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Two novel short C-type lectin from Chinese mitten crab, *Eriocheir sinensis*, are induced in response to LPS challenged

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ABSTRACT

The basic mechanism of host fighting against pathogens is pattern recognition receptors recognized pathogen-associated molecular patterns. However, the specificity of recognition within the innate immune molecular of invertebrates remains largely unknown. For this reason, we investigated the immune functionality of two pattern recognition receptors, C-type lectin EsLecA and EsLecG, post lipopolysaccharides (LPS) challenge in Chinese mitten crab (*Eriocheir sinensis*), which is a commercially important and disease vulnerable aquaculture species. The cloning of full-length EsLecA and EsLecG cDNA were based on the initial expressed sequence tags (EST) isolated from a hepatopancreatic cDNA library via PCR. The EsLecA cDNA contained a 480-bp open reading frame that encoded a putative 159-amino-acid protein, while EsLecG cDNA contained a 465-bp open reading frame that encoded a putative 154-amino-acid protein. Comparison, with other reported invertebrate and vertebrate sequences, revealed the presence of carbohydrate recognition domains that were common among C-type lectin superfamilies. EsLecA and EsLecG mRNA expression in *E. sinensis* were (a) both detected in all tissues, including the hepatopancreas, gills, hemocytes, testis, accessory gland, ovary, muscle, stomach, intestine, heart, thoracic ganglia and brain, and (b) responsive in hepatopancreas, gill, hemocytes post-LPS immuno-challenge all appeared dramatically variation. Collectively, the data presented here demonstrate the successful isolation of two novel C-type lectins from the Chinese mitten crab, and their role in the innate immune system of an invertebrate.

1. Introduction

Invertebrate animals do not truely have an adaptative immune response that is generated by memory and targeted immunoglobulin production, as in vertebrates [1]. Nonetheless invertebrate, such as crustaceans, are capable of effective innate immune responses for protection against intruding pathogens [2]. In the event of pathogens intruding, their conserved pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides (LPS), peptidoglycans and β-1, 3-glucans, i.e., which are essential and unique components of virtually all microorganisms, but absent in higher organisms [3] could be discriminated by a wide range of pattern recognition receptors (PRRs) that are highly conserved in evolution [4], then encountered a variety of defense mechanisms.

Lectins, an important member of PRRs, existed as transmembrane receptors or as soluble proteins in circulating fluids [5]. They plays crucial roles in innate immunity such as nonself-recognition and clearance of invading microorganisms [6], via recognizing and non-covalently binding to specific sugar moieties and agglutinate pathogens by binding to cell surface glycoproteins and glycoconjugates [7]. C-type lectins are the most diverse and well studied among the lectin families. The term C-type lectin was originally used to distinguish a group of Ca^{2+}-dependent (C-type) carbohydrate-binding proteins from the other types of lectins [8]. This big gene family mediate sugar binding with diverse architecture contained homologous carbohydrate recognition domains (CRDs) by which discriminate specific oligosaccharides at cell surfaces, attach to circulating proteins and in the extracellular matrix [9–11]. Although C-type lectin has been studied in vertebrates for many years, they have not been well characterized in invertebrates.

The mitten crab (*Eriocheir sinensis*) (Henri Milne Edwards 1854), which belongs to *Crustacea*, *Decapoda*, *Varunidae*, *Eriocheir,* is native to China. This crab is a traditional savory food especially in the

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Yangtze River Area, and comprises one of the most economically important freshwater aquatic species of China [12]. With the development of intensive *E. sinensis* culture which has expanded rapidly over the last two decades, reached a yield of 4.0×10^5 t in China in 2005 [13], various diseases caused by bacteria (especially Gram-negative bacteria), viruses, or rickettsia-like organisms have also begun to emerge and have resulted in enormous losses [14,15]. Therefore, an improved understanding of the innate immune ability of crabs and their bio-defense mechanisms has become a research priority. With this in mind, several research labs, including our own, have begun screening immune-related genes from Chinese mitten crab by constructing cDNA libraries [16–19], with the aim of designing efficient strategies for disease control. Among our cDNA library [18], two EST sequences identified as EsLecA and EsLecG. (partial CDS) were found to be homologus to C-type lectin. In this study we examined two novel C-type lectin mRNA expression patterns in different tissues after cDNA full-length cloning, and detected their transcription variation in three major crustacean immune organs induced after LPS (*Escherichia coli* 0111:B4 origin) challenge.

2. Materials and methods

2.1. Animal immune challenge and sample collection

Healthy adult Chinese mitten crabs ($n = 140$; 100 ± 20 g wet weight) were collected from the Tongchuan aquatic product market in Shanghai, China. After acclimated for one week at 20–25 °C in filtered, aerated freshwater, crabs were placed in an ice bath for 1–2 min until each was lightly anesthetized. Hemolymph was draw from the hemocoel in arthrodial membrane of the last pair of walking legs using a syringe (approximately 2.0 ml per crab) with an equal volume of anticoagulant solution (glucose: 2.05 g, citrate: 0.8 g, NaCl: 0.42 g, double distilled water: add to 100 ml) added, and centrifuged at 800 *g* at 4 °C to isolate hemocytes. The other tissues (hepatopancreas, gills, testis, accessory gland, ovary, muscle, stomach, intestine, heart, thoracic ganglia and brain) were harvested, snap frozen in liquid nitrogen, and stored at −80 °C prior to nucleic acid analysis. For cloning and expression analysis, tissues from 10 crabs were pooled, and ground with a mortar and pestle prior to extraction.

For LPS stimulation, 120 crabs were divided equally into two groups: experimental group crabs were injected into the arthrodial membrane of the last pair of walking legs with approximately 100 μl of LPS (Sigma-Aldrich, L2630) resuspended (500 μg/ml) in PBS, while control group with 100 μl PBS (pH = 7.4). Five crabs were randomly selected at each time interval of 0 (as blank control), 2, 4, 8, 12, 24, 48 and 72 h post-injection. Hepatopancreas, gills and hemocytes were harvested according to methods above, and were stored at −80 °C, after the addition of 1 ml Trizol reagent (Invitrogen, CA, USA) for subsequent RNA extraction. Except the 40 crabs were sacrificed for tissue collection respectively, experimental group had 11 individuals death, and then control group had 0 individuals death until 72 h post challenged.

2.2. Total RNA extraction and first-strand cDNA synthesis

Total RNA was extracted from *E. sinensis* tissues sampled from Section 2.1 using Trizol® reagent (RNA Extraction Kit, Invitrogen, CA, USA) according to the manufacturer's protocol. The total RNA concentration and quality were estimated using spectrophotometry at an absorbance at 260 nm and agarose-gel electrophoresis respectively.

Total RNA (5 μg) isolated from hepatopancreas was reverse transcribed using the SMARTer™ RACE cDNA Amplification kit (Clonetech, USA) for cDNA cloning. For RT-PCR and qRT-PCR expression analysis, total RNA (4 μg) was reverse transcribed using the PrimeScript™ Real-time PCR Kit (TaKaRa, Japan).

2.3. EST analysis and cloning of full-length Es-lectin cDNA

A cDNA library was previously constructed using the hepatopancreas of the Chinese mitten crab, from which 3355 successful sequencing reactions were obtained using a T3 primer [18]. The *E. sinensis* C-type lectin partial cDNA sequence was extended using 5′ and 3′ RACE (SMARTer™ RACE cDNA Amplification kit, Clontech), and a total of two gene-specific primers (Table 1) based on the original EST sequence. The 3′ RACE PCR reaction was carried out in a total volume of 50 μl containing 2.5 μl (800 ng/μl) of the first-strand cDNA reaction as a template, 5 μl of 10× Advantage 2 PCR buffer, 1 μl of 10 mM dNTPs, 5 μl (10 μM) gene-specific primer (EsLecA-3′RACE, Table 1), 1 μl of Universal Primer A Mix (UPM; Clonetech, USA), 34.5 μl of sterile deionized water, and 1 U 50× Advantage 2 polymerase mix (Clonetech, USA). For the 5′ RACE, UPM was used as forward primers in PCR reactions in conjunction with the reverse gene-specific primers (EsLecA-5′RACE, EsLecG-5′RACE, Table 1). PCR amplification conditions for both the 3′ and 5′ RACE were as follows: 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 3 min; 20 cycles at 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min. PCR amplicons were size separated and visualized on an ethidium bromide stained 1.2% agarose gel. Amplicons of expected sizes were purified with Wizard® SV Gel and PCR Clean-Up System (Promega, USA), and inserted into a pMD19T Vector (Takara, Japan). Positive clones containing inserts of an expected size were sequenced using T7 and SP6 primers (Table 1).

2.4. Sequence analysis and phylogenetic analysis

Eriocheir sinensis C-type lectin full-length cDNAs and deduced amino acid sequences were compared against sequences from other representative vertebrates and invertebrates reported in the GenBank of NCBI, using the BLAST program (http://blast.ncbi.nlm.nih.gov). These analyses were completed by multiple sequence alignment using ClustalX and ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). An unrooted neighbor-joining phylogenetic tree was constructed with MEGA5.0. The homologous conserved domains and signal peptides were identified by SMART

Table 1
Primer sequences.

Primers name	Sequences (5′–3′)
RACE	
EsLecA-3′RACE	TTTCTGAACGGTGACCCTGTGCC
EsLecA-5′RACE	CACCGTTCAGAAACTCCCAGGCAC
EsLecG-5′RACE	CTGAGGGTGTAGTTGAGGAGTGGGATG
LongUP	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
ShortUP	CTAATACGACTCACTATAGGGC
qRT-PCR	
EsLecA-F	ATGGGTGGAAGCGGTAGCC
EsLecA-R	TCGGGTGCCAGAAGGGAAT
EsLecG-F	TCTCGTTGAAGGACAGTGGAAGTG
EsLecG-R	CTGACAGATGGCGTAGTG
β-actin-F	CTCCTGCTTGCTGATCCACATC
β-actin-R	GCATCCACGAGACCACTTACA
Sequencing	
T7	TAATACGACTCACTATAGG
SP6	ATTTAGGTGACACTATAGAA