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Molecular cloning, characterization and expression of a C-type lectin cDNA in Chinese mitten crab, *Eriocheir sinensis*

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ABSTRACT

C-type lectins are pattern-recognition proteins which are functionally important for pathogen recognition and immune regulation in vertebrates and invertebrates. In this study, a lectin cDNA named as Es-Lectin was cloned and characterized from the Chinese mitten crab, *Eriocheir sinensis*. The full-length sequence of this Es-Lectin cDNA was 651 bp, including an open reading frame of 483 bp encoding 160 amino acids. The predicted molecular weight of the Es-Lectin was 11.8 kDa. A typical signal peptide of 21 amino acids was deduced at the N-terminus of the predicted protein. This Es-Lectin belongs to a C-type lectin and contains six cysteines, a conserved EPN motif (Glu-Pro-Asn) and an imperfect WND (Trp-Asn-Asp) motif (FND, Phe-Asn-Asp). This Es-Lectin had 55% and 32% identity with other two C-type lectins in *E. sinensis*, and 29–36% homology with decapods. Although the Es-Lectin was also expressed in gill, hepatopancreas, intestine, muscle and stomach, its expression in haemocytes was the greatest. The expression of Es-Lectins in haemocytes increased at 1.5 h after the *Aeromonas hydrophila* challenge. After a slight decrease, the Es-Lectin expression in haemocytes significantly increased at 48 h post-challenge. The diverse distribution of Es-Lectin and its enhancement by bacterial challenge indicate that C-type lectins are important in the innate immune response to bacterial infection, and can be activated for innate immune response in crab at the initial stage after pathogen infection.

1. Introduction

Most invertebrates lack acquired immunity since these animals have no immunoglobulins, or memory following the first challenge of a pathogen [1]. Therefore, the innate immune system plays a major role in defending antipathogens [2]. The mechanism to defend the invading microorganisms is to recognize the characteristic of carbohydrate structures of a pathogen and to effectively discriminate pathogens [3]. During this recognition process, the innate recognition is mediated by a special protein known as pattern-recognition receptors (PRRs) [4]. Proteins containing a C-type lectin-like domain are among the groups of PRRs in vertebrates to regulate the entire immune system [5–7].

The C-type lectins are a large group of extracellular metazoan proteins and have a function to recognize oligosaccharides in cell surfaces [1]. These lectins contain a Ca^{2+}-dependent carbohydrate recognition domain (CRD) with two or three pairs of disulfide bonds to bind the carbohydrate residues of foreign pathogens [3,8,9]. The classification of the C-type lectin family is based on the architecture of the C-type lectin domain-containing proteins [10]. For example, the bovine conglutinin which was the first lectin discovered from animals is a member of the C-type lectin family [11]. In vertebrates, the C-type lectin is currently classified into 17 groups [3]. However, in invertebrates, little information is available for the classification of the C-type lectin family at gene and protein levels, though several reports on C-type lectins in invertebrates have been published [12–14].

Current literature suggests that the member of the C-type lectin domain in invertebrates is more abundant and diverse than that in vertebrates [3,15]. In crustaceans, many C-type lectins have been identified [9,16,17], and most of these lectins display anti-virus and anti-bacteria activities [18–20]. In *Fenneropenaeus merguiensis*, a Ca^{2+}-dependent lectin in hemolymph contributes to the defense response to potential pathogenic bacteria [21]. A natural lectin from the serum of the shrimp *Litopenaeus vannamei* plays a significant role in host immune response against bacterial infections [22]. In *Penaeus japonicus*, an N-acetylglucosamine (GlcNAc) specific lectin isolated from serum has an opsonic activity against bacterial infection [23]. Based on cDNA sequence, more structural information is available in decapods. Two C-type lectin-like domain (CTLD)-containing proteins

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were identified in *Eriocheir sinensis* and showed different roles in innate immune response to bacterial infection [24]. In *Portunus trituberculatus*, a CTLD-containing protein with one CRD was isolated and designated as PtLP [9]. A C-type lectin known as PmLec with one CRD was purified from the serum of *Penaeus monodon*. The PmLec is specific for bacterial lipopolysaccharide and serves a pattern-recognition protein and opsonin [25]. In *L. vannamei*, two putative CRDs were found in a C-type lectin (named LvLT) against virus infection [19]. The dual-CRD structure also exists in the C-type lectin in *P. monodon* (named PmLT), and its expression in haemocytes suggests a possible function as a pattern-recognition protein to defend viral and bacterial pathogens [26].

Chinese mitten crab, *E. sinensis*, is an important freshwater crustacean for aquaculture and has brought significant revenue to rural economy. In the past few years, attempts have been made to understand the immunological mechanism in the Chinese mitten crab because various diseases caused by bacteria, viruses and *Rickettsia*-like organisms have threatened the sustainability for Chinese mitten crab farming [24,27–29]. In this study, we aimed to identify the C-type lectin cDNA from *E. sinensis* and characterize its expression patterns in various tissues following an *Aeromonas hydrophila* challenge on this crab. The characterization and molecular expression of the C-type lectins from *E. sinensis* will provide an insight into the understanding of pathogen recognition responses in crustacean and will be useful to develop techniques to control disease outbreak in aquaculture.

2. Materials and methods

2.1. Animals

Adult Chinese mitten crabs *E. sinensis* were obtained from a fishing ground in Shanghai and acclimatized in the laboratory for 2 weeks before carrying out the experiment. The crabs weighing 150–200 g were used for the bacterial challenge test.

Haemocytes were collected in an equal volume of anticoagulant solution (NaCl 510 mM; Glucose 115 mM; Na-citrate 30 mM; EDTA-Na_2 mM; pH 7.55) and immediately centrifuged at 800 rpm for 10 min at 4 °C. After haemocytes had been sampled, other crab tissues (gill, hepatopancreas, intestine, muscle and stomach) were also collected and immediately preserved in liquid nitrogen for RNA extraction.

2.2. Preparation of bacteria

The Gram-negative bacterium *A. hydrophila* obtained from Guangzhou Microbiology Research Institute was chosen to challenge the crab because it was the pathogenic bacterium causing aeromonosis or haemorrhagic septicemia in crab and other aquatic animals [30]. The experimental bacteria were grown in the Luria–Bertani nutrient agar, and then diluted with sterile 0.85% NaCl to reach the density of 7.2×10^8 CFU/ml using by plate counts [30–33].

2.3. RNA isolation

Total RNA was extracted from the haemocytes and other tissues using the Unizol Reagent kit (Biostar, Shanghai, China) according to the manufacturer's instructions. The concentration of the total RNA was quantified using spectrophotometry at a wavelength of 260 nm.

2.4. The full-length cDNA cloning

Based on the ESTs sequence in the haemocyte cDNA library of *E. sinensis* [34], the special primers were designed for the gene clone (Table 1).

The full-length cDNA sequence of the lectin gene was obtained by using the SMART™ RACE cDNA amplification kit (Clonetech, USA). For 5′-RACE, the primers of LEC 5-R and the universal primer A mix (UPM) were used in a PCR reaction (1 circle of 94 °C for 4 min; 30 circles of 94 °C for 30 s, 66 °C for 30 s and 72 °C for 3 min; 1 circle of 72 °C for 7 min). A touchdown PCR was used for 3′-RACE with the primers of LEC3-F and the abridged universal amplification primer (AUAP). The PCR reaction conditions were followed the manufacturer of Advantage™ 2 PAC kit (Clonetech, USA). The expected PCR products were eluted from the agarose gel and ligated to the pGEM-T easy vector (Promega, USA). The ligation product was transformed to *Escherichia coli*. Clones were sequenced with universal primers T7 and SP6. After splicing the two fragments, the full-length cDNA sequence was validated using the primers UPM and LEC AS. All the primers used in this study are shown in Table 1.

2.5. Phylogenetic analysis

The lectin amino acid sequences from various species were downloaded from NCBI. A phylogenetic tree was constructed using the neighbour-joining method in the Molecular Evolutionary Genetics Analysis (MEGA 4) package [35]. The conserved domains CRD were searched with SMART program (http://smart.embl-heidelberg.de/).

2.6. Es-Lectin expression

The expressions of Es-Lectin mRNA in crab tissues were detected by quantitative real time PCR. Total RNA from haemocytes, hepatopancreas, muscles, gills, intestine, and stomach was separately extracted. Before reverse transcription, the RNA samples were treated with DNAase (Promega, USA). The first strand cDNA was synthesized using a cDNA first strand synthesis kit with superscript™ III RNAse H-reverse transcriptase (Invitrogen, USA) using 5 μg total RNA. According to the cDNA full-length sequence, a pair of gene-specific primers (LEC S-RT and LEC AS-RT) was designed to amplify the 279 bp fragment, and the primers β-actin F and β-actin R were used to amplify the 266 bp fragments as the internal standard gene control.

The SYBR Green quantitative real time PCR assay was conducted to determine the Es-Lectin mRNA expression in an iQ™ 5 Multicolor Real Time PCR Detection System. The PCR temperature profile and PCR reaction conditions were followed the manufacturer of SYBR Premix Ex Taq (TaKaRa, Dalian, China), and the PCR product was sequenced to verify the specificity of quantitative real time PCR. The expression level of Es-Lectin was calculated by $2^{-\Delta\Delta CT}$ [36], and the expression in haemocytes was used as the calibrator. Comparisons were made in different tissues using the analysis of variance (SPSS 14.0 package, SPSS Inc., New York, USA).

2.7. Infection response

In the bacterial challenge group, 100 μl *A. hydrophila* (7.2×10^8 CFU/ml) was injected into the arthrodial membrane of the

Table 1
Primers used in this study for the analysis of the Es-Lectins in *E. sinensis*.

Primer name	Primer sequence 5′–3′
UPM	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
AUAP	GGCCACGCGTCGACTAGTAC
LEC5-R	CGACCTTGGGGCTTGGCAGAT
LEC3-F	GTGGGCGGCGGCTGCTTCTA
LEC AS	AGGTGCTATAACAACAACTTCAAGG
LEC S-RT	GGGCGGCGGCTGCTTCTA
LEC AS-RT	CCATCATTGTTGGGCTCGTT
β-actin F	TAGGTGGTCTCGTGGATGCC
β-actin R	GAGACCTTCAACACCCCCGC