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Characterization and functional analysis of a novel gC1qR in the swimming crab *Portunus trituberculatus*

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ABSTRACT

The receptor for the globular head of complement component C1q, gC1qR, is a multifunctional and multiligand binding protein with a crucial role in host defense. In the present study, a full-length cDNA sequence of a gC1qR homolog (PtgC1qR) in *Portunus trituberculatus* was identified. PtgC1qR was a 268-amino-acid polypeptide with a conserved MAM33 domain and a mitochondrial targeting sequence in the first 56 amino acids. The transcripts of PtgC1qR were detected in all examined tissues with the highest level detected in the hepatopancreas. Compared with other early embryonic stages, PtgC1qR was highly expressed in the fertilized eggs and embryos at the cleavage stage, which suggest PtgC1qR may be a maternal gene. The transcripts of PtgC1qR in hemocytes exhibited time-dependent response expression pattern after challenged with bacteria (*Vibrio alginolyticus*, *Micrococcus luteus*) and fungi (*Pichia pastoris*). Moreover, the recombinant PtgC1qR (rPtgC1qR) exhibited strong antibacterial activity and microbial-binding activity, suggesting its crucial role in immune defense and recognition. Further phenoloxidase (PO) assay showed that rPtgC1qR could suppress the crab PO activity *in vitro* in a dose-dependent manner, and it could result in nearly 100% inhibition of PO activity under the concentration of 11.65 μM. Knockdown of PtgC1qR could significantly enhance the expression of serine protease related genes (*PtSP1-3* and *PtSPH*), proPO-associated genes (*PtproPO* and *PtPPAF*) and C3-like genes (*Pta2M1* and *PtTEP*). However, the phagocytosis related genes (*PtMyosin*, *PtRab5* and *PtArp*) and *Pta2M2* were significantly down-regulated in the PtgC1qR silenced crabs. These findings together demonstrate that PtgC1qR might function in crab immune response via its antibacterial activity, immune recognition or regulating the proPO system, complement pathway and phagocytosis.

1. Introduction

The complement component C1q is the first subcomponent of the classical complement pathway that act as a bridge to connect the innate immunity and acquired immunity [1]. The receptor for the globular head of complement component C1q, gC1qR, can bind specifically to C1q [2,3]. This protein has been detected in various tissues and cellular compartments except in erythrocytes [4,5]. As a potentially multifunctional protein, gC1qR on the cell surface can function as molecular patterns and docking receptors for numerous extra- or intracellular proteins [6–8]. Although a growing number of gC1qRs have been recently reported in invertebrates [9–11], their potential functions and molecular mechanisms in host defense remain to be further elucidated.

The versatility of gC1qR as a multiligand binding protein has been confirmed [5,7,12]. After binding to a ligand, gC1qR can induce proinflammatory by-products and activate early defense responses against microbial and viral invasion through the complement and kinin/kallikrein pathways [5,13,14]. In crustaceans, gC1qR, such as FcgC1qR from *Fenneropenaeus chinensis* and MrgC1qR from *Macrobrachium rosenbergii*, could bind to microorganisms and pathogen-associated molecular patterns (PAMPs) [9,11]. The recombinant PmC1qBP from *Penaeus monodon* could even bind to the mouse C1q [10]. In addition, gC1qR is proved to play important roles in promoting viral infection and maintaining virus persistence through binding to the core proteins of hepatitis virus and adenovirus [14,15]. Whereas white spot syndrome virus replication could be inhibited by the attachment of gC1qR to its envelope proteins [7].

In arthropods, an important characteristic of the innate immune

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Table 1
Primer sequences used in this study.

Primers Name	Sequence (5′–3′)	PCR objective
PtgC1qR-F	CTCACTTCTCCCTTAATTTTCGC	Gene cloning
PtgC1qR-R	TTAGTAGGTCGTACATCATCCCATC	Gene cloning
PtgC1qR-3′F	GAGCCAAGACACTCTCCTTTACCTGC	Gene cloning
3′ROP	GACTCGAGTCGATCGATTTTTTTTTTTTTTTTTT	Gene cloning
PtgC1qR-F′	CGCGGATCCATGTTGCGTGGAGCCCTG	Recombinant expression
PtgC1qR-R′	CCGCTCGAGCACGAAATCCTGAAGGCGC	Recombinant expression
PtgC1qR-RTF	ACGTGTCTTCAGGGGCGTGT	Real-time PCR
PtgC1qR-RTR	CACGAGAGGTGACCGGGAGC	Real-time PCR
PtSP1-RTF	ACTATGTCCAGCCAGCGTGT	Real-time PCR
PtSP1-RTR	GGAAGGACTCGCGCTCATAG	Real-time PCR
PtSP2-RTF	TAAGGACATCGGACAGGAGACACT	Real-time PCR
PtSP2-RTR	TAAGGAAGTGAACGCTATCTCT	Real-time PCR
PtSP3-RTF	AAGCCAGTCGAAATACAGGAG	Real-time −PCR
PtSP3-RTR	CAGCATCTCCTTCCCAATTCC	Real-time PCR
PtSPH-RTF	CATCCTTGACCAGCCAGCA	Real-time PCR
PtSPH-RTR	CCCACCCAGACACAACACA	Real-time PCR
PtMyosin-RTF	CGTTGGCGAAGTAGGAGAGT	Real-time PCR
PtMyosin-RTR	GAACAAGAGGCGTAATGAGGT	Real-time PCR
PtRab5-RTF	AACCCAGCATCCAGTCACCC	Real-time PCR
PtRab5-RTR	TACCCCTAAGCCCCTCAACC	Real-time PCR
PtArp-RTF	GCTCCACCATCACCACTCTG	Real-time PCR
PtArp-RTR	TTAGCCATCTCCTTCCCTGC	Real-time PCR
PtproPO-RTF	CCTCTTCTTCACGACACTCAACTG	Real-time PCR
PtproPO-RTR	TCACGAGATAACACAAAACGCC	Real-time PCR
PtPPAF-RTF	GGACAGGACCAAGACCCAGT	Real-time PCR
PtPPAF-RTR	GATTTGAGAAGGAACAAGCGTG	Real-time PCR
Ptα2M1-RTF	TGTGCCTCCTACCGCCTTCC	Real-time PCR
Ptα2M1-RTR	GGTGTCCCTCTCTCTCAACTCATTT	Real-time PCR
Ptα2M2-RTF	GTGGTTGGCTACGGGACGGGT	Real-time PCR
Ptα2M2-RTR	ACGGCAATGTCATCACTGGGGAT	Real-time PCR
PtTEP-RTF	CTCTTCTCGCTGCTTTCCTTCATC	Real-time PCR
PtTEP-RTR	TTTTTGGGACTTTGCCACCACTT	Real-time PCR
β-actin-F	TCACACACTGTCCCCATCTACG	Real-time PCR
β-actin-R	ACCACGCTCGGTCAGGATTTTC	Real-time PCR
M13-47	CGCCAGGGTTTTCCCAGTCACGAC	Sequencing
RV-M	GAGCGGATAACAATTTCACACAGG	Sequencing
T7 promoter	TAATACGACTCACTATAGGG	Sequencing
T7 terminator	GCTAGTTATTGCTCAGCGGT	Sequencing

The *Bam* HI and *Xho* I sites were underlined.

system is activation of serine proteinase (SP) cascade pathways in hemolymph, which results in activation of prophenoloxidase (proPO) in a manner similar to vertebrate complement system [16,17]. Recent studies have shown that some complement components, such as mannose-binding lectin (MBL) and alpha-2-macroglobulin (α2M), are involved in activation of the proPO activating system [18,19]. Due to lack of the late complement components (C5−C9), MBL in *Halocynthia roretzi* and α2M in *Ixodes ricinus* perform the final immune function mainly via phagocytosis [20,21]. The C1qR in human is also reported to play a specific role in modulating phagocytosis [22]. However, little is known about the regulation of immune signaling by gC1qR in crabs.

The swimming crab *Portunus trituberculatus*, a commercially important species in aquaculture, often suffers various diseases caused by bacterias and viruses resulting in catastrophic economic losses to crab aquaculture [23,24]. Hence, it is imperative to further illustrate the innate immune defense mechanism of crabs and explore new strategies for controlling the diseases. In this study, a gC1qR homolog, PtgC1qR, was cloned and characterized. The expression patterns following pathogen challenge, antimicrobial activity, microorganisms binding assay and RNA interference assay were carried out in order to reveal its immune functions in host defense.

2. Materials and methods

2.1. Crabs, immune challenge and samples collection

Healthy crabs (140 ± 5 g) purchased from a commercial farm in Qingdao, China, were reared in filtered seawater (15 ± 2 °C) for one week before processing. During the experiment, crabs were fed with clam meat once daily at night. To analyze the expression patterns after pathogens challenge, crabs were randomly separated into four groups and each individual of the challenge group was injected with 100 μl of live *Vibrio alginolyticus* (3×10^8 CFU/ml), *Pichia pastoris* (3×10^8 CFU/ml) or *Micrococcus luteus* (3×10^8 CFU/ml) suspended in phosphate buffered saline (PBS). The crabs receiving an injection of 100 μl PBS were served as the control group. At 0, 2, 4, 8, 12, 24, 48 and 72 h post injection, five individuals were randomly sampled to collect hemocytes. Briefly, hemolymph was harvested from the last walking leg with an equal volume of ice-cold anticoagulant buffer (27 mM sodium citrate, 336 mM NaCl, 115 mM glucose, 9 mM EDTA, pH 7.0) [25]. Then the hemolymph immediately centrifuged at 800 g, 4 °C for 5 min to isolate the hemocytes. For tissue-specific expression analysis, the hemocytes, gill, hepatopancreas, eyestalk, muscle, heart, intestine, stomach, ganglia thoracalia, ovary, testis and brain from five untreated crabs were also collected for RNA isolation.

The ovigerous crabs (*P. trituberculatus*) were collected from a market in Qingdao, Shandong Province, China. The fertilized eggs (Fe) were collected immediately after discharge, and the embryos at the cleavage stage (Cs), blastula stage (Bs), gastrula stage (Gs) and heart beating stage (Hs) were collected separately in 1.5 ml tubes from the same crab, then immediately frozen in liquid nitrogen until RNA extraction. The developmental stages of embryos were monitored under a dissecting microscope, and samples at each stage were collected from five identical crabs.

2.2. RNA isolation, cDNA synthesis and gene cloning of PtgC1qR

The total RNA was extracted from the hepatopancreas using TRIzol reagent according to the manufacture's protocol (Invitrogen). The concentration of RNA was measured using Nanodrop 2000 (Thermo), and 1% agarose gel electrophoresis was done to test RNA integrity. The first-strand cDNA was synthesized for the quantitative real-time PCR (qRT-PCR) analysis using a PrimeScript™ first Strand cDNA Synthesis Kit (Takara, Dalian, China) with an oligo dT primer. To amplify the 3′ end of PtgC1qR cDNA sequence, the first-strand cDNA was synthesized using the Clonetech SMARTer™ RACE cDNA Amplificationkit (Takara, Dalian, China) with 3′ CDS primer.

A gene specific forward primer PtgC1qR-3′F was designed based on the unigene sequence obtained from our transcriptome data. 3′ fragment was amplified using PtgC1qR-3′F and 3′ RACE Outer Primer (3′ ROP). And then a pair of primers, PtgC1qR-F and PtgC1qR-R (Table 1) were designed to obtain the 5′ and middle fragment of the cDNA sequence. The polymerase chain reaction (PCR) was conducted under the following parameters: 94 °C for 32 min; 35 cycles at 94 °C for 30 s, 58 °C for 50 s, and 72 °C for 1 min; and finally, 72 °C for 10 min. The PCR products were gel-purified and cloned into pMD19-T simple vector (TaKaRa). After being transformed into the competent cells of *Escherichia coli* DH5a, the positive recombinants were identified through anti-Amp selection and PCR screening with M13-47 and RV-M primers (Table 1). Three of the positive clones were sequenced by a commercial company (Sangon, China). The complete PtgC1qR cDNA sequence was obtained by overlapping the two fragments.

2.3. Sequence analysis and construction of the phylogenetic tree

The similarities of PtgC1qR were performed using the online BLAST program (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi). Signal peptide prediction was carried out using SignalP 4.0 program (http://www.cbs.dtu.dk/services/SignalP). The putative domain and the mitochondrial targeting sequence were predicted by the SMART (http://www.smart.embl-heidelberg.de/) and MITOPROT programs (http://www.ihg2.helmholtz-muenchen.de/ihg/mitoprot.html), respectively. The cell attachment sequence (arginyl–glycyl–aspartic acid, RGD) and glycosylation sites were predicted using ExPASy PROSITE (http://www.expasy.ch/prosite/). MEGA 7.0 was used to construct a phylogenetic tree.

2.4. Quantitative real-time PCR analysis of PtgC1qR

qRT-PCR was applied to detect the mRNA expression of PtgC1qR in different tissues, embryos at early developing stages and hemocytes after pathogens challenge. The primers used for the qRT-PCR analysis were listed in Table 1, and β-actin was chosen as the reference. The prepared cDNA was diluted 50 times by DEPC-treated water first. Reactions were carried out on an ABI PRISM 7300 Sequence Detection System (Applied Biosystems) using SYBR green II as fluorescent dye. For each PCR reaction, a total volume of 20 μl, containing 10 μl of 2 SYBR Premix Ex Taq (TaKaRa), 0.4 μl 50 ROX Reference Dye, 4 μl of the diluted cDNA, 0.4 μl of each primer (10 μM), and 4.8 μl of sterile distilled H_2O. The PCR program was 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 35 s. The data was calculated using $2^{-\Delta\Delta Ct}$ methods. All data were given in terms of relative mRNA expression as mean ± S.D. The results were subjected to one-way analysis of variance using SPSS 16.0, and significant differences were considered if $P < 0.05$.

2.5. Expression and purification of recombinant PtgC1qR

A pair of gene-specific primers, PtgC1qR-F′ and PtgC1qR-R′, were designed to amplify the sequence encoding mature peptide of PtgC1qR (*Bam*H I and *Xho* I sites are underlined in Table 1). The purified PCR products were inserted into pMD19-T simple vector and then were sequenced to ensure the correct coding sequence. The recombinant plasmid pMD19-T-PtgC1qR digested completely by restriction enzymes *Bam*H I and *Xho* I (NEB), then cloned into pET-32a (+) vector (Novagen). Afterward, the recombinant plasmid pET-32a-PtgC1qR was transformed into competent *E. coli* BL21(DE3) cells (Novagen) for over-expression. The pET-32a vector without insert fragment was selected as a negative control, which could express a thioredoxin (Trx) with 6 × His-tag in the prokaryotic expression system.

After induction expression with isopropyl-β-D-thiogalactosidase (IPTG) at 28 °C, the bacteria pellets were harvested by centrifugation at 8000*g* for 5 min at 4 °C, and resuspended in buffer I (50 mM sodium phosphate, 300 mM NaCl, pH 7.0), then sonicated at 4 °C for 30 min in a combination of 2 s sonication and 2 s interval under 180 W power. The cell lysates were centrifuged at 8000 g for 10 min at 4 °C to collect inclusion bodies. The inclusion bodies were washed twice with buffer I, then washed twice with buffer II (50 mM sodium phosphate, 300 mM NaCl, 2 M urea, pH 7.0) and dissolved in buffer III (50 mM sodium phosphate, 300 mM NaCl, 8 M urea, pH 7.0). The recombinant PtgC1qR (rPtgC1qR) and rTrx proteins were purified by TALON Metal affinity resins (Clontech) under denaturing conditions.

The purified proteins were refolded in gradient urea-TSB glycerol buffer (50 mM Tris-HCl, 50 mM NaCl, 10% glycerol, 1% glycine, 1 mM EDTA, 0.2 mM oxidized glutathione, 2 mM reduced glutathione, urea concentration of 6, 5, 4, 3, 2, 1, 0 M urea in each gradient, pH 8.0; each gradient at 4 °C for 12 h). The resultant proteins were separated by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The purified protein solutions were concentrated with Microsep Advance Centrifugal Devices (3 kD, Pall corporation) based on the manufacturer's instructions. The concentration of rPtgC1qR was measured by BCA (bicinchonininc acid) Protein Assay Kit (Beyotime), respectively.

2.6. Antimicrobial activity assay

The antimicrobial activity of recombinant PtgC1qR proteins was tested against the Gram-positive bacteria (*M. luteus* and *Staphylococcus aureus*), Gram-negative bacteria (*V. alginolyticus* and *Pseudomonas aeruginosa*) and yeast (*P. pastoris*) by liquid growth inhibition assay [26]. Briefly, microorganisms were grown to mid-logarithmic phase and diluted with 50 mM Tris-HCl buffer (pH 8.0) to 10^3 CFU/ml. In sterile 96-well plate, 50 μl of recombinant proteins in 1/2-fold serial dilution with 50 mM Tris-HCl buffer (pH 8.0) were added into the wells. The group with 50 μl of Tris-HCl buffer was used as blank. Then 50 μl of cell suspension (1×10^3 CFU/ml) were added into the wells and incubated at 37 °C for 2 h after that, 150 μl of corresponding medium was added and incubated overnight at suitable temperatures. Absorbance was measured at 600 nm using a precision microplate reader (Emax). The assay was performed with triplicates. The minimum inhibitory concentration (MIC) value was expressed as the range between the highest concentration of the protein where microorganisms were growing ($P > 0.05$) and the lowest concentration that caused 100% growth inhibition ($P < 0.05$).

2.7. Microorganism binding activity of rPtgC1qR

The Gram-positive bacteria (*M. luteus* and *S. aureus*), Gram-negative bacteria (*V. alginolyticus* and *P. aeruginosa*) and yeast (*P. pastoris*) were used to test the binding activity of the recombinant proteins by the previous method described [27]. Bacteria and yeast were cultured to the logarithmic growth phase, and fixed with 37% formaldehyde by gently shaking at 37 °C for 1 h to destroy the proteinase activity of microorganisms. 0.5 ml of microbial cell suspension (3×10^8, suspended in PBS) and 0.5 ml of purified PtgC1qR proteins (final concentration, 1 mg proteins) were incubated with gentle rocking at 4 °C for 30 min. After centrifugation at 800 × *g* and 4 °C for 5 min, the supernatant was removed, and the pellets were washed twice with PBS.

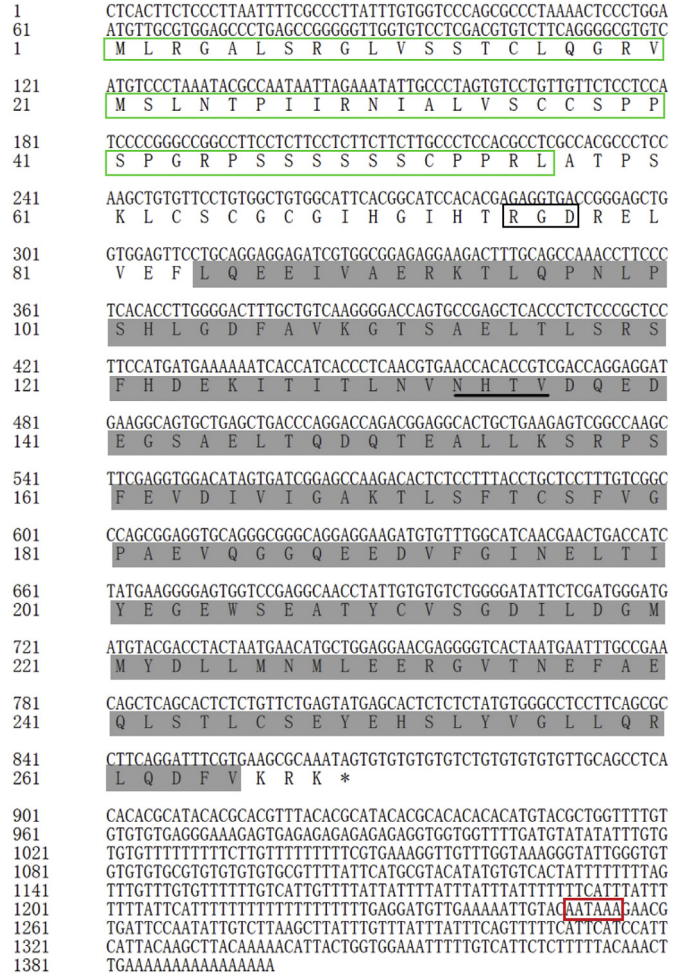

Fig. 1. The full-length cDNA and deduced amino acid sequences of PtgC1qR from *P. trituberculatus*. The MAM33 domain is shadowed in gray. The mitochondrial cleavage site, RGD domain and polyadenylation signal are shown in green, black and red frames, respectively. The stop codon is indicated by the asterisk, and the N terminal glycosylation site is underlined. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Bound proteins were subsequently eluted with 1 × SDS-PAGE sample loading buffer. Microorganisms incubated with PBS were used as control. The supernatant, washed and eluted fractions were run on 15% (w/v) SDS-PAGE.

2.8. Prophenoloxidase inhibitory assay

The hemocytes were obtained using the above mentioned method, washed twice and resuspended in 0.1 M PBS (pH 7.0), then homogenized (20 kHz/100 W, 4 × 30 s, 4 °C) in an ultrasonic homogenizer (Cole Parmer). The hemocyte lysate supernatant (HLS) was separated by centrifugation at 12,000 rpm for 10 min at 4 °C.

The phenoloxidase (PO) assay was performed according to the previous method with slightly modification [28]. Briefly, 40 μl HLS was mixed with 50 μl rPtgC1qR to the final concentrations of 11.61, 5.81 and 2.90 μM in a sterile 96 well plate. The 10 μl V. *alginolyticus* (OD560 = 0.4) were added into the wells and incubated at room temperature for 5 min. The reaction was started by adding 100 μl of L-3, 4-dihydroxyphenylalanine (2 mg/ml, Sigma) in 50 mM Tris-HCl (pH 8.0). The PO activity was monitored at 0, 5, 10, 15, 20, 30, 40 and 50 min by measuring the absorbance at 490 nm. The HLS with *V. alginolyticus* and 50 mM Tris-HCl (pH 8.0) was set as the negative control. The curves of absorbance at 490 nm were plotted against the reaction time.

2.9. Synthesis of siRNAs and RNAi assay

Based on the sequence of PtgC1qR gene, small interfering RNA (siRNA) for RNA interference (RNAi) assays of crab was synthesized *in vitro* using a commercial kit according to the manufacturer's instructions (Takara, Japan). The sequence-specific PtgC1qR-siRNA (5′—CUC GAAGCUUGGCCGACUCUU-3′) was synthesized to silence the expression of PtgC1qR gene, and the sequence of siRNA was scrambled to generate the random-siRNA sequence (5′- GUACUCGCUCGUCGUAGU CAC -3′). The formation of double stranded RNAs was monitored by determining the size in agarose gel electrophoresis. The synthesized siRNAs were dissolved in siRNA buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl) and quantified by spectrophotometry. The synthesized siRNA was diluted with PBS to a final concentration of 0.5 g/l before injection.

The RNAi assay was conducted in crab by the injection of siRNA into the foot of the third appendage using a syringe. In details, 50 μg of siRNA (PtgC1qR-siRNA or siRNA-scrambled) was injected at a volume of 100 μl per crab, and another 50 μg siRNA was injected using the same method at 24 h after the first injection. At the same time, PBS only was injected into crabs as a black control. The amount of injected siRNA was chosen according to our pre-experiment and the RNAi assay in *Eriocheir sinensis* [29]. For each treatment, the hemocytes from five crabs were collected at 12, 24 and 48 h after the second injection. Then the expression of PtgC1qR gene was further determined by qRT-PCR to detect the efficiency of the RNAi.

2.10. The expression patterns of immune-related genes in the PtgC1qR silenced crab

After setting up the RNAi assay, the expression of twelve immune-related genes, including *PtSP1-3* (JF412648, JF412649 and JF412650), *PtSPH* (JF412651), *PtproPO* (FJ215871.1), *PtPPAF* (proPO-activating factor, GQ914996.1), *PtMyosin* (POR|c99862_g4), *PtRab5* (small GTP-binding protein, POR|c94716_g1), *PtArp* (actin related protein, POR|c49398_g1), *Pta2M1-2* (alpha-2-macroglobulin, POR|c84480_g1, POR|c98111_g4) and *PtTEP* (thioester-containing protein, POR|c98947_g3), involved in the serine protease cascades, phagocytosis, proPO activating system and complement pathway was detected in the PtgC1qR silenced crabs. Primer sequences were listed in Table 1.

3. Results

3.1. Sequence analysis of PtgC1qR

The complete cDNA of PtgC1qR gene was 1398 bp in length, including a 5′-untranslated region (UTR) of 60 bp, an open reading frame (ORF) of 804 bp encoding a 268-amino-acid-long protein without signal peptide and a 3′ UTR of 534 bp with the polyadenylation signal (AATAAA) and poly(A) tail. The nucleotide and deduced amino acid sequences are shown in Fig. 1. The theoretical mass of PtgC1qR mature protein was calculated to 29.36 kDa with an isoelectric point of 4.81. The conserved mitochondrial acidic matrix protein (MAM33) domain (residues 84–265) was found in the deduced protein sequence. Using the MITOPROT program, a 56-amino-acid-long mitochondrial targeting sequence was found at the N-terminal end of this predicted protein sequence. An RGD motif and one glycosylation site were also forecasted via ExPASy PROSITE.

3.2. Comparative similarity and phylogenic analysis

BLAST analysis demonstrated that PtgC1qR shared more similarities with other invertebrate gC1qRs (71% identity with *Eriocheir sinensis* ANN46490.1, 73% with *Palaemon carinicauda* AFY05651.1, 68% with

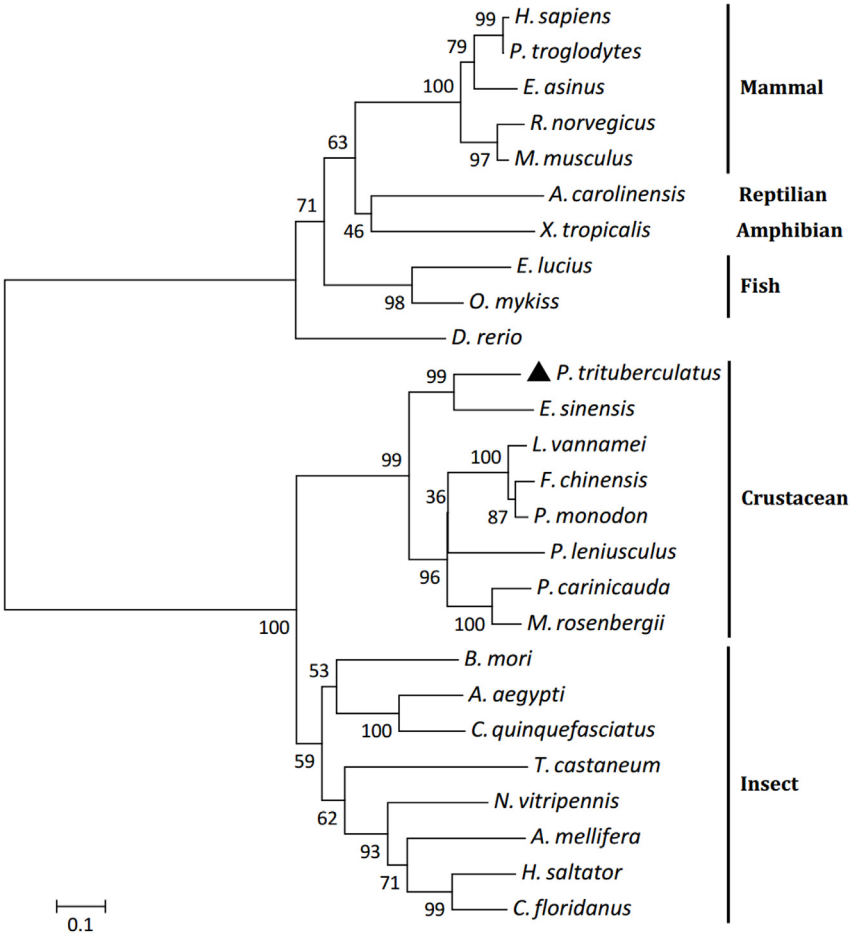

Fig. 2. Phylogenetic analysis of C1qBP/gC1qR proteins. Neighbor-joining tree was constructed with Mega 7.0 software. The species and corresponding GenBank accession numbers were as follows: *Eriocheir sinensis* (ANN46490.1), *Litopenaeus vannamei* (AFY05650.1), *Fenneropenaeus chinensis* (AFJ59951.1), *Palaemon carinicauda* (AFY05651.1), *Penaeus monodon* (ADV18978.1), *Macrobrachium rosenbergii* (AJE28353.1), *Pacifastacus leniusculus* (AEC50078.1), *Bombyx mori* (ABD36320.1), *Aedes aegypti* (ABF18301.1), *Culex quinquefasciatus* (EDS32443.1), *Tribolium castaneum* (XP_967206.1), *Nasonia vitripennis* (XP_001607503.1), *Apis mellifera* (XP_397201.2), *Harpegnathos saltator* (EFN75748.1), *Camponotus floridanus* (EFN62645.1), *Homo sapiens* (CAC82720.1), *Pan troglodytes* (XP_514549.2), *Equus asinus* (XP_014713475.1), *Rattus norvegicus* (NP_445835.1), *Mus musculus* (NP_034870.1), *Anolis carolinensis* (XP_003220073.1), *Xenopus tropicalis* (XP_002936962.2), *Danio rerio* (XP_005159016.1), *Esox lucius* (XP_010877746.1), *Oncorhynchus mykiss* (CCJ27811.1). PtgC1qR from the swimming crab was marked with the black triangle.

Pacifastacus leniusculus AEC50078.1, 51% with *Aedes aegypti* ABF18301.1 and *Bombyx mori* XP_012553378.1) than vertebrate gC1qRs (38% identity with *Homo sapiens* AAB53110.1, 38% with *Pan troglodytes* XP_514549.2, 34% with *Mus musculus* AAH38075 and 33% with *Danio rerio* XP_005159016.1).

A phylogenetic tree was constructed using the complete amino acid sequences of 26 gC1qRs from representative invertebrates and vertebrates (Fig. 2). The tree topology can be separated into two clusters. PtgC1qR had a closer relationship with crustacean gC1qRs, especially with EsgC1qR from *E. sinensis*, while the other invertebrate gC1qRs formed insect cluster. The gC1qRs from mammals, reptilian, amphibian, and fishes were clustered into a big branch of vertebrates.

3.3. Expression profiles of PtgC1qR in adult tissues and embryos

PtgC1qR was a widely distributed gene and was highly expressed in hepatopancreas, muscle and gonads, whereas the lowest expression level was detected in normal hemocytes of both male and female crabs (Fig. 3A). Besides, the expression of PtgC1qR in hepatopancreas and gonads of female crabs were significantly higher than those in males. As shown in Fig. 3B, PtgC1qR was detected and highly expressed in Fe and Cs. The transcripts of PtgC1qR exhibited a linear decrease after Cs and the lowest level was found in Hs. The mRNA level of PtgC1qR in Hs was less than one tenth of that in Fe.

3.4. Expression pattern analysis after pathogen challenge

The expression level of PtgC1qR was increased 2.9-fold at 2 h post *V. alginolyticus* infection and 1.8-fold at 4 h post-inection (Fig. 4A). Then PtgC1qR transcripts declined and gradually recovered to the initial level at 8 h post-injection. After *M. luteus* and *P. pastoris* challenge, the transcripts of PtgC1qR rose to their respective highest levels at 8 and 12 h post-injection. Subsequently, the PtgC1qR transcripts in both *M. luteus* and *P. pastoris* challenged crab recovered at 24 h post-injection (Fig. 4B and C).

3.5. Recombinant expression and purification of rPtgC1qR

The recombinant plasmid pET-32a-PtgC1qR was transformed into *E. coli* BL21 for protein expression. The whole cell lysate of the positive clones was analyzed using SDS-PAGE after IPTG induction, and recombinant protein rPtgC1qR was expressed as a soluble protein. As shown in Fig. 5, a distinct 45 kDa band was observed, which is in accordance with the predicted molecular mass of the recombinant PtgC1qR. The concentration of the rPtgC1qR proteins was 2.34 mg/ml.

3.6. Antimicrobial activity of rPtgC1qR

Antimicrobial activities and minimal growth inhibition concentrations (MIC) of rPtgC1qR were analyzed by a liquid growth inhibition assay and shown in Table 2. The purified rPtgC1qR displayed antibacterial activities against the tested Gram-negative bacteria *V. alginolyticus* L59 and *P. aeruginosa* P25, Gram-positive bacteria *M. luteus* M2 and *S. aureus* S7. The highest antibacterial activity was against *V. alginolyticus* L59 with MIC value of 1.45–2.90 μM. No obvious inhibitory activity was detected against fungus *P. pastoris* GS115.

3.7. Binding activity to microorganisms

A microbial-binding assay was performed to test whether the

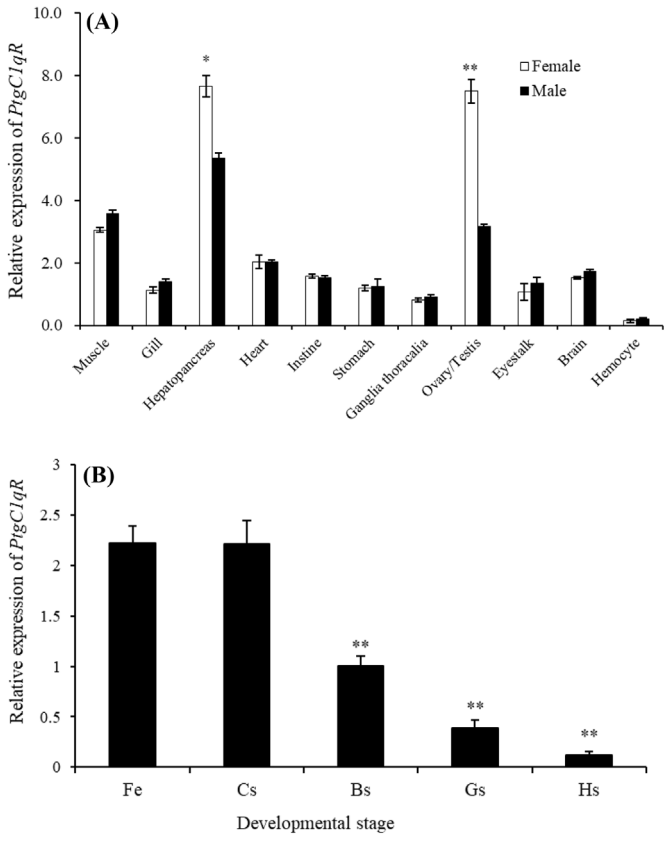

Fig. 3. Relative expression of PtgC1qR in different tissues (A) and early embryos (B) of *P. trituberculatus*. The data determined by qRT-PCR. Each column represented the mean of quadruplicate assays within ± S.D. The significant differences were represented with asterisk (*$P < 0.05$, **$P < 0.01$).

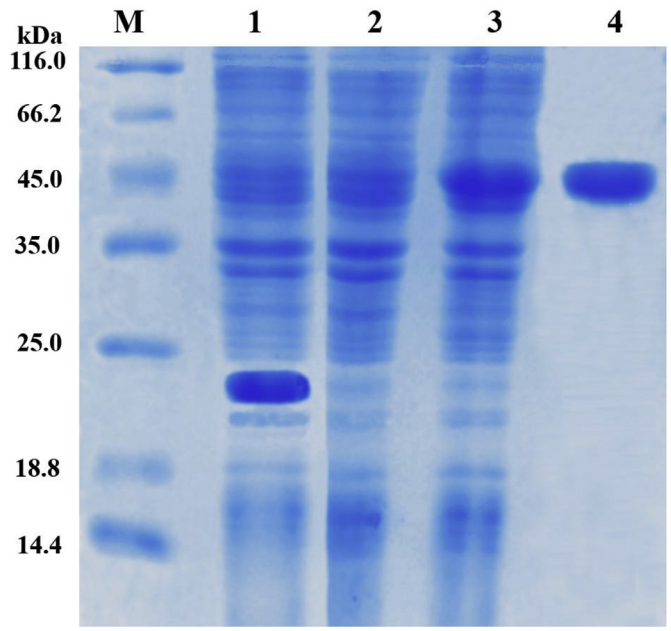

Fig. 5. SDS-PAGE analysis of the recombinant PtgC1qR. Lane M: protein molecular standard; lane 1: IPTG induced rTrx; lane 2: negative control without IPTG induction; lane 3: IPTG induced recombinant proteins; lane 4: purified recombinant proteins.

recombinant protein rPtgC1qR could bind to microorganisms. As shown in Fig. 6, rPtgC1qR was detected in the eluted fractions whereas no band was detected in the supernatant or washed fractions, which suggest PtgC1qR could firmly attach to the bacteria *M. luteus*, *S. aureus*, *P. aeruginosa* and *V. alginolyticus*. While the recombinant PtgC1qR were found in the eluted and supernatant fractions but not observed in the washed fraction, indicating PtgC1qR slightly bound to *P. pastoris*.

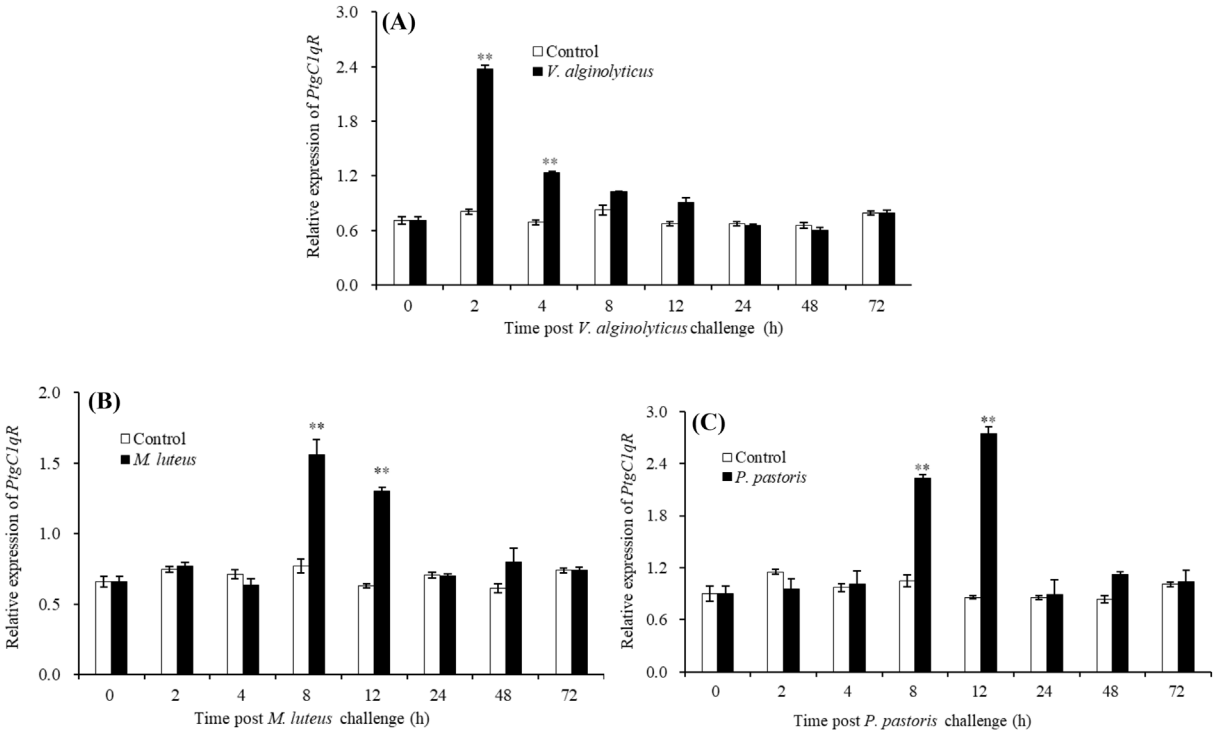

Fig. 4. Temporal expression profiles of PtgC1qR in hemocytes after challenged with *V. alginolyticus* (A), *M. luteus* (B), and *P. pastoris* (C) revealed by qRT-PCR. Each column represented the mean of quadruplicate assays within ± S.D. The significant differences were represented with asterisk (*$P < 0.05$, **$P < 0.01$).

Table 2
Antimicrobial activities and MIC of rPtgC1qR.

Microorganisms	MIC (μM)
Gram-negative bacteria:	
Vibrio alginolyticus L59	1.45–2.90
Pseudomonas aeruginosa P25	2.90–5.81
Gram-positive bacteria:	
Micrococcus luteus M2	2.90–5.81
Staphylococcus aureus S7	5.81–11.61
Fungus:	
Pichia pastoris GS115	Na

MIC was expressed as the interval a–b, where a was the highest concentration tested at which microorganisms were growing ($P > 0.05$) and b was the lowest concentration that caused 100% growth inhibition ($P < 0.05$). Na indicated no antimicrobial activity.

3.8. Inhibition of rPtgC1qR on prophenoloxidase system

The involvement of rPtgC1qR in regulating the proPO system was verified by measuring PO activity in the *V. alginolyticus*-incubated HLS (Fig. 7). Time course analysis showed that PO activity in HLS increased and reached a plateau after 30 min reaction time. At concentrations of 2.90, 5.81 and 11.61 μM, rPtgC1qR was able to inhibit the activation of proPO system by about 7.59–98.62% at 40 min reaction time point as compared to negative control.

3.9. Expression analysis of potential immune-related genes after PtgC1qR silenced by RNAi

The expression of PtgC1qR in siRNA interference group was shown to be suppressed significantly ($P < 0.01$) in the hemocytes at 24 h when compared with that of the control group (Fig. 8A). The silencing efficiency of the siRNA reached up to 92.7%, then 24 h was selected as the optimal time point of PtgC1qR knockdown for further experiments.

Under the conditions where the expression of PtgC1qR gene was knocked down, the expression levels of serine protease related genes (*PtSP1*, *PtSP2*, *PtSP3* and *PtSPH*), proPO-associated genes (*PtproPO* and *PtPPAF*) and C3-like genes (*Ptα2M1* and *PtTEP*) were significantly up-regulated ($P < 0.01$). By contrast, the expression of phagocytosis-related genes (*PtMyosin*, *PtRab5* and *PtArp*) and *Ptα2M2* were remarkably suppressed ($P < 0.05$) in the PtgC1qR silenced carbs (Fig. 8B).

4. Discussion

The complement system comprises numerous vital components of innate immunity and serves as one of major effector mechanisms of the innate defense system. Complement represents an evolutionarily ancient component of host defense, which can be traced back from

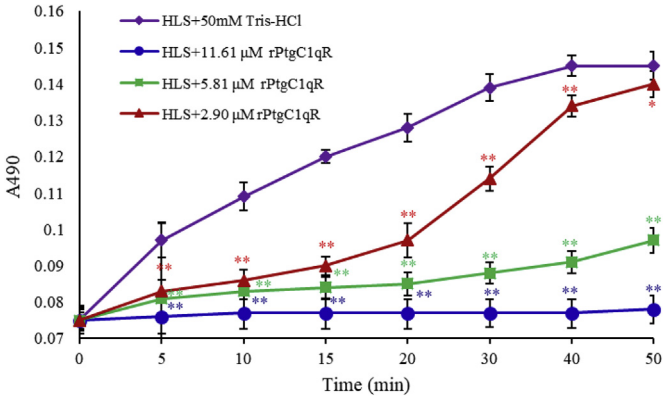

Fig. 7. The inhibition of PO activity by rPtgC1qR. The hemocyte lysate supernatant (HLS) was added the rPtgC1qR to the final concentrations of 11.61 μM, 5.81 μM and 2.90 μM. Tris-HCl buffer (50 mM, pH 8.0) was added as the negative control. The PO activity was monitored by measuring the absorbance at 490 nm. Error bars represented the mean ± SD (n = 3). Significant differences between control and the tested groups at the same sampling point were represented with asterisk (*$P < 0.05$, **$P < 0.01$).

Cephalochordata [30], Urochordata [31], Echinodermata [32], and even more ancient protostome, such as horseshoe crab [33] and sea anemone [34]. The specificity and strength of the complement components are regulated by partner or chaperone molecules [10], but molecular studies on the complement pathway in crab are limited. Therefore, the molecular characterization of a gC1qR homolog in the swimming crab and its roles in immune response were investigated in this study.

PtgC1qR contained a common MAM33 domain at its C-terminal, and shared higher similarities with the gC1qRs from crustacean and insect, indicating it is a clear member of gC1qR family. As many other gC1qRs in crustaceans [4,7,9–11]. PtgC1qR had a mitochondrial targeting sequence at its N terminal, suggesting PtgC1qR could be directed to the mitochondrial matrix. Furthermore, an RGD motif was also found in PtgC1qR. RGD motif in several structural proteins of white spot syndrome virus (WSSV) could help enhance WSSV infection [8,35]. However, the RGD motif in *PlgC1qR* from *P. leniusculus* could prevent WSSV replication [7]. As suggested in FcgC1qR of *F. chinensis*, PtgC1qR contains an RGD motif that might serve as binding sites or receptors for integrin [9,36].

The PtgC1qR transcripts were widely distributed in various tested tissues, with the highest expression level in hepatopancreas that is a key organ involved in immune response of crab. Besides, the fixed phagocytes distributed in hepatopancreas are responsible for eliminating pathogens [37]. Interestingly, the expression of PtgC1qR in the hepatopancreas and gonads of female crabs was significantly higher than

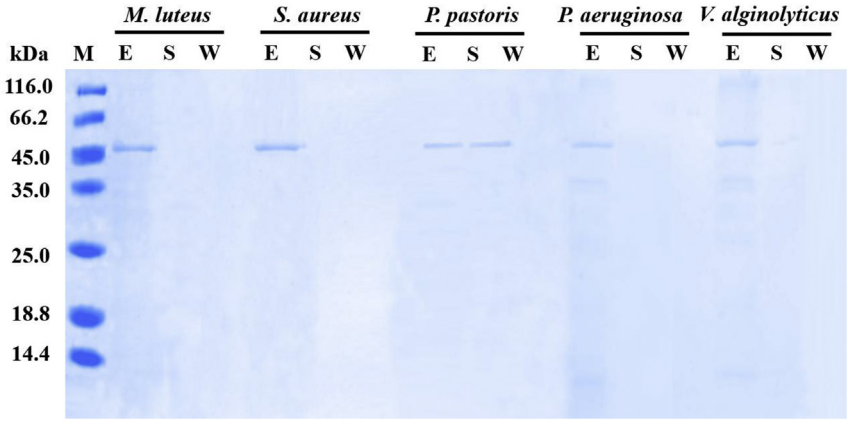

Fig. 6. Binding activity of the recombinant PtgC1qR to microorganisms. The recombinant proteins were incubated with formaldehyde-fixed microorganisms at 4 °C for 30 min. After incubation, the supernatants were separated by centrifugation. The pellets were washed with PBS buffer and the bound proteins were eluted with SDS-PAGE sample loading buffer. The eluted (E), supernatants (S) and washed (W) fractions were examined by SDS-PAGE.

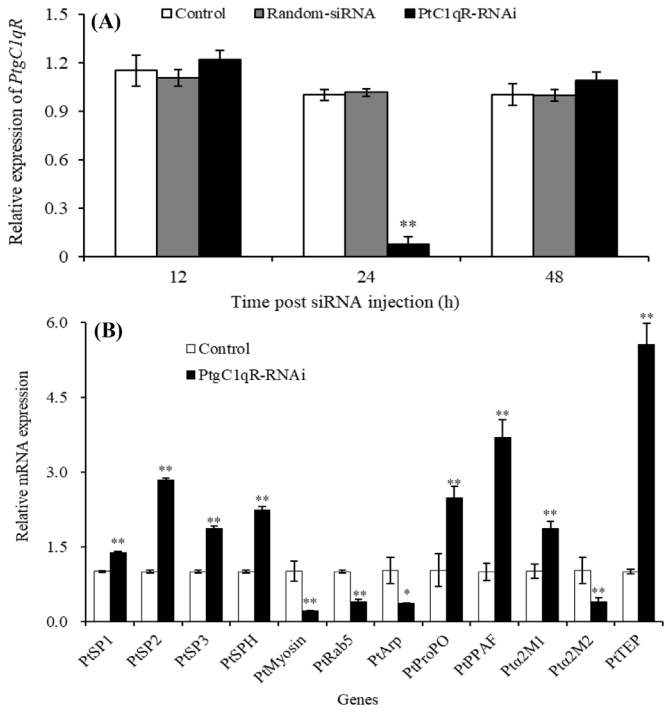

Fig. 8. Silencing efficiency of siRNA and immune-related genes expression analysis in the PtgC1qR silenced crab. (A) The effect of siRNA silencing on transcription level of PtgC1qR in crab hemocytes. (B) The expression of immune-related genes in hemocytes of the PtgC1qR silenced crabs. Each column represented the mean of triplicate assays within ± S.D. The significant differences were represented with asterisk (*$P < 0.05$, **$P < 0.01$).

that in male crabs, which suggest the sex differences might exist in immune defense of the swimming crab. Furthermore, the PtgC1qR transcripts remarkably decreased after the cleavage stage and reached the lowest level at the heart beating stage, suggesting that PtgC1qR may act as an important maternal complement component that transferred from mother to offspring and involved in the early defense against pathogens in developing embryos. It is agreed with the maternal transmission of other complement components demonstrated in fishes, such as C3, C4, C5 and factor B [38,39].

The current study had shown that the PtgC1qR transcripts were remarkably upregulated after bacterial and fungal infection. Furthermore, PtgC1qR exhibited an earlier response to *V. alginolyticus* infection compared with *M. luteus* and *P. pastoris*. Similar results have been reported in FcgC1qR and PmC1qBP, which are more sensitive to Gram-negative bacteria stimulation than Gram-positive bacteria [9,10]. Taken together, these findings suggest PtgC1qR may play an essential role in the immune responses of the swimming crab against pathogenic invasion.

Recently, a growing reports have revealed that gC1qR could recognize and bind to numerous bacterial pathogens. For instance, FcgC1qR from *F. chinensis* could tightly bind to Gram-positive bacteria but slightly attach to Gram-negative bacteria [9]. However, in the present work, the fusion protein rPtgC1qR, as reported in MrgC1qR of *M. rosenbergii* [11], could tightly attach to both Gram-positive bacteria and Gram-negative bacteria. These results suggest that PtgC1qR might act as pattern recognition receptors (PRRs). In addition, we revealed that the rPtgC1qR exhibited strong antimicrobial activities against both Gram-positive and Gram-negative bacteria, suggesting rPtgC1qR is a potential antibacterial protein. Further study should be carried out to decipher the antibacterial mechanism of PtgC1qR.

As described in previous study, the proPO system of *P. trituberculatus* could be activated by bacterial infection [40], which was also observed in this study. Here, the recombinant PtgC1qR inhibited PO activity in crab HLS by a dose-dependent manner, and similar inhibition has been also found in serine protease inhibitors (SPIs), such as Esserpin from *E. sinensis* [41] and alpha-2-macroglobulin from *L. vannamai* [18]. Though the target protease is unclear, we presume PtgC1qR might act as a co-factor involved in the proPO system. The significant upregulation of serine protease related genes and proPO-associated genes in the PtgC1qR silenced crab favors this view as well.

The primitive complement system is a crucial innate immune factor that often cooperates with phagocytosis [21,42]. Herein, phagocytosis-related were significantly suppressed when the expression of PtgC1qR gene was knocked down, indicating PtgC1qR might perform its function by regulating phagocytosis. PtgC1qR could also regulate C3-like genes in the swimming crab with similarities to the activation of the classical complement pathway in vertebrates. Nevertheless, invertebrates lack antibody–antigen complexes, hence also absent the classical complement pathway. C1q in the classical system and mannose-binding lectin in the lectin pathway are closely related with respect to the structures and functions [21,43]. Therefore, we speculate that PtgC1qR might perform its complement immune function through the lectin pathway.

In conclusion, gC1qR is a ubiquitous, multiligand binding protein with versatile physiological functions in vertebrates and invertebrates. The characteristics and protein functions of PtgC1qR in *P. trituberculatus* were first reported in this study. PtgC1qR is remarkably upregulated after bacterial and fungal infection, and more sensitive to Gram-negative bacteria. The recombinant PtgC1qR exhibited strong antibacterial activity and microbial-binding activity. Meanwhile, PtgC1qR may modulate the proPO system, phagocytosis and complement pathway. Altogether, it could be speculated that PtgC1qR plays a crucial role in the innate immune of the swimming crab.

Acknowledgments

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