrated the involvement of CTLs in the prophenoloxidase activating system [26]. In *Litopenaus Vannamei*, *LvCTL4*, via the NF-kB signaling pathway, participates in the antibacterial immune response [27], and *LvLT* has a role in defense against white spot syndrome virus (WSSV) infection [28]. In *Eriocheir sinensis*, Chinese mitten crab, thirteen CTLs have been reported, and some of them promote the encapsulation of hemocytes and enhances the clearance of bacteria in the innate immune response [29–36].

E. sinensis is one of the most vital aquaculture crustacean species in China. In this study, a novel CTL with a single CRD, named *EsLecB*, was identified from *E. sinensis*. *EsLecB* was characterized and phylogenetically analyzed, and its tissue distribution, temporal expression pattern of pathogen- and PAMPs stimulant-challenged in hemocytes were studied. Furthermore, we expressed and purified the recombinant *EsLecB* protein (rEsLecB), which was used to explore its potential function in the microorganisms binding activities, agglutination, antibacterial activity, prophenoloxidase activation as well as in vitro cellular encapsulation response.

2. Materials and methods

2.1. Experimental animals

Healthy adult Chinese mitten crabs (n = 200; about 80 g wet weight) were purchased from the Tongchuan aquatic product market in Shanghai, China. Crabs were maintained for one week at 25 °C in filtered, aerated freshwater before processing every day.

2.2. Immune challenge and sample collection

In each challenge, the crabs were divided to two groups: the experimental group (n = 60) and the control group (n = 60). Pathogens were injected into the arthrodial membrane of last pair of the crabs' walking legs. For PAMPs challenge, 100 ul of lipopolysaccharide (LPS) (Sigma–Aldrich, Shanghai, China) (500 ug/ml) from *E. coli* 0111:B4 or peptidoglycan (PG) from *S. aureus* (Sigma–Aldrich) (500 ug/ml) in phosphate buffered saline (PBS; KH_2PO_4, 0.24 g; Na_2HPO_4; 1.44 g) were injected into each crab. For bacterial challenge, four bacteria: *V. parahemolyticus*, *S. aureus*, *B. subtilis*, *A. hydrophilla* obtained from the National Pathogen Collection Center for Aquatic Animals (Shanghai Ocean University, China), cultured overnight in Luria–Bertani medium, and collected by centrifugation (5000 × g, 5 min), washed three times by PBS, and resuspended in PBS. The bacteria plated for colony counting and then the bacterial suspension was adjusted to 1×10^6 CFU/ml, and then injected into the crabs as 100 μl of bacterial suspension (1×10^6 CFU/ml). The control groups were injected with 100 μl PBS. At each time point (0, 6, 12, 24, 36 and 48 h after injection) five crabs were randomly selected. Hemolymph was separated from the hemocoel in each crabs arthrodial membrane of the last pair of walking legs using a syringe with an equal volume of anticoagulant solution (0.1 M glucose, 30 mM citrate, 26 mM citric acid, 0.14 M NaCl, 10 mM EDTA). The samples were centrifuged (700 × g, 10 min, 4 °C) to obtain hemocytes. Hemocytes of each interval challenged crab were sampled and stored at −80 °C. Tissues such as heart, hepatopancreas, gill, stomach, ovary, gland, spermatheca, sperm, muscles, intestines, and hemocytes were also isolated from the control group crabs, and stored at −80 °C for cloning and expression analysis.

2.3. Total RNA extraction and first-strand cDNA synthesis

According to the manufacturer's protocol, total RNA from *E. sinensis* tissues and hemocytes was extracted using the Trizol reagent (RNA Extraction Kit, Invitrogen, Carlsbad, CA, USA). Spectrophotometry (OD_{260} nm) and 1.2% agarose-gel electrophoresis were used to estimate the total RNA concentration and quality. cDNA was synthesized from total RNA (5 μg) using a SMARTer™ RACE cDNA Amplification kit (Clontech, Mountain View, CA, USA), according to the manufacturer's instructions used as templates for full-length cDNA cloning. Total RNA (4 μg) extracted from each *E. sinensis* tissue was reverse transcribed for RT-PCR or SYBR Green fluorescent quantitative real-time RT-PCR (qRT-PCR) using the PrimeScript™ Real-time PCR Kit (TaKaRa, Dalian, China).

2.4. Cloning of full-length EsLecB cDNA

A partial cDNA sequence (expressed sequence tag (EST)) of the *E. sinensis* C-type lectin (*EsLecB*) was obtained from the hepatopancreas cDNA library of Chinese mitten crab, which was used to design specific primers (Table 1) for extended the full-length cDNA using 3′ rapid amplification of cDNA ends (RACE) (SMARTer™ RACE cDNA Amplification kit, Clontech). The polymerase chain reaction (PCR) procedure was: 5 cycles at 94 °C for 30 s, 72 °C for 3 min; 5 cycles at 94 °C for 30 s, 70 °C for 30 s, and 72 °C for 2 min; 20 cycles at 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 2 min; and finally, 72 °C for 10 min. The PCR fragments were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), and inserted into a pZeroBack/Blunt vector (Tiangen, Shanghai, China) to construct the recombinant plasmid, and then transformed into *E. coli* Top 10 cells (Tiangen). Positive clones containing inserts of the anticipated size were sequenced on both strands using 23-mer and 24-mer primers (Table 1). The cloned fragments were sequenced and confirmed as CTL sequences by comparisons with other CTLs from other representative vertebrates and invertebrates reported in the GenBank database of NCBI, using the online BLAST program (http://blast.ncbi.nlm.nih.gov).