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A galectin from *Eriocheir sinensis* functions as pattern recognition receptor enhancing microbe agglutination and haemocytes encapsulation

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ABSTRACT

Galectins are a family of β-galactoside binding lectins that function as pattern recognition receptors (PRRs) in innate immune system of both vertebrates and invertebrates. The cDNA of Chinese mitten crab *Eriocheir sinensis* galectin (designated as EsGal) was cloned via rapid amplification of cDNA ends (RACE) technique based on expressed sequence tags (ESTs) analysis. The full-length cDNA of EsGal was 999 bp. Its open reading frame encoded a polypeptide of 218 amino acids containing a GLECT/Gal-bind_lectin domain and a proline/glycine rich low complexity region. The deduced amino acid sequence and domain organization of EsGal were highly similar to those of crustacean galectins. The mRNA transcripts of EsGal were found to be constitutively expressed in a wide range of tissues and mainly in hepatopancreas, gill and haemocytes. The mRNA expression level of EsGal increased rapidly and significantly after crabs were stimulated by different microbes. The recombinant EsGal (rEsGal) could bind various pathogen-associated molecular patterns (PAMPs), including lipopolysaccharide (LPS), peptidoglycan (PGN) and glucan (GLU), and exhibited strong activity to agglutinate *Escherichia coli*, *Vibrio anguillarum*, *Bacillus subtilis*, *Micrococcus luteus*, *Staphylococcus aureus* and *Pichia pastoris*, and such agglutinating activity could be inhibited by both D-galactose and α-lactose. The *in vitro* encapsulation assay revealed that rEsGal could enhance the encapsulation of haemocytes towards agarose beads. These results collectively suggested that EsGal played crucial roles in the immune recognition and elimination of pathogens and contributed to the innate immune response against various microbes in crabs.

1. Introduction

Galectins are a large evolutionally conserved protein family and universally present in a wide variety of eukaryotic organisms ranging from fungi to mammals [1]. All known vertebrate galectins contain at least one carbohydrate recognition domain (CRD) and exhibit β-galactoside binding activity [2,3]. According to their molecular structural features, they can be generally classified into proto type (mono-CRD type), tandem-repeat type (bi-CRD type) and chimera type (formed by an N-terminal proline and glycine rich domain and a C-terminal CRD) [4]. Additionally, novel type galectins with quadruple-CRD have been also found in some species, including scallop, oyster and abalone [5–8]. So far, at least fifteen distinct subtypes of galectins have been identified in mammals, and these various and ubiquitous galectins are proposed to mediate diverse biological processes, such as host-pathogen interactions, immunomodulation and so on [9].

Recently, galectins from marine invertebrates have attracted increasing attention of immunologists, and they are proved to be involved in innate immune defense system [10,11]. Among them, most of the molluscan galectins so far identified are tandem-repeat type and quadruple-CRD type. For examples, a tandem-repeat galectin from the Manila clam *Ruditapes philippinarum*, RpGal, could be induced upon infection with the protozoan parasite *Perkinsus olseni* and it could directly bind to the surface of both *P. olseni* and *Vibrio tapetis* [12]. While another tandem-repeat galectin from the blood clam *Tegillarca granosa*, TgGal, could be induced by *Vibrio*

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parahaemolyticus, lipopolysaccharide (LPS) and peptidoglycan (PGN) [13]. Moreover, a canonical quadruple CRD galectin, CvGal, was found responsible for recognition of the protozoan parasite *Perkinsus marinus* in the eastern oyster *Crassostrea virginica* [14]. The quadruple-CRD galectins from bay scallop *Argopecten irradians* (AiGal1 and AiGal2) and red abalone (HrGal) could also be induced by invading microbes or simulating foreigners and AiGal2 exhibited strong activity to agglutinate various microbes [5–7]. Additionally, a quadruple-CRD galectin has been identified in the pearl oyster *Pinctada fucata*, and its mRNA expression levels all increased after *Vibrio alginolyticus* stimulation [8]. While in marine crustaceans, a galectin from the kuruma shrimp *Marsupenaeus japonicas*, MjGal, functioned as an opsonin and promoted bacterial clearance from haemolymph [15], and galectins from white shrimp *Litopenaeus vannamei*, LvGal1 and LvGal2, were proved to be involved in immune recognition and bacteria phagocytosis [16,17]. However, compared with other PRRs in marine crustaceans, the knowledge of the biological roles of marine crustacean galectins in innate immunity is still very limited and fragmentary.

The Chinese mitten crab *Eriocheir sinensis* is one of the most important aquaculture species in South-East Asia [18,19]. With the development of intensive culture and environmental deterioration in the last decades, various diseases caused by fungi, bacteria or viruses had frequently occurred in cultured *E. sinensis* populations [20]. Crabs lack an adaptive immune system, and mainly employ innate immune system to recognize and eliminate invading microbes [21]. To date, a large variety of immune related molecules, such as pattern recognition receptors (PRRs) and immune effectors have been characterized in crabs [18,19,21]. However, rare information of galectin was available in this specie. The main objectives of the present research were (1) to clone the full-length cDNA of galectin from *E. sinensis* (designated as EsGal), (2) to investigate the tissue distribution of EsGal mRNA transcripts, and their temporal expression profile after microbes stimulation, and (3) to validate the potential activity of EsGal protein in the immune responses of crabs.

2. Materials and methods

2.1. Crabs, microbe stimulation and haemocytes collection

Approximately two hundred crabs were collected from a local farm in Qingdao, Shandong, China. After acclimated for two weeks, fifty crabs were kept in tanks containing live *Vibrio anguillarum* strain M3 (kindly provided by Prof. Zhaolan Mo, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences) at final concentration of 8×10^6 CFU mL^{-1} as Gram-negative bacteria stimulation group. Other fifty crabs were transferred to the tanks containing live *Micrococcus luteus* (28001, Microbial Culture Collection Center, China) at final concentration of 8×10^6 CFU mL^{-1} as Gram-positive bacteria stimulation group. The third fifty crabs were transferred to the fungi-containing tanks with live *Pichia pastoris* strain GS115 (PA17237, Lifetechnologies, USA) at final concentration of 8×10^6 CFU mL^{-1} as fungi stimulation group. Five individuals from each group were randomly sampled at 0, 3, 6, 12, 24, 48 and 96 h post stimulation. The haemolymph was collected from chelipeds using a syringe with an equal volume of anticoagulant (27 mmol L^{-1} sodium citrate, 336 mmol L^{-1} NaCl, 115 mmol L^{-1} glucose, 9 mmol L^{-1} EDTA, pH 7.0), and centrifuged at 800 g, 4 °C for 10 min to harvest the haemocytes for RNA preparation. Haemocytes, heart, muscle, gill, haepatopancreas and gonad from five untreated crabs were collected to determine the distribution of EsGal mRNA transcripts in various tissues.

2.2. RNA preparation and cDNA synthesis

Total RNA was extracted using RNAiso plus reagent (9108, Takara, Japan). The first-strand synthesis was carried out with M-MLV RT (M5313, Promega, USA) and dNTPs Mix (U1515, Promega, USA) using the DNaseI (RQ-1, M6101, Promega, USA) treated total RNA as template and adaptor primer-oligo (dT) as primer (Table 1). The reactions were performed at 42 °C for 1 h, terminated by heating at 95 °C for 5 min and then stored at −80 °C.

2.3. Cloning the full-length cDNA of EsGal

One expressed sequence tag (EST) sequence (CMCES_A_0959) homologous to previously identified galectins was selected for further cloning of EsGal [21]. Two gene-specific primers, EsGal-RACE-F1/2, were designed based on this EST to clone the full-length cDNA of EsGal via rapid amplification of cDNA ends (RACE) technique (Table 1). All PCR amplifications were performed in a PCR Thermal Cycler (TP-600, Takara, Japan), and the PCR products were gel-purified using MiniBest Agarose Gel DNA Extraction Kit Ver. 4.0 (9762, Takara, Japan) and then cloned into the pMD19-T simple vector (3271, Takara, Japan). After being transformed into the competent cells *Escherichia coli* strain Top10 (CB104, Tiangen, China), the positive recombinants were identified through anti-ampicillin selection and PCR screening with M13-47 and RV-M primers (Table 1). Three of the positive clones were sequenced using a PRISM 3730XL automated sequencer (Appliedbiosystems, USA).

2.4. Sequence characterization and multiple sequence alignment

The searches for protein sequence similarities were conducted

Table 1
Oligonucleotide primers used in the current study.

Primer	Sequence (5′-3′)	Brief information
EsGal-RACE-F1	TTTATGAGGGAAGGGACCAGGAC	gene specific primer for RACE
EsGal-RACE-F2	CTTCGGTCCAGGCAAGATTCTC	gene specific primer for RACE
Adaptor-oligo(dT)	GGCCACGCGTCGACTAGTACT$_{17}$VN	adaptor primer
EsGal-qRT-F	CAACCAGAATCACTTCGCA	gene specific primer for real-time PCR
EsGal-qRT-R	TTATCCTCGATCCAGACACAG	gene specific primer for real-time PCR
Esactin-qRT-F	GCATCCACGAGACCACTTACA	internal control for real-time PCR
Esactin-qRT-R	CTCCTGCTTGCTGATCCACATC	internal control for real-time PCR
EsGal-recombiant-F	ATGGGATCCCCAATATATAAT	gene specific primer for recombinant
EsGal-recombiant-R	CTAGAACCTTGGACCTACACC	gene specific primer for recombinant
M13-47	CGCCAGGGTTTTCCCAGTCACGAC	vector primer for sequencing
RV-M	GAGCGGATAACAATTTCACACAGG	vector primer for sequencing
T7	ACATCCACTTTGCCTTTCTC	vector primer for sequencing
T7-ter	TGCTAGTTATTGCTCAGCGG	vector primer for sequencing