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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

with blast algorithm at the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/blast/). The deduced amino acid sequences of EsGal were analyzed with the EditSeq module of DNAStar Lasergene suite 12.3.1. SignalP 4.1 program was utilized to predict the presence and location of signal peptide (http://www.cbs.dtu.dk/services/SignalP/). The protein domain features of EsGal were predicted by Simple Modular Architecture Research Tool (SMART) 7.0 (http://smart.embl-heidelberg.de/). Multiple sequence alignment of EsGal and other galectins was performed with ClustalW multiple alignment program 2.1 (http://www.ch.embnet.org/software/ClustalW.html) and multiple alignment show program 2.0 (http://www.bioinformatics.org/sms2/).

2.5. Real-time PCR analysis of EsGal mRNA expression

The mRNA transcripts of EsGal in different tissues and their temporal expression profile in haemocytes of crabs stimulated with various microbes were determined via quantitative real-time PCR (qRT-PCR). All qRT-PCR reactions were performed with the SYBR premix ExTaq (Tli RNaseH plus) (RR420, Takara, Japan) on a 7500 Real-Time Detection System (Appliedbiosystems, USA). The information of all primers used in this assay was shown in Table 1. The mRNA expression level of EsGal was normalized to that of β-actin for each sample. The comparative C_t method ($2^{\Delta\Delta Ct}$ method) was used to analyze the mRNA expression level of EsGal [22]. All data were given in terms of relative mRNA expression level as mean ± S.D. (n = 5). The data were subjected to one-way analysis of variance (one-way ANOVA) followed by a multiple comparison (S–N–K), and the *p* values less than 0.05 were considered statistically significant.

2.6. Recombinant, overexpression and purification of EsGal in E. coli

The cDNA fragment encoding the mature peptide of EsGal was amplified with two gene-specific primers, EsGal-recombiant-F/R (Table 1), and ligated to the expression vector p*EASY*-E1 (CE101, Transgen, China). The recombinant plasmids, p*EASY*-E1/EsGal, were isolated by MiniBest Plasmid Purification Kit Ver. 4.0 (9760, Takara, Japan) and transformed into *Escherichia coli* strain BL21 (DE3) (CD601, Transgen, China). The parent pET-32a(+) vector without inserts was employed as a negative control. The positive transformants of *E. coli* BL21 (DE3)/p*EASY*-E1/EsGal and *E. coli* BL21 (DE3)/pET-32a(+) were incubated in LB medium containing 100 mg L^{-1} ampicillin at 37 °C with shaking at 220 rpm. When the culture media reached OD_{600} of 0.5–0.7, the cells were incubated for 4 additional hours with the induction of isopropyl-beta-D-thiogalactopyranoside (IPTG, 776687, AiKB, China) at the final concentration of 1 mmol L^{-1}. The recombinant proteins (designated as rEsGal) and negative control (recombinant thioredoxin, designated as rTRX) were purified by a Ni^{2+} chelating sepharose column (71-5027-67, Novagen, USA) under denatured condition (8 mol L^{-1} urea). The purified protein was refolded in gradient urea-TBS glycerol buffer (50 mmol L^{-1} Tris-HCl, 50 mmol L^{-1} NaCl, 5% glycerol, 2 mmol L^{-1} reduced glutathione, 0.2 mmol L^{-1} oxide glutathione, a gradient urea concentration of 6, 4, 3, 2, 0 mol L^{-1}, pH 8.0, each gradient at 4 °C for 12 h) using Slide-A-Lyzer dialysis cassettes (66830, Thermofisher, USA). Then, the resultant proteins were separated by reducing 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized with coomassie brilliant blue R-250.

2.7. Preparation of antibody and western blotting analysis

For preparation of polyclonal antibody, the re-natured protein rEsGal was continued to be dialyzed against ddH_2O using Slide-A-Lyzer dialysis cassettes overnight and then was freeze concentrated. The rEsGal was immuned to six-week old rats to acquire polyclonal antibody using the water soluble adjuvant QuickAntibody (KX0210043, KBQbio, China) according to the usage information. The blood of the immuned rat was collected from the heart and allowed to clot at 4 °C overnight. The clotted blood was centrifuged at 3000 g for 20 min and the serum was tested via western blotting. Briefly, The membrane was blocked with 10 mg mL^{-1} albumin from bovine serum (BSA, 771407, AiKB, China) in PBS at 37 °C for 1 h, incubated with antibody at 37 °C for 1 h, and washed three times with PBS containing 0.05% Tween-20 (PBS-T). Then, the membrane was incubated with goat-anti-rat Ig-horseradish peroxidase (HRP) conjugate (AS028, Abclonal, USA) diluted 1:1000 in PBS at 37 °C for 1 h, and washed three times with PBS-T. Protein bands were stained with sediment 3,3′,5,5′-Tetramethylbenzidine (TMB) solution (PA-108, Tiangen, China) for 5 min and stopped by washing with distilled water.

2.8. ELISA based PAMP binding assay

The binding activity of rEsGal to PAMPs was examined by enzyme linked immuno sorbent assay (ELISA) based PAMP binding assay. Briefly, 20 μg of LPS from *E. coli* 0111:B4 (L2630, Sigma-Aldrich, USA), PGN from *Staphylococcus aureus* (77140, Fluck, USA), glucan from baker's yeast *Saccharomyces cerevisiae* (GLU, G5011, Sigma-Aldrich, USA) in 100 μL of carbonate-bicarbonate buffer (50 mmol L^{-1}, pH 9.6) were coated to 96-well micro titer plate (Costar/Corning, USA) at 25 °C for 12 h, respectively. After the plate was washed three times with PBS-T and blocked using 1 mg mL^{-1} BSA in PBS, 100 μL serial concentrations of rEsGal were added to the wells in the presence of 0.1 mg mL^{-1} BSA, respectively. After incubated at 18 °C for 3 h, the plate was washed three times with PBS-T. One hundred microliter of mouse anti-His tag antibody (372900, Lifetechnologies, USA) diluted 1:1000 in PBS was added to the wells as the first antibody. After incubation at 37 °C for 1 h, the plate was washed again and 100 μL of goat-anti-mouse Ig-HRP conjugate (HS201, Transgen, China) diluted 1:1000 in PBS was added as second antibody and incubated at 37 °C for 1 h. After washing with PBS-T for three times, 50 μL of soluble TMB solution (PA-107, Tiangen, China) was added to each well and incubated at room temperature for 5 min in dark. After the reaction stopped by adding 50 μL per well of 2 mol L^{-1} H_2SO_4, the absorbance was measured with an automatic ELISA reader (Synergy H1M, BioTek, USA) at 450 nm. The rTRX was employed as negative control. Each experiment was repeated in triplicate. The results were expressed as ELISA index (EI) according to the following formula: $EI = OD_{sample}/\text{cut off}$, where the cut off was established as the mean OD of negative controls plus three standard deviations at every point. Samples with EI > 1.0 were considered positive [23].

2.9. Microbe agglutination assay and agglutination inhibition assay

The fluorescein isothiocyanate (FITC)-labeled Gram-negative bacteria *E. coli* strain Top10 and *Vibrio anguillarum* strain M3, Gram-positive bacteria *Micrococcus luteus*, *Staphylococcus aureus* (kindly provided by Dr. Changkao Mu, School of Marine Science, Ningbo University) and *Bacillus subtilis* (1.2428, General Microbiological Culture Collection Center, China), and fungi *Pichia pastoris* strain GS115 were suspended in TBS buffer (50 mmol L^{-1} Tris-HCl, 50 mmol L^{-1} NaCl, pH 8.0) at 1.0×10^9 CFU mL^{-1}. Ten microliter microbe suspension was added to 25 μL rEsGal (25 nm mol L^{-1}) or 25 μL rTRX (25 nm mol L^{-1}) dissolved in TBS buffer. The mixtures were incubated at room temperature for about 45 min and cells were then observed by a fluorescence microscopy (BX51, Olympus,

Japan). To test the carbohydrate binding specificity of rEsGal, 12.5 μL of various carbohydrates were premixed with 12.5 μL of rEsGal at room temperature for 30 min before adding the microbe suspension. The carbohydrates tested in this experiment included D-fructose (F4892, Sigma-Aldrich, USA), D-galactose (92403, Sigma-Aldrich, USA), D-glucose (G7018, Sigma-Aldrich, USA), D-maltose (D8110, Sigma-Aldrich, USA), D-mannose (M3655, Sigma-Aldrich, USA), L-fucose (G4401, Sigma-Aldrich, USA), sucrose (S9378, Sigma-Aldrich, USA) and α-lactose (L8783, Sigma-Aldrich, USA) with a series of 2-fold diluted concentration ranging from 200 mmol L^{-1} to 25 mmol L^{-1}. The inhibitory effect was expressed as the minimum concentration required for complete inhibition of the agglutinating activity against FITC-labeled *E. coli* strain Top10.

2.10. In vitro encapsulation assay

Totally 50 μL suspensions of rEsGal-coated or rTRX-coated nickel agarose beads were added to 450 μL of haemocytes (1×10^7 cells mL^{-1} suspended in anticoagulant as described above) and incubated overnight at room temperature in a 1.5 mL tube with slow

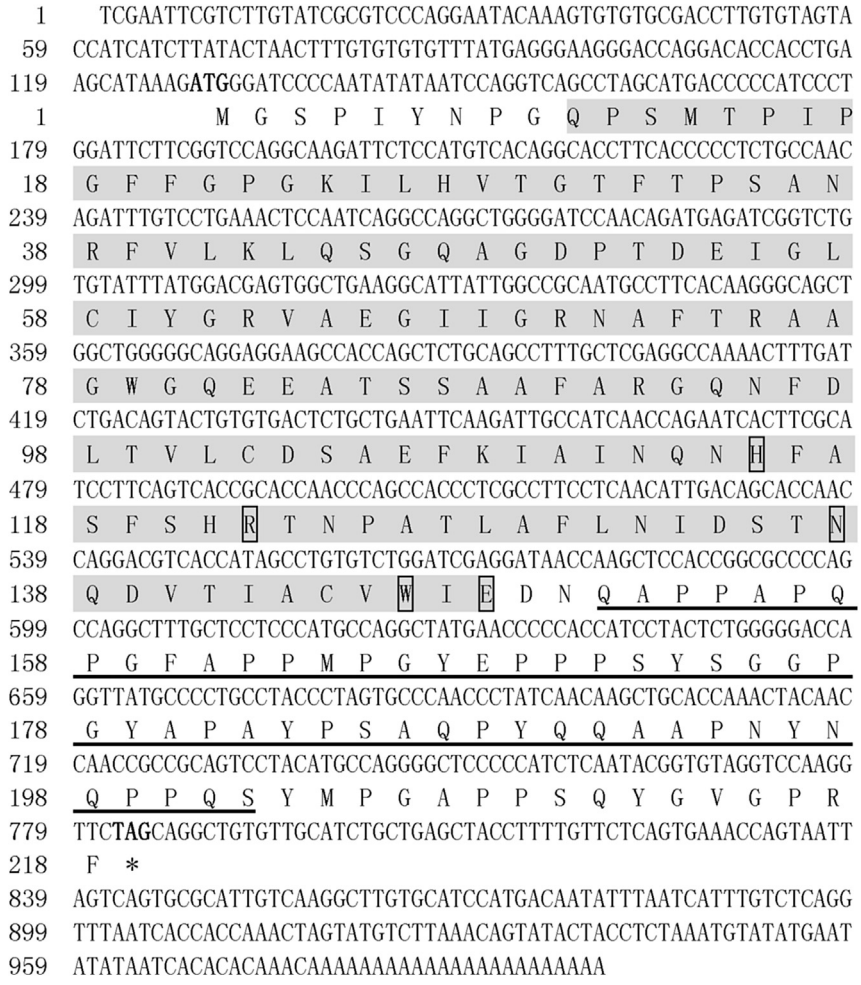

Fig. 1. Nucleotide and deduced amino acid sequences of EsGal. The nucleotides and deduced amino acids are numbered along the left margin. The GLECT/Gal-bind_lectin domain was in shade. The proline/glycine rich low complexity region was underlined. Conserved amino acids involved in sugar binding activity are boxed. The asterisk indicated the stop codon.

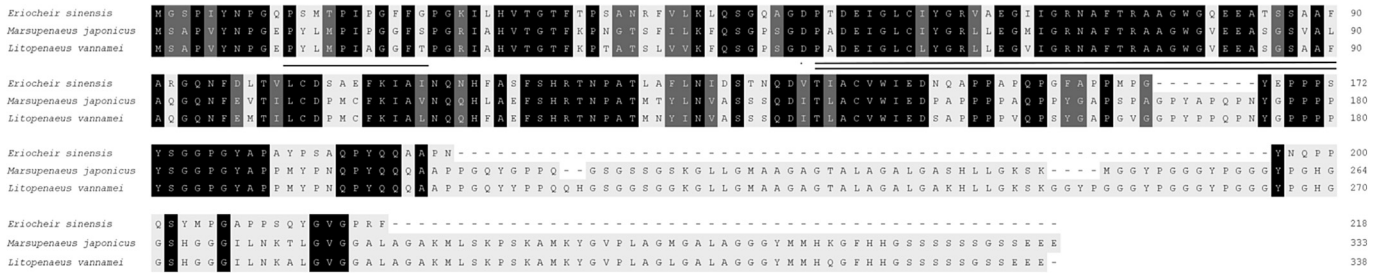

Fig. 2. Multiple alignments of marine crustacean galectins. The black shadow region indicated positions where all sequences share the same amino acid residue. Similar amino acids are shaded in grey. Gaps are indicated by dashes to improve the alignment. Amino acids involved in dimerization were underlined. Amino acids involved in sugar recognition were double underlined. Species and gene accession numbers are as follows: *Eriocheir sinensis* (ADF32023), *Litopenaeus vannamei* (AGV04659) and *Marsupenaeus japonicas* (AFJ59948).

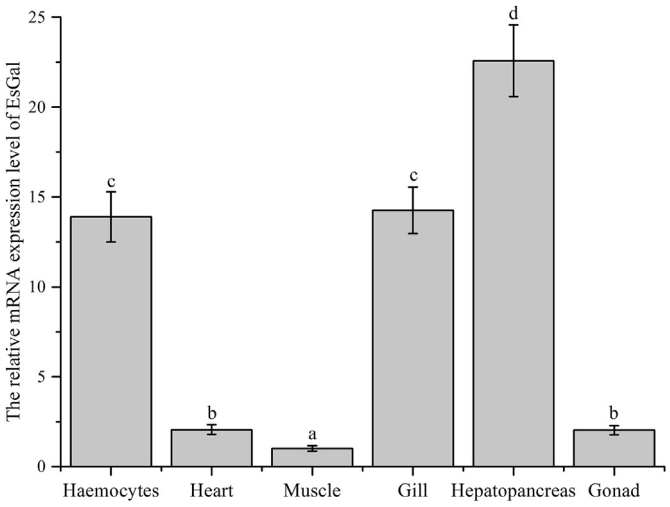

Fig. 3. Tissue distribution of EsGal mRNA transcripts detected by qRT-PCR. EsGal mRNA expression level in haemocytes, heart, muscle, gill, haepatopancreas and gonad of five adult crabs was normalized to that of muscle. The β-actin gene was used as an internal control to calibrate the cDNA template for all the samples. Vertical bars represented mean ± S.D. (n = 5), and bars with different characters indicated significantly different ($p < 0.05$).

rotation. After incubation, the haemocytes were removed, and the agarose beads were washed with 1 mL TBS buffer for four times, each for 5 min. The beads were re-suspended in TBS buffer and observed by microscopy. To verify the encapsulation enhancing specificity of rEsGal, antibody against EsGal protein or mouse anti-His tag antibody diluted 1:1000 was added to the incubation mixture with rats' pre-immune serum or nonspecific mouse Ig as negative control, respectively.

3. Result

3.1. The molecular features, sequence alignment and phylogeny relationship of EsGal

An EST CMCES_A_0959 from the Chinese mitten crab cDNA library was homologous to galectins identified previously [21]. A fragment of 816 bp at the 3′ end of EsGal cDNA was obtained by RACE technique. After overlapping the EST with amplified fragments, a 999 bp nucleotide sequence representing the complete sequence of EsGal cDNA was obtained and deposited in GenBank under the accession number GQ240296. The complete sequence of EsGal consisted of a 5′ untranslated regions (UTR) of 127 bp, a 3′-UTR of 215 bp with a poly (A) tail and an open reading frame (ORF) of 657 bp. The ORF encoded a polypeptide of 218 amino acid residues with a calculated molecular mass of approximately 23.14 kDa and a theoretical isoelectric point of 5.20. No signal peptide was predicted in the deduced amino acid sequence of EsGal by SignalP program. A GLECT/Gal-bind_lectin domain (from Q^{10} to E^{148}) and a proline/glycine rich low complexity region (from Q^{151} to S^{202}) were found in the amino acid sequence of EsGal (Fig. 1). The alignment of the amino acid sequence of EsGal with other known crustacean galectins revealed the conserved amino acids involved in dimerization and sugar recognition of EsGal (Fig. 2). The deduced amino acid sequence of EsGal exhibited as high as 67% similarity with MjGal and LvGal1.

3.2. The distribution of EsGal mRNA in different tissues

The qRT-PCR technique was employed to detect the distribution

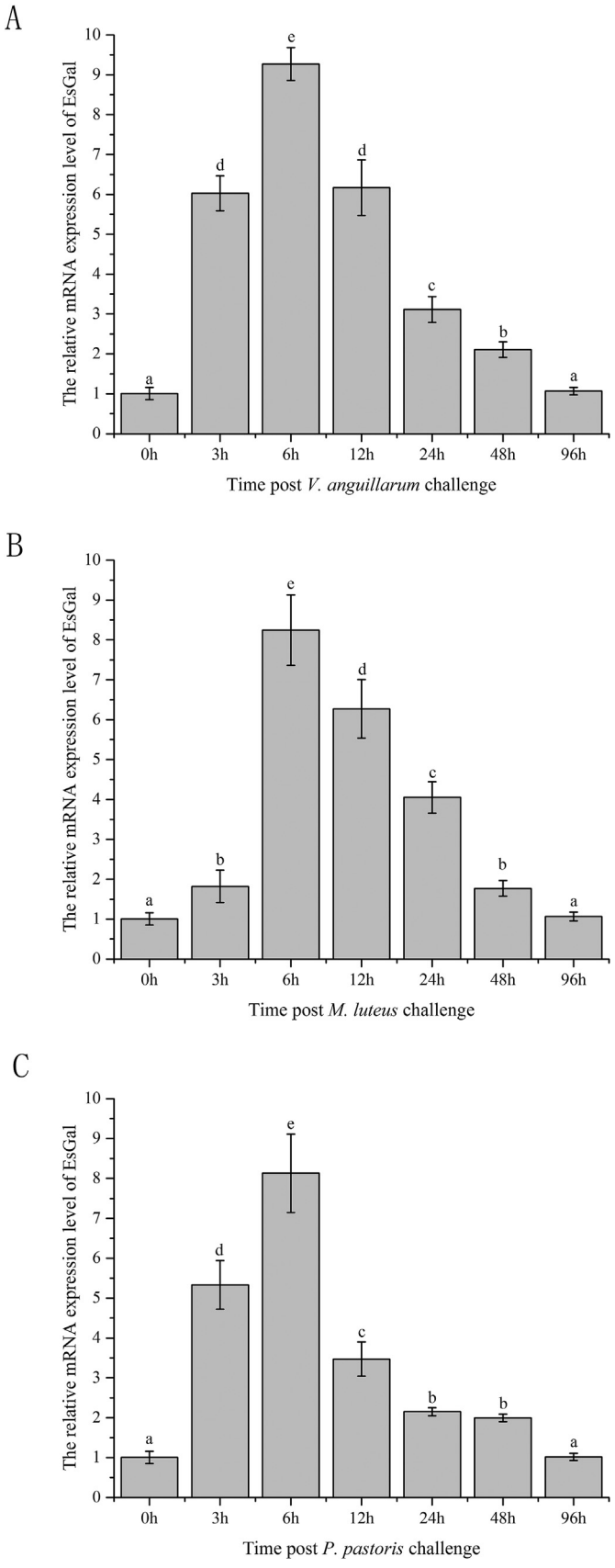

Fig. 4. Temporal mRNA expression profiles of EsGal detected by qRT-PCR in crab haemocytes at 3, 6, 12, 24, 48 and 96 h post different microbe stimulations (A: *V. anguillarum*, B: *M. luteus*, C: *P. pastoris*). The β-actin gene was used as an internal control to calibrate the cDNA template for all the samples. Each values was shown as mean ± S.D. (n = 5), and bars with different characters indicated significantly different ($p < 0.05$).

of EsGal mRNA transcripts in different tissues with β-actin gene as internal control (Fig. 3). For both EsGal and β-actin, there was only one peak at the corresponding melting temperature in the dissociation curve analysis, indicating that the PCR products were specifically amplified (data not shown). The highest mRNA expression level of EsGal was found in hepatopancreas, which was 22.57-fold ($p < 0.05$) of that in muscle, while the expression level in gill and haemocytes was 14.26-fold ($p < 0.05$) and 13.89-fold ($p < 0.05$) of that in muscle, respectively.

3.3. The temporal expression profile of EsGal mRNA post microbe stimulation

The temporal mRNA expression profile of EsGal in haemocytes after invading microbe stimulation was examined via qRT-PCR. The mRNA transcripts of EsGal in haemocytes all increased after the stimulation of *V. anguillarum*, *M. luteus* and *P. pastoris*. After *V. anguillarum* stimulation, the mRNA expression of EsGal increased significantly during 3–48 h ($p < 0.05$), and decreased to the original expression level at 96 h (Fig. 4A). In the *M. luteus* stimulation group, its mRNA transcripts increased to the peak level at 6 h post stimulation (8.24-fold, $p < 0.05$), kept at a high level till 48 h (1.77-fold, $p < 0.05$) and then decreased to the original expression level at 96 h (Fig. 4B). Similarly, its mRNA transcripts were significantly up-regulated at 3 h post *P. pastoris* stimulation (5.33-fold, $p < 0.05$) and reached the peak level at 6 h (8.13-fold, $p < 0.05$) (Fig. 4C).

3.4. Recombinant expression of EsGal in E. coli and preparation of antibody

To investigate the potential activities of EsGal, the recombinant plasmid p*EASY*-E1/EsGal was transformed in *E. coli* strain BL21 (DE3). After IPTG induction, the whole-cell lysate was separated by SDS-PAGE, and a distinct band of rEsGal was revealed with a molecular mass of approximately 23 kDa (Fig. 5B), while the pET-32a(+) vector without insert fragment as a negative control produced a distinct band of rTRX protein of approximately 20 kDa (Fig. 5A). The rEsGal and rTRX proteins were purified from the IPTG induced whole cell lysate. The purified protein was used to obtain immune serum, and western blotting was carried out to identify

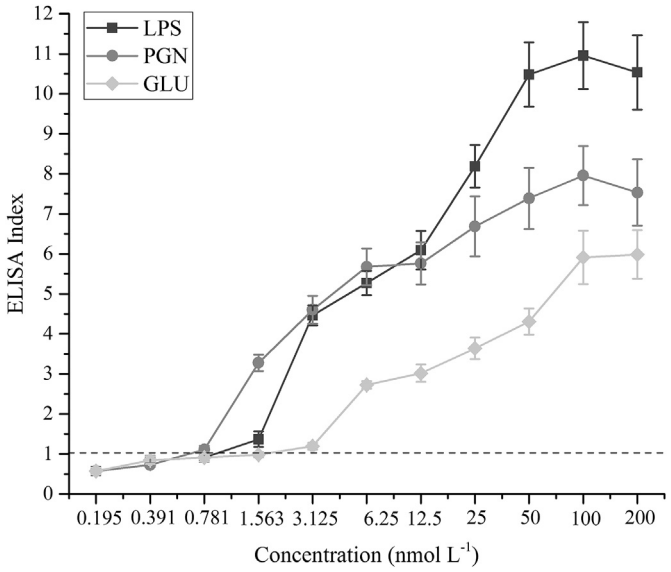

Fig. 6. ELISA analysis of the interaction between rEsGal and PAMPs. Samples with ELISA Index (EI) > 1.0 were considered positive. Results are representative of the mean ± S.D. (n = 3).

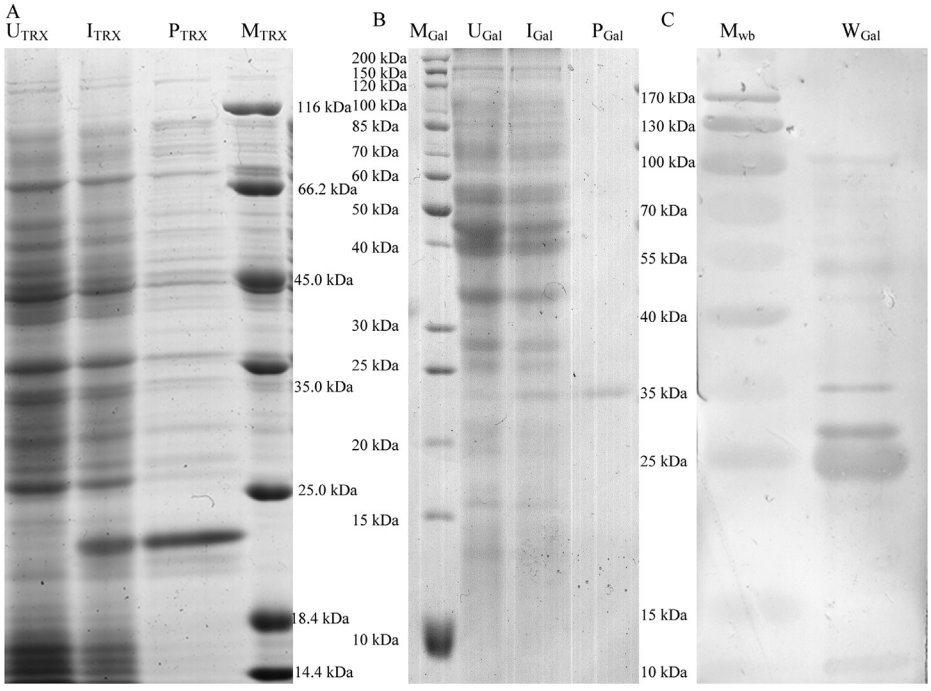

Fig. 5. SDS-PAGE analysis of the rEsGal and rTRX protein in *E. coli* strain BL21 (DE3) and western blotting analysis of rEsGal protein. A: rTRX. Lane U_{TRX} was the supernatant of non-induced bacteria lysate of rTRX protein. Lane I_{TRX} was the supernatant of IPTG-induced bacteria lysate of rTRX protein. Lane P_{TRX} was purified rTRX protein. Lane M_{TRX} was the unstained protein molecular weight marker (26610, Fermentas, USA). B: rEsGal. Lane M_{Gal} was the unstained protein ladder (26614, Fermentas, USA). Lane U_{Gal} was the supernatant of non-induced bacteria lysate of rEsGal protein. Lane I_{Gal} was the supernatant of IPTG-induced bacteria lysate of rEsGal protein. Lane P_{Gal} was purified rEsGal protein. C: western blotting analysis. Lane M_{wb} was the pre-stained protein ladder (26616, Fermentas, USA). Lane W_{Gal} was the specificity of the antiserum tested via western blotting.

the specificity of antibody. A clear reaction band about 25 kDa with high specificity was revealed, and a few non-specific bands were also visible (Fig. 5C). As negative control, no visible reaction band was detected in group of rats' pre-immune serum (data not shown).

3.5. PAMPs binding activity of recombinant EsGal protein

The binding activity of rEsGal to various PAMPs was examined by ELISA based PAMP binding assay. The binding activity was recorded as EI, and the samples with EI > 1.0 were considered as positive. The results showed that the rEsGal could bind three typical PAMPs in a dose-dependent manner and exhibited binding activity even at rather low concentration (Fig. 6).

3.6. Microbial agglutinating activity of rEsGal

The FITC-labeled Gram-negative bacteria *E. coli* strain Top10 and *V. anguillarum* strain M3, Gram-positive bacteria *M. luteus*, *S. aureus* and *Bacillus subtilis*, and fungi *P. pastoris* strain GS115 were used to test the microbial agglutinating activity of rEsGal. The rEsGal protein could agglutinate all the tested microorganisms in the presence of Ca^{2+}, but it exhibited no agglutination activity in the absence of Ca^{2+}. No agglutination was observed in control groups (Fig. 7). Additionally, the agglutinating activity of rEsGal towards *E. coil* strain Top10 was inhibited after the addition of 100 mmol L^{-1} D-galactose and 50 mmol L^{-1} α-lactose, while no significant change of this agglutinating activity was observed after the incubation of other carbohydrates even at their maximum tested concentration (Fig. 8).

3.7. In vitro encapsulation assay

In the *in vitro* encapsulation assay, almost all the agarose beads coated with rEsGal were encapsulated by variable numbers of haemocytes (encapsulation ratio 88.34%), while few or no haemocytes were attached to the control beads (15.64%). Additionally, after the addition of the antibody against EsGal protein and mouse anti-His tag antibody, the encapsulation ratio of haemocytes

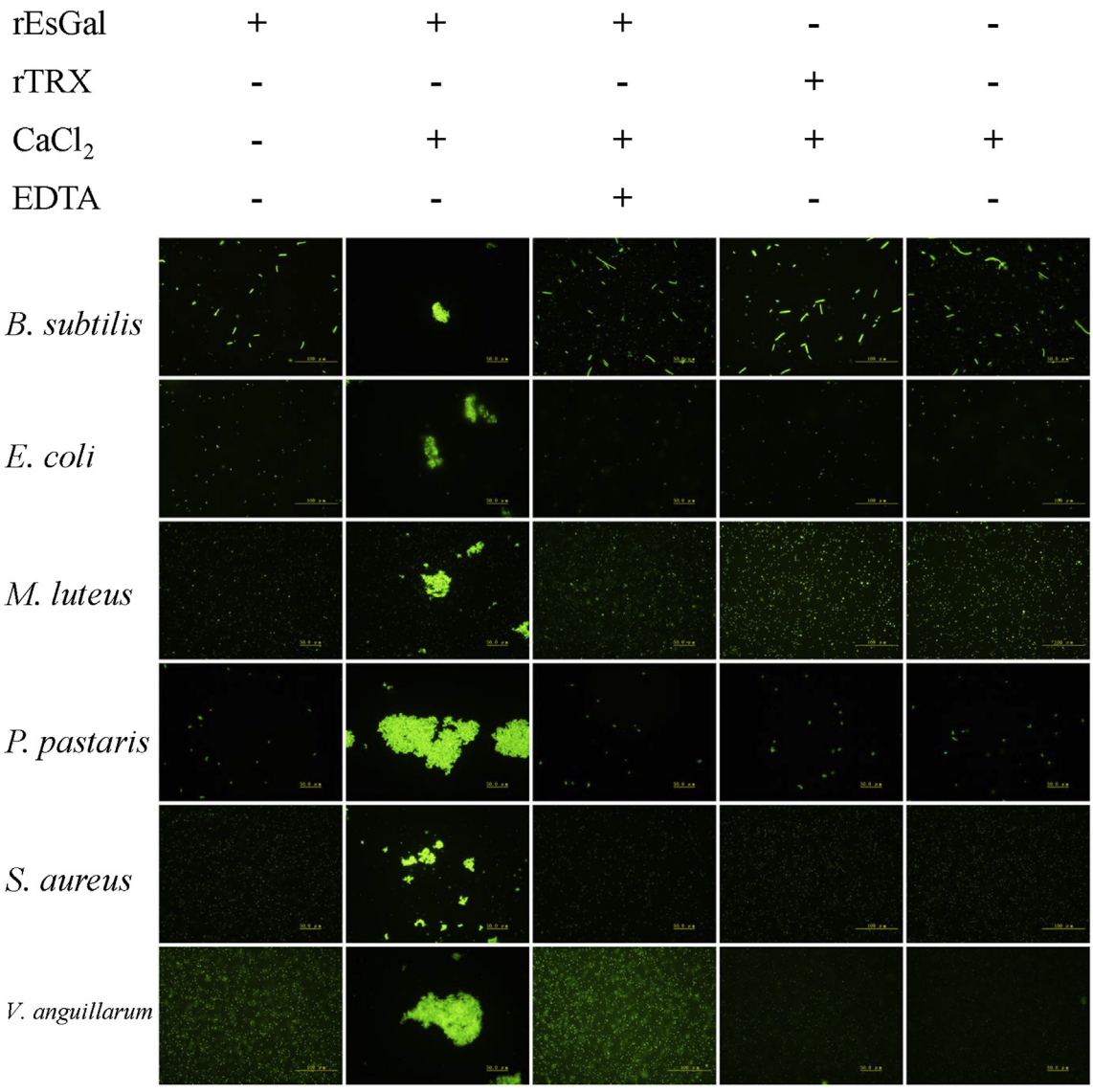

Fig. 7. Microbial agglutinating activity of rEsGal against FITC-labeled *B. subtilis*, *E. coli* strain Top10, *M. luteus*, *P. pastoris* strain GS115, *S. aureus* and *V. anguillarum* strain M3. +: presence. −: absence. The concentration of rEsGal, rTRX, $CaCl_2$ and EDTA were 25 nmol L^{-1}, 25 nmol L^{-1}, 10 mmol L^{-1} and 10 mmol L^{-1}, respectively.

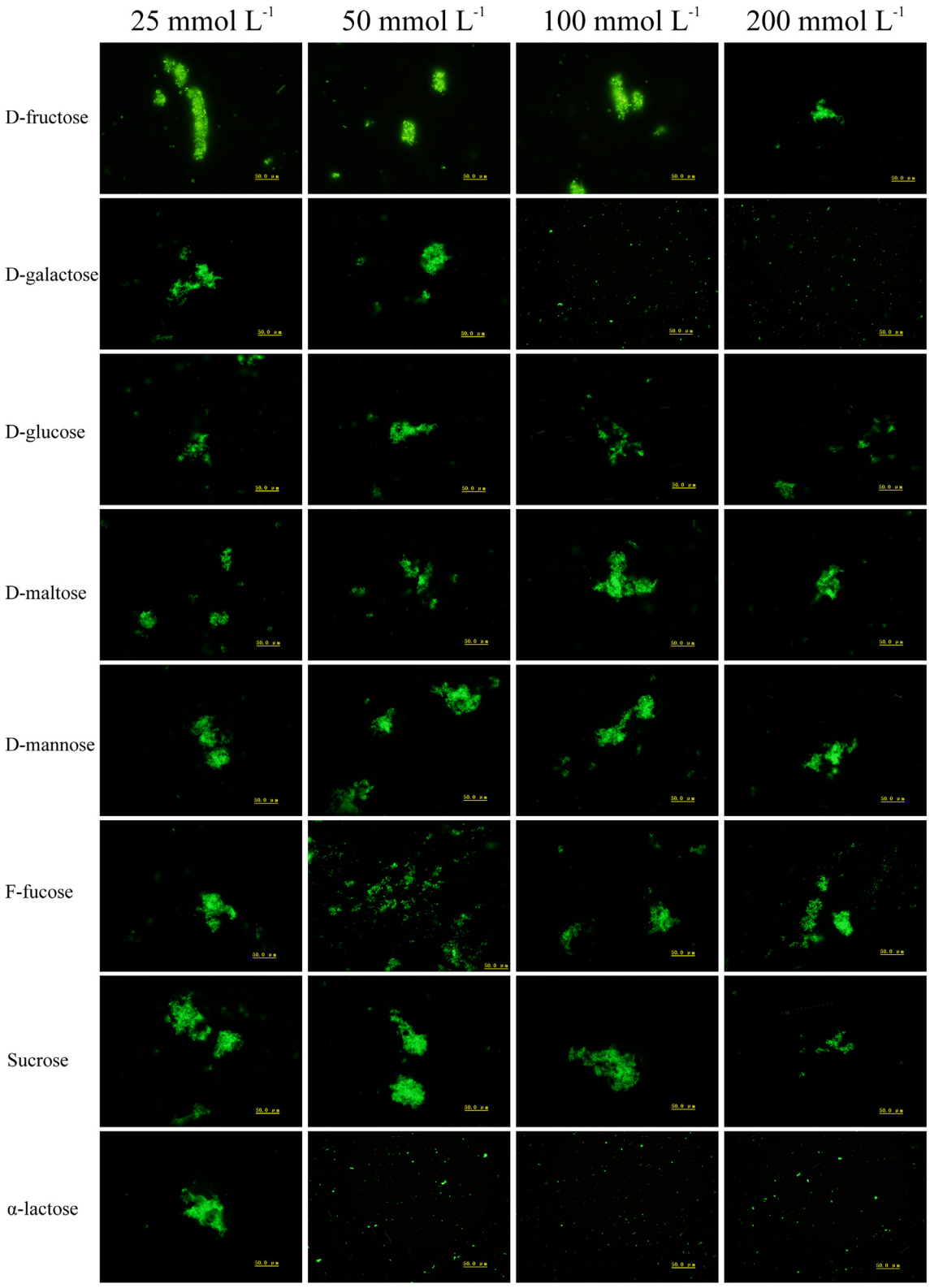

Fig. 8. Inhibition of agglutinating activity of rEsGal against *E. coli* strain Top10 by various compounds. FITC-labeled *E. coli* strain Top10 was incubated in TBS buffer with 25 nmol L^{-1} rEsGal and 10 mmol L^{-1} $CaCl_2$. The scale bars were auto generated and represented 50 μm.

decreased to 16.24% and 14.04%, respectively, while the encapsulation ratio of haemocytes in rats' pre-immune serum added group and nonspecific mouse Ig added group was 83.67% and 85.62%, respectively (Fig. 9).

4. Discussion

Galectins represent a large evolutionally conserved protein family with carbohydrate binding specificity primarily to β-

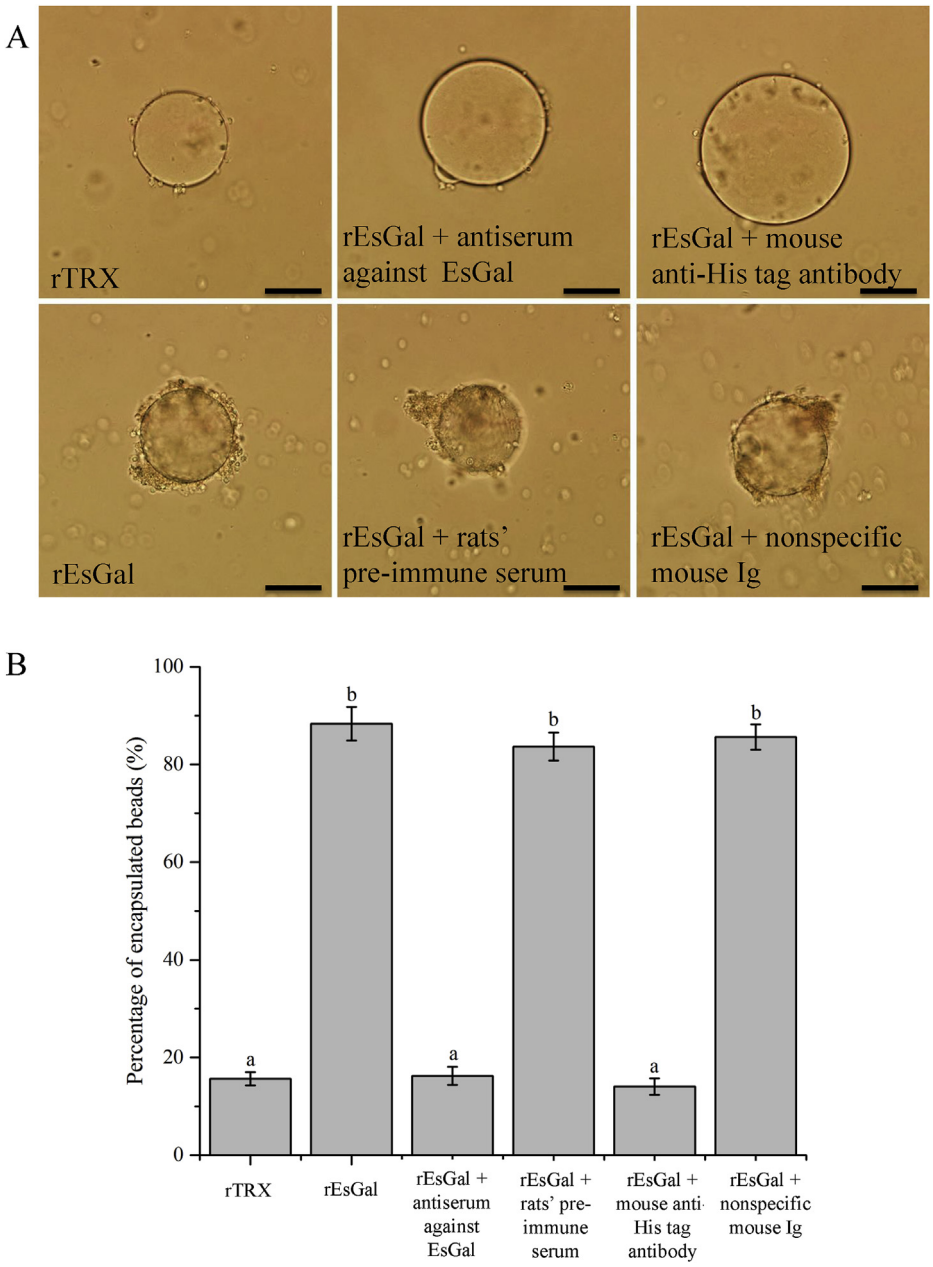

Fig. 9. The enhancement of haemocytes encapsulation by rEsGal. A: Nickel agarose beads coated with rTRX or rEsGal. The concentration of both rEsGal and rTRX was 25 nmol L^{-1}, and all the antibody was diluted 1:1000. B: The ratio of beads encapsulated by haemocytes. Vertical bars represent mean ± S.D. (n = 3), and bars with different characters indicated significantly different ($p < 0.05$). The scale bars represented 50 μm.

galactoside residues [24]. They play important roles in diverse immunological and pathological processes and function as receptors and effectors in innate immune system [3]. In the present research, the full-length cDNA of galectin was cloned from Chinese mitten crab *E. sinensis*. Consistent with other identified crustacean galectins, no classical signal peptides were revealed in EsGal (Fig. 1), indicating it might be secreted to the extracellular space via an endoplasmic reticulum (ER)/Golgi independent pathway [15–17]. The amino acid sequence of EsGal shared high similarities with other identified galectins from marine crustaceans (Fig. 2). There were only one GLECT/Gal-bind_lectin domain and a proline/glycine rich low complexity region observed at the N-terminus and C-terminus of the amino acid sequence of EsGal, respectively (Fig. 1). But it was worth noting that EsGal could not be classified as chimera-type galectins, as the CRD of typical chimera-type galectins was located at the C-terminus. Such phenomenon has also been observed in MjGal, LvGal1 and LvGal2 [15–17], indicating that galectins from marine crustaceans might represent a new type, besides the four known ones. The conserved function domains of EsGal and high similarity with other identified galectins collectively suggested that EsGal was a novel member of invertebrate galectin family, and it could share similar functions with those from other invertebrates.

In the present research, the mRNA transcripts of EsGal were constitutively detected in all the tested tissues (Fig. 3), which was similar to that of MjGal, LvGal1 and LvGal2 [15–17], indicating that EsGal could be involved in many important physiological processes of crabs. Most invertebrate C-type lectins involved in immune responses were mainly expressed in the hepatopancreas and released to the circulation [16]. Similarly, the highest mRNA expression level

of EsGal was also observed in hepatopancreas, followed by gill and haemocytes, which was speculated to be related with the potential function of these tissues. The hepatopancreas was regarded as the central immune related organ in crustaceans and mollusks [10,25], while gill was believed to be the first defense line against invading microbes in fish and lower animals [26]. The crustacean haemocytes are involved in various aspects of surveillance and cellular immune responses [27], so they were selected as candidate for investigating the temporal mRNA expression profile of EsGal post various microbe stimulations. It was reported that the mRNA transcripts of LvGal1 and MjGal could respond to Gram-negative bacteria *V. anguillarum*, but not Gram-positive bacteria *Micrococcus lysodeikticus* [15,17], while those of AiGal1 and AiGal2 could be induced by Gram-negative bacteria *V. anguillarum* and Gram-positive bacteria *M. luteus* but not the fungi *P. pastoris* [5,7]. In the present research, the mRNA transcripts of EsGal in haemocytes increased significantly after the stimulation of *V. anguillarum*, *M. luteus* and *P. pastoris* (Fig. 4), indicating EsGal might exhibit a wider ligand spectrum. Additionally, the EsGal responded to invading bacteria more intensely than fungi, indicating that it might be mainly involved in the immune response against bacterial pathogen. Moreover, EsGal mRNA was fleetly increased within the first 3 h, and reached to the peak level at 6 h after different microbe stimulations, which was earlier than those of LvGal1, LvGal2, MjGal, AiGal1 and AiGal2 in the immune response [5,7,15,17], indicating that EsGal could serve as an acute protein in the immune response of crabs against various invading microbes.

Galectins function as PRRs in the immune defense against invading microbes, which makes them indispensable components in the innate immune response [28,29]. In the present research, the rEsGal could bind LPS, PGN and GLU, in a dose-dependent manner even at rather low concentration (Fig. 6). It was reported that rMjGal could also bind to several polysaccharides, including LPS and lipoteichoic acid (LTA), but not PGN [15]. The activity of rEsGal to bind various PAMPs indicated that EsGal could serve as a PRR to recognize various invading microbes. Galectin-glycan interactions are essential to diverse immune processes, including those relevant to pattern recognition, immunomodulation and immune response [28]. Recently, galectins have been observed to interact directly with the β-galactosides on the surface of viruses, bacteria, fungi and parasites [30]. In the present research, rEsGal displayed high Ca^{2+}-dependent binding/agglutinating activity to Gram-negative bacteria *E. coli* strain Top10 and *V. anguillarum* strain M3, Gram-positive bacteria *M. luteus*, *S. aureus* and *B. subtilis*, and fungi *P. pastoris* strain GS115 (Fig. 7), suggesting that EsGal was involved in immune defense against a broad-spectrum of microbes by recognizing and binding to their surface. Furthermore, the agglutinating activity could be inhibited by D-galactose and α-lactose (Fig. 8), both of which were β-galactosides containing oligosaccharides. It was demonstrated that the binding mechanism of galectins was related with certain residues of CRD [5]. Most of these residues were conversed in EsGal, including H^{116}, N^{122}, N^{137}, W^{146} and E^{148} (Fig. 1), which supported that EsGal were involved in immune defense by recognizing and binding bacteria in a β-galactoside manner. Additionally, galectins were once considered as calcium-independent S-type lectins [31]. However, it was reported that some galectin did exhibit Ca^{2+}-dependent carbohydrates binding activity, for example, galectin from the tunicate *Polyandrocarpa misakiensis* (PmGal) [32]. Similarly, in the present research, rEsGal displayed Ca^{2+}-dependent binding/agglutinating activity to various microbes. Although the Ca^{2+}-dependency was rarely investigated in other marine invertebrate galectins, these phenomena observed in PmGal and EsGal indicated calcium-independent binding activity may not be the indispensable feature for galectin, especially for the ancient ones.

Besides pathogen recognition, galectins could also mediate host-pathogen interaction and cell-cell adhesion to neutralize invading microbes [28]. In the crustacean cellular response, encapsulation refers to a process by which haemocytes attach to nonself components and eliminate the particle, and it always accompanies with melanization through the activation of prophenoloxidase (proPO) system [33,34]. As the main cellular immune response against large invading particles, this process requires the coordination of both cellular and humoral factors [25]. Galectins are one of the most effective humoral factors to activate cellular interaction, and to trigger and then enhance cellular encapsulation in invertebrates [10]. In the present research, the rEsGal could promote haemocytes encapsulation *in vitro*, and this promotion could be inhibited by both antibody against EsGal protein and mouse anti-His tag antibody. Similar function in cellular adhesion and the enhancement of encapsulation was also identified in the rAiGal2 from *Argopecten irradians* [5]. The significant activity of rEsGal to enhance haemocytes encapsulation might forebode the involvement of EsGal in the activation of proPO system.

In conclusion, a novel galectin has been identified from *E. sinensis*. Its mRNA transcripts were found to be significantly induced after microbe stimulation. It could not only bind to various PAMPs and agglutinate various microbes in a β-galactoside manner, but also enhance the encapsulation of haemocytes. All these results suggested that EsGal functioned as a resourceful PRR involved in the innate immune defense against invading microbes in crabs.

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