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A novel globular C1q domain containing protein (C1qDC-7) from *Crassostrea gigas* acts as pattern recognition receptor with broad recognition spectrum

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ABSTRACT

The globular C1q domain containing (C1qDC) proteins are a family of versatile pattern recognition receptors (PRRs) to bind various ligands by their globular C1q (gC1q) domain. In the present study, a novel globular C1qDC (*Cg*C1qDC-7) was characterized from Pacific oyster *Crassostrea gigas*. The open reading frame of *Cg*C1qDC-7 was of 555 bp, encoding a polypeptide of 185 amino acids. Phylogenetic analysis indicated that *Cg*C1qDC-7 shared high homology with C1qDCs from *Crassostrea virginica*, *Mytilus galloprovincialis,* and *Mizuhopecten yessoensis*. The mRNA transcripts of *Cg*C1qDC-7 were widely expressed in all the tested tissues including mantle, gonad, gills, adductor muscle, hemocytes, hepatopancreas and labial palps, with the highest expression level in hemocytes and gills. The recombinant protein of *Cg*C1qDC-7 (r*Cg*C1qDC-7) exhibited binding activity towards Gram-negative bacteria (*Vibrio splendidus*, *V. anguillarum*, *Escherichia coli*, *V. alginolyticus,* and *Aeromonas hydrophila*), Gram-positive bacteria (*Micrococcus luteus* and *Staphylococcus aureus*) and fungi (*Pichia pastoris* and *Yarrowia lipolytica*), and displayed strongest binding affinity towards Gram-negative bacteria *V. splendidus* and *V. anguillarum.* It also exhibited affinity to vital pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), peptidoglycan (PGN), mannan (MAN) and Poly (I:C) with high affinity towards LPS and PGN, and low affinity to MAN and Poly (I:C). These results collectively indicated that *Cg*C1qDC-7 was a novel PRR in *C. gigas* with high binding affinity towards LPS and PGN as well as Gram-negative bacteria.

1. Introduction

It is generally recognized that invertebrates lack the lymphocyte-mediated adaptive immunity and rely solely on the innate immunity to eliminate invading pathogens [1]. The initial step of the innate immune response appears to be the discrimination of non-self, generally known as immune recognition [2]. Being the most important molecules responsible for immune recognition, pattern recognition receptors (PRRs) can recognize various pathogen-associated molecular patterns (PAMPs) and depict a complicated scene of host-pathogen interaction [3]. So far, massive expansion of PRRs, majority of which contain recognition domains of Ig, leucine-rich repeat (LRR), C-type lectin (CTL), C1q and scavenger receptor (SR), have been annotated and identified in invertebrates by screening the available genomic sequence [4]. As one of the largest groups in the animal kingdom with abundant biodiversity, molluscs also have evolved a series of PRRs families [5,6]. In oyster *Crassostrea gigas*, there is significant expansion of C1q, CTL and Ig recognition domains, and 321 C1qDCs, 266 C-type lectin (CTLs) and 190 fibrinogen-related proteins (FREPs) are annotated in its genome [7]. These recognition domain containing proteins play an indispensable role in the first step of immune defense, and the investigation on these PRRs will expand our understanding of pattern recognition mechanisms.

C1qDCs are a family of proteins containing a globular head C1q domain (gC1q) in C-terminus, which recognize a broad range of ligands and trigger a serial of immune responses, such as the classical pathway of complement [8]. In vertebrates, there are two major domains with distinct functions. One domain is the globular "heads" domain gC1q, which is considered to act as the crucial part of C1q to recognize a wide range of PAMPs [9–11]. The other one is collagen-like domain (cC1q),

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which assembles a bouquet-like structure participating in the interaction and activation of C1r and C1s, the important downstream molecules of classical complement pathway [12,13]. Recently, various C1qDCs have also been identified from mollusc, such as *Mytilus galloprovincialis* (*Mg*C1qDCs) [14], pearl mussel *Hyriopsis cumingii* (*Hc*C1qDCs) [15], *Chlamys farreri* (*Cf*C1qDCs) [16], *Argopecten irradians* (*Ai*C1qDCs) [17,18], and *C. gigas* (*Cg*C1qDCs) [19,20]. All of these molluscan C1qDCs contain a typical gC1q domain with a 10-stranded β-sandwich jelly roll topology [11,21], indicating that they cannot interact with the other downstream protease to activate complement pathway [22,23]. To our best knowledge, only one C1q-like protein has been identified with a typical vertebrate type cC1q [24].

C1qDCs identified from mollusc have been reported to exert diverse biological functions in innate immunity, especially in microbe recognition, microbe binding, and phagocytosis enhancement. It was reported that the recombinant *Cf*C1qDC (r*Cf*C1qDC) and *Ai*C1qDC-2 (r*Ai*C1qDC-2) proteins both bound LPS, PGN, β-glucan and poly (I:C), while the latter could also bind mannan and yeast-glucan [16,18]. *Cg*C1qDCs in oyster (*Cg*C1qDC-2 ~ 4) could enhance the phagocytosis of oyster hemocytes, and the enhancements towards Gram-negative bacteria were significantly higher than that towards Gram-positive bacteria, indicating the important roles of molluscan C1qDC as an opsonin in the clearance of invading microbes [20]. Interestingly, *Cg*C1qDC-3 with higher LPS binding affinity and stronger ability to enhance phagocytosis could be more rapidly and persistently up-regulated during secondary challenge with homologous *Vibrios*, which might provide new evidences for the possible involvements of C1qDCs as PRRs in immune specificity [20]. However, the comparative studies on the binding specificity of these molluscan C1qDCs are still very limited. Further investigation on the functional divergence of the expanded C1qDCs in oyster will contribute to the better understanding of the immune recognition mechanism, the complex stress adaptation patterns in mollusc, and the evolution of complement system.

As many other marine molluscs, oysters live in the intertidal zones and have to cope with a series of biotic threats caused by infections of various pathogens including bacteria, virus, and protozoan [25]. The expanded C1qDCs in oyster imply their significant roles in immune defense. Since some C1qDCs have been identified from other molluscan species, it is meaningful to compare the binding spectrum and reveal the major functions of C1qDCs in the immune recognition. The major purposes of the present study are to (1) identify new C1qDC from oyster, (2) illustrate its recognition and binding activities towards various microbes, and (3) compare the binding specificity with C1qDCs from other molluscan species.

2. Material and methods

2.1. Oysters, mice and microbes

Pacific oyster (*C. gigas*), about 12 cm in length and 180 g in weight, were collected from an aquaculture farm in Dalian, Liaoning Province, China and cultured in aerated water for one week prior to use. Female mice (six-week old) were purchased from Dalian Institute of Drug Control. Gram-negative bacteria *Vibrio splendidus* and *V. anguillarum* were grown in 2216E medium at 18 °C and 28 °C for 20 h, respectively; *Escherichia coli*, *V. alginolyticus* and *Aeromonas hydrophaila* were grown in LB medium both at 37 °C for 20 h. Gram-positive bacteria *Staphylococcus aureus* and *Micrococcus luteus* were cultured in LB medium both at 37 °C for 12 h. Fungi *Pichia pastoris* and *Yarrowia lipolytica* were inoculated in yeast liquid medium at 30 °C with shaking at 200 rpm. The microorganisms were collected by centrifugation at 6000 rpm for the following experiments.

2.2. Sample collection

After an acclimatization of seven days, nine oysters were randomly divided into three groups with three individuals in each group. The hemolymph from three oysters was mixed as a single sample. The hemolymph was collected and centrifuged at 800 g, 4 °C for 10 min to harvest the hemocytes. All the samples were stored at −80 °C in Trizol reagent (Invitrogen, California, USA) for the subsequent RNA extraction.

Various tissues including adductor muscle, hepatopancreas, labial palps, gonad, mantle, gills and hemocytes were obtained from nine oysters as parallel samples (three individuals in each parallel). Hemolymph was centrifuged at 800 g, 4 °C for 10 min to harvest the hemocytes.

2.3. RNA extraction and cDNA synthesis

Total RNA from oyster tissues including adductor muscle, hepatopancreas, labial palps, gonad, mantle, gills and hemocytes were extracted by Trizol reagent (Invitrogen, California, USA). Briefly, about 50–100 mg tissue samples were added into 1 mL TRIzol™ Reagent, and homogenized with lapping rod (hemocytes were broken by 1 mL syringe). Then 0.2 mL chloroform was added into the tissue homogenate to separate total RNA. After precipitated by isopropanol, the total RNA was washed by 75% ethanol twice, and resuspended in RNase-free water for cDNA synthesis.

The cDNA synthesis was performed using PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China) according to the manufacturer's instruction. The first-strand cDNA synthesis was performed with primer oligo (dT). One microgram of total RNA was added into the 20 μL reaction system, and the mixture was incubated in 37 °C for 15 min, and then 85 °C for 15 s. The cDNA mix was diluted to 1:40 and stored at −80 °C for next processing.

2.4. Gene cloning of *Cg*C1qDC-7 and sequence analysis

The open reading frame (ORF) sequence of *Cg*C1qDC-7 was cloned using the cDNA library of hemocytes. The PCR amplification was performed by using gene-specific primer *Cg*C1qDC-7-F and *Cg*C1qDC-7-R (Table 1) with the following protocols: 5 min at 95 °C; 30 cycles at 94 °C for 30s, 55 °C for 45s, 72 °C for 40s; and 72 °C for 10 min. The PCR product was gel-purified, cloned into PMD 19-T simple vector (Takara, Dalian, China), and confirmed by DNA sequencing.

ClustalW Multiple Alignment program was used to conduct the multiple sequence alignment, and Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/) was employed to predict the conserved domain. A Neighbor-Joining (NJ) phylogenetic tree was constructed using the MEGA 6.0 package with 1000 pseudo-replicates of bootstrap resampling to test the reliability of the branching.

2.5. Determination of tissue distribution of *Cg*C1qDC-7 mRNA

The mRNA expression levels of *Cg*C1qDC-7 in different tissues

Table 1
Sequences of the primers used in this study.

Primer	Sequence (5′-3′)
Clone primers	
*Cg*C1qDC-7-F	TATGCTTGCCTATGTTCGACTGT
*Cg*C1qDC-7-R	TCGTCTTTGTCGTGCAATTTT
Recombinant expression	
*Cg*C1qDC-7-ExF	CCGGAATTCGCCCAGTTCATGGACGAC
*Cg*C1qDC-7-ExR	CCGCTCGAGAATCGAATTAATCTTGCT
RT-PCR primers	
CgC1qDC-7-RTF	TACAAACAACATCTCCCGCC
CgC1qDC-7-RTR	ATACTCCGTCTTCTGGTGCTG
CgEF-RT-F	AGTCACCAAGGCTGCACAGAAAG
*Cg*EF-RT-R	TCCGACGTATTTCTTTGCGATGT

including adductor muscle, hepatopancreas, labial palps, gonad, mantle, gills and hemocytes were determined by SYBR Green fluorescent qRT-PCR using ABI PRISM 7500 Sequence Detection System (Applied Biosystems, USA). One pair of gene-specific primers for *Cg*C1qDC-7, *Cg*C1qDC-7-RTF and *Cg*C1qDC-7-RTR (Table 1), were employed to amplify a fragment of 208 bp. The oyster Elongation Factor (EF, GenBank accession NM_001305313) fragment, amplified with primers *Cg*EF-RT-F and *Cg*EF-RT-R (Table 1), was chosen as internal reference. Dissociation curve analysis of amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. The relative expression levels of *Cg*C1qDC-7 was analyzed by comparative Ct method ($2^{-\triangle\triangle Ct}$ method) [26].

2.6. Recombinant expression and purification of CgC1qDC-7 protein

Recombinant protein of *Cg*C1qDC-7 was expressed in *E. coli.* The ORF sequence of *Cg*C1qDC-7 was amplified using the primers *Cg*C1qDC-7-ExF and *Cg*C1qDC-7-ExR (Table 1). The PCR products were gel-purified and cloned into pET-30a expression vector with a His tag. The forward positive clones were screened by PCR, and further confirmed by nucleotide sequencing. The valid recombinant plasmid was extracted and transformed into *E. coli* Transetta DE3. Positive transformants were cultured in LB medium at 37 °C shaking at 200 rpm. When OD_{600} of the culture medium reached 0.4–0.6, the bacteria were incubated for additional 8 h with the induction of IPTG at the final concentration of 1 mM. The recombinant protein of *Cg*C1qDC-7 with two six-His (6 × His) tags were purified by Ni^+ affinity chromatography, and desalted by extensive dialysis. The purified protein was concentrated and stored at −80 °C.

2.7. Preparation of polyclonal antibodies against CgC1qDC-7

Six-week-old mice were immunized with r*Cg*C1qDC-7 to acquire polyclonal antibodies following the description in previous report [27]. Briefly, 100 μL r*Cg*C1qDC-7 (0.3 mg/mL) was emulsified with 100 μL complete Freund's adjuvant (Sigma, USA) to immunize each mouse by subcutaneous implantation. The second and third injections were performed on the 14th and 21st day with incomplete Freund's adjuvant (Sigma, USA). The fourth injection was performed with 100 μL r*Cg*C1qDC-7 on the 28th day. The anti-r*Cg*C1qDC-7 serum was obtained on the 36th day and stored at −80 °C before use.

2.8. Western blot analysis of CgC1qDC-7

The specificity of polyclonal antibody against r*Cg*C1qDC-7 was verified by western blot assay. Briefly, r*Cg*C1qDC-7 was separated by 12% SDS-PAGE, and electrophoretically transferred onto a nitrocellulose membrane. The membrane was washed three times with TBS containing 0.1% Tween 20 (TBST), blocked by 5% skimmed milk (100 mL TBST, 5 g skimmed milk) at 4 °C overnight, and then incubated with 1:1000 diluted polyclonal antibody against r*Cg*C1qDC-7 at 37 °C for 3 h. After three times of washing with TBST, the membrane was incubated with 1:3000 diluted secondary antibody Goat-anti-mouse IgG conjugated with HRP (ABclonal, Boston, USA) at 37 °C for 2 h. After the final three times of washing with TBST, the protein bands were developed by using Super ECL Detection Reagent (Sigma-Aldrich, USA) for 2 min, and captured image by Amersham Imager 600 system (GE Healthcare, Bonston, USA).

2.9. PAMPs binding assay

The PAMPs binding activity of r*Cg*C1qDC-7 was examined by enzyme-linked immunosorbent assay (ELISA) [28]. Briefly, the 96-well microliter plates (Costar) were coated with 10 μg of lipopolysaccharide (LPS), peptidoglycan (PGN), mannan (MAN) and Poly (I:C) in 100 μg of carbonate-bicarbonate buffer (100 mmol/L, pH 9.6) at 4 °C overnight, respectively. The plate was blocked with 3% bovine serum albumin (BSA) in TBS at 37 °C for 1 h. After three times of washing with TBST, 100 μL r*Cg*C1qDC-7 (protein concentration were 90 μg/mL) diluted by 2 times were added in the wells and incubated at 24 °C for 2 h. The same concentration of rTrx was used as positive control. After three times of washing with TBST, 100 μL of 1:2000 diluted anti-r*Cg*C1qDC-7 polyclonal antibodies was added and incubated at 37 °C for 1 h. Then, the plate was washed with TBST for three times, and 100 μL of 1:2000 diluted HRP Goat-anti-mouse Ig-alkaline phosphatase conjugate (ABclonal, Boston, USA) was added as second antibody to detect the polyclonal antibody against r*Cg*C1qDC-7. After the final washes, 100 μL of TMB (dihydrochloride) (Solarbio, California, USA) was added to each well and incubated at room temperature in dark for 15 min. The reaction was stopped by adding 100 μL of 1 mol/L HCl per well and the absorbance at OD_{450} was measured by Infinite M1000 PRO (Tecan, Sweden). TBS was used as blank, and non-immune serum instead of immune serum as first antibody was set as negative control. Each experiment was repeated in triplicate.

2.10. Microorganism binding assay

Microorganism binding activities of r*Cg*C1qDC-7 towards Gram-positive bacteria (*M. luteus* and *S. aureus*), Gram-negative bacteria (*V. splendidus*, *V. anguillarum*, *E. coli*, *V. alginolyticus*, and *A. hydrophila*) and fungi (*P. pastoris* and *Y. lipolyticd*) were examined according to the method reported previously with some modification [29]. In brief, the microbes were harvested by centrifugation at 6000 *g* for 10 min, and suspended in TBS ($OD_{600} = 1$). They were incubated with r*Cg*C1qDC-7 (200 μL, 300 μg/mL) and rTrx under rotation at room temperature for 45 min, respectively. The microorganisms combined with proteins were centrifuged, washed three times with TBS, and resuspended in 100 μL TBS for SDS-PAGE. The microorganism binding spectrums of r*Cg*C1qDC-7 were examined by western blot. The anti-r*Cg*C1qDC-7 polyclonal antibodies previously prepared from mouse were used as primary antibody, and secondary antibodies were HRP goat anti-mouse IgG (ABclonal, Boston, USA). After extensive washing, the protein bands were visualized by using Super ELC Detection Reagent (Sigma-Aldrich, USA). The purified r*Cg*C1qDC-7 was employed as positive controls and rTrx was set as negative control.

2.11. Statistical analysis

All the data were shown as mesn ± S.D. The two-samples Student's test was performed for the comparisons between groups. Multiple group comparisons were executed by one-way ANOVA and followed by a Tukey multiple group comparison test using Statistical Package for Social Sciences (SPSS) 16.0 Software. The difference was considered as significant at $p < 0.05$.

3. Results

3.1. The sequence features and phylogenitic relationship of CgC1qDC-7

The ORF of *Cg*C1qDC-7 gene was of 555 bp, encoding a putative polypeptide of 185 amino acids. The recombinant plasmid (pET-30a-*Cg*C1qDC-7) was constructed by inserting *Cg*C1qDC-7 into pET-30a, and transformed in *E. coli* Transetta (DE3). The r*Cg*C1qDC-7 including a 19 kDa for the polypeptide of *Cg*C1qDC-7 and 6 kDa for His-tag (pET-30a plasmid) was consistent with the predicted molecular mass of a predicted molecular weight of 24.88 kDa and isoelectric point (PI) of 6.64, (Fig. 1A). The sequence was deposited in GenBank with the accession number of XP_011425599.1. SMART analysis revealed that there was a signal peptide (residues 1–25) and a C1q domain (residues 49–181) in *Cg*C1qDC-7 (Fig. 1B).

A total of 15 C1qDC genes from different species were chosen to build a phylogenetic tree (Fig. 1C). All the selected C1qDC genes were

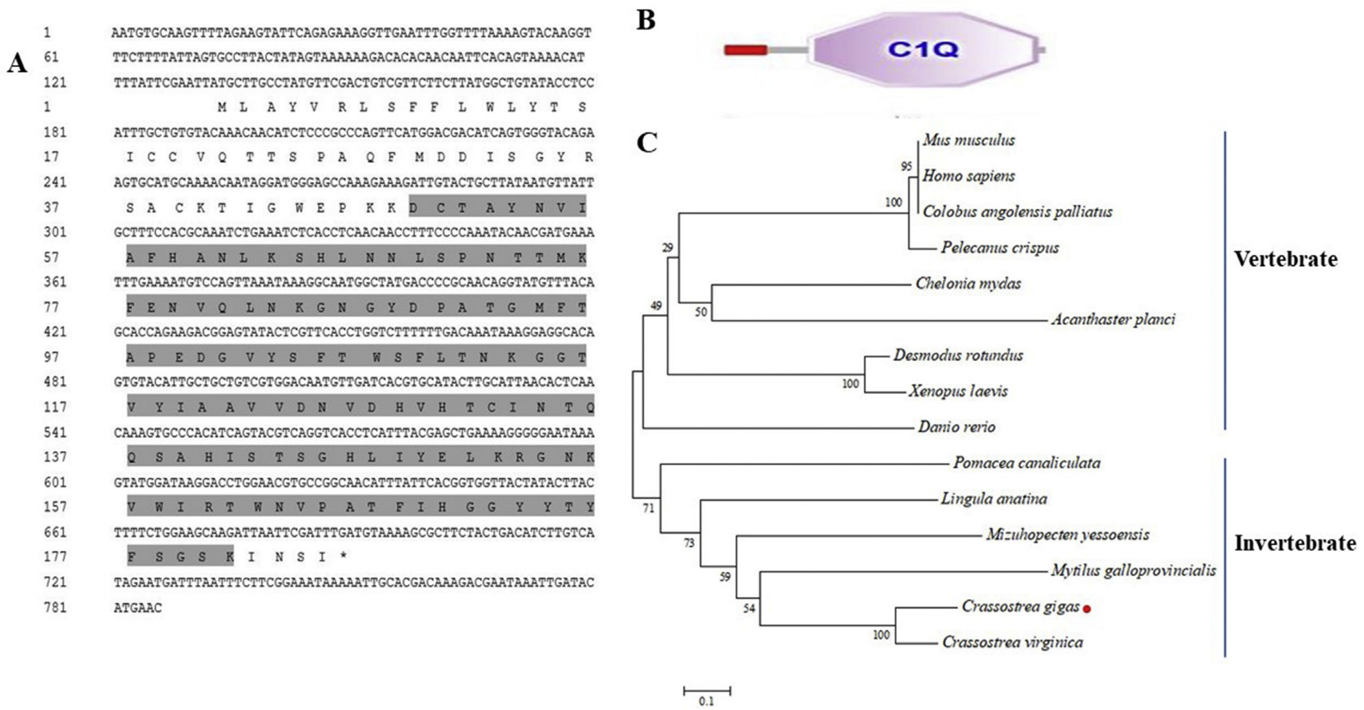

Fig. 1. The domain characters of *Cg*C1qDC-7. A: Complete nucleotide and deduced amino acid sequence of *Cg*C1qDC-7. The C1q domain is gray. B: Protein domains of *Cg*C1qDC-7 were predicted by SMART (http://smart.embl.de/). C: Phylogenetic relationship of C1qDCs protein sequences. Mega 6.0 program was used to construct the neighbor-joining (NJ) tree, algorithm based on the multiple sequence alignment of C1qDCs protein sequences. C1qDCs protein from other species: *Mus musculus* (NP_112150.1); *Homo sapiens* (NP_852100.3); *Colobus angolensis palliatus* (XP_011807967.1); *Pelecanus crispus* (KFQ49687.1); *Chelonia mydas* (XP_007060808.1); *Acanthaster planci* (XP_022111598.1); *Desmodus rotundus* (XP_024425520.1); *Xenopus laevis* (XP_018122782.1); *Danio rerio* (XP_009296972.1); *Pomacea canaliculata* (PVD38877.1); *Lingula anatina* (XP_013406650.1); *Mytilus galloprovincialis* (CBX41675.1); *Crassostrea virginica* (XP_022296808.1); *Mizuhopecten yessoensis* (XP_021340550.1); *Crassostrea gigas* (XP_011425599.1).

distinctly divided into vertebrate and invertebrate branches, and *Cg*C1qDC-7 was firstly clustered with *Cv*C1qDC, and then grouped in the mollusc branch.

3.2. Multiple alignment of CgC1qDC-7 with other C1qDC proteins

C1qDCs from diverse organisms gained from NCBI were aligned with *Cg*C1qDC-7 (Fig. 2), and BLAST analysis revealed that the deduced amino acid sequence of *Cg*C1qDC-7 shared homology with C1qDC from other organisms, such as 19.3% identity with C1qDC from *Pelecanus crispus*, 20.8% identity with C1qDC from *Danio rerio*, 26% identity with C1qDC from *Mytilus galloprovincialis*, and 13.4% identity with C1qDC from *Homo sapiens*.

3.3. Tissue distribution of CgC1qDC-7 mRNA transcripts

The distribution of *Cg*C1qDC-7 mRNA in different tissues was investigated by quantitative real-time PCR. The mRNA transcript of *Cg*C1qDC-7 was ubiquitously detected in the seven tissues including adductor muscle, hepatopancreas, labial palp, gonad, mantle, gills, and hemocytes (Fig. 3A). The highest expression level of *Cg*C1qDC-7 was observed in hemocytes and gills, which were approximately 8-fold and 4-fold of that in adductor muscle, respectively ($p < 0.05$). There were no significant differences between the other groups and the control group ($p < 0.05$).

3.4. The recombinant protein of CgC1qDC-7 and its polyclonal antibodies

The recombinant plasmid (pET-30a-*Cg*C1qDC-7) was expressed in *E.coli* Transetta (DE3) and the recombinant protein (r*Cg*C1qDC-7) was analyzed by 12% SDS-PAGE. A distinct band was observed at 25 kDa, which was coincident with the predicted molecular weight of *Cg*C1qDC-7 (Fig. 4A, Lane 2). After purification, a single band about 25 kDa representing r*Cg*C1qDC-7 was revealed (Fig. 4A, Lane 3). The purified r*Cg*C1qDC-7 was used to prepare the polyclonal antibody, and the specificity of polyclonal antibody was examined by western blot assay. A distinct band with the similar molecular weight as r*Cg*C1qDC-7 was revealed (Fig. 4B, Lane 4), indicating the high specificity and efficiency of the polyclonal antibodies.

3.5. The PAMPs binding activity of CgC1qDC-7

ELISA assay was conducted to quantify the binding activity of *Cg*C1qDC-7 towards different PAMPs. r*Cg*C1qDC-7 exhibited the highest affinity to LPS (P/N = 30.94) and PGN (P/N = 28.86), while low affinity to MAN (P/N = 15.78) and Poly (I: C) (P/N = 8.13) in a dose-dependent manner with a saturable process (Fig. 5). When the protein concentration increased from 2.81 to 90.00 μg/mL, the P/N values increased from 10.16 to 30.94 and 9.40 to 28.86 for LPS and PGN, respectively. No obvious binding affinity was observed in the rTrx control groups.

3.6. The microorganism binding activity of CgC1qDC-7

The microorganism binding affinity of *Cg*C1qDC-7 was determined by western bolt assay. A distinct band with the similar molecular weight as r*Cg*C1qDC-7 was revealed in nitrocellulose membrane, indicating that r*Cg*C1qDC-7 could bind Gram-negative bacteria (*V. splendidus*, *V. anguillarum*, *E. coli*, *V. alginolyticus,* and *A. hydrophila*), Gram-positive (*M. luteus* and *S. aureus*), and fungi (*P. pastoris* and *Y. lipolytica*) (Fig. 6). It displayed strong binding affinity towards Gram-negative bacteria *V. splendidus* and *V. anguillarum*. No binding affinity

Fig. 2. Multiple sequence alignment of the C1qDC proteins. Multiple sequence alignment of *Cg*C1qDC-7 with known C1qDCs from other species: *Pelecanus crispus* (KFQ49687.1); *Homo sapiens* (NP_852100.3); *Danio rerio* (XP_009296972.1); *Mytilus galloprovincialis* (XP_021340550.1); *Crassostrea gigas* (XP_011425599.1).

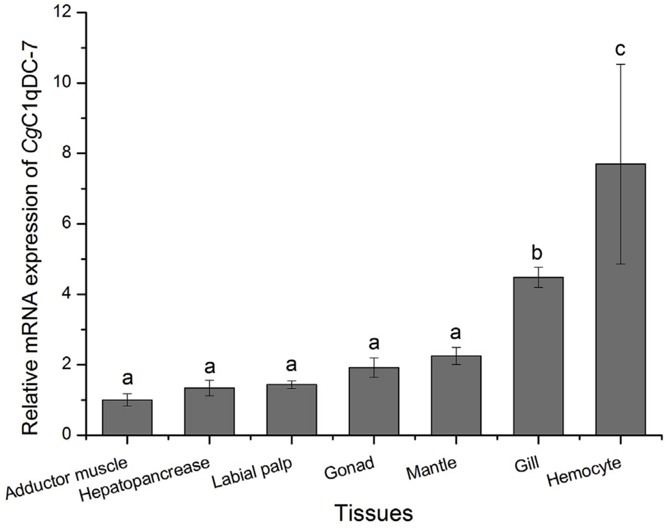

Fig. 3. Tissue distribution of *Cg*C1qDC-7 mRNA. *Cg*C1qDC-7 mRNA expression levels in different tissues of oyster *C. gigas* detected by qRT-PCR. *Cg*C1qDC-7 transcript levels in hemocytes, gills, mantle, gonad, labial palp and hepatopancreas were normalized to that of adductor muscle. Vertical bars represent the mean ± S. D. (N = 3), the letters (a, b, c) were used to present significant differences.

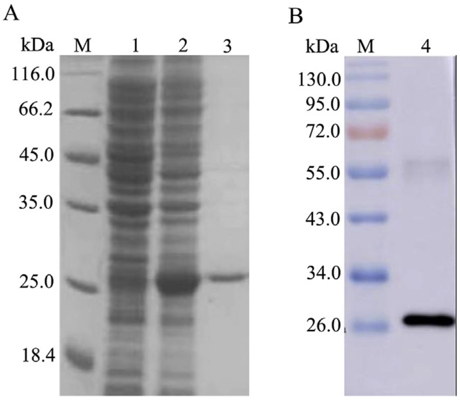

Fig. 4. The recombination protein of *Cg*C1qDC-7 and the specificity of its polyclonal antibody. A: SDS-PAGE analysis of r*Cg*C1qDC-7. Lane M: standard protein molecular weight marker; Lane 1: negative control (without induction); Lane 2: induced r*Cg*C1qDC-7; Lane 3: purified r*Cg*C1qDC-7. B: Western-blot of r*Cg*C1qDC-7 against its polyclonal antibody (Lane 4).

was detected in the rTrx group.

4. Discussion

As the recognition subunit of the immune system, C1q recognizes immunoglobulins through the gC1q domain and connects complement classical pathway-driven innate immunity to antibody-mediated acquired immunity [30,31]. Through the gC1q domain, C1q can recognize a variety of non-self ligands including bacteria, fungi and PAMPs on the pathogens [32]. So far, gC1q domain has been widely

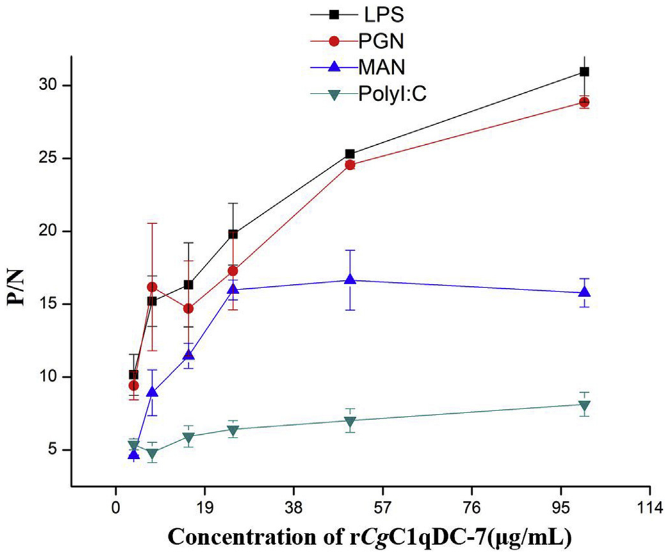

Fig. 5. The affinity of r*Cg*C1qDC-7 to LPS, PGN, MAN and Poly (I:C) was analysis by ELISA. Samples with P/N > 2.1 were considered positive. Results are representative of average three such experiments.

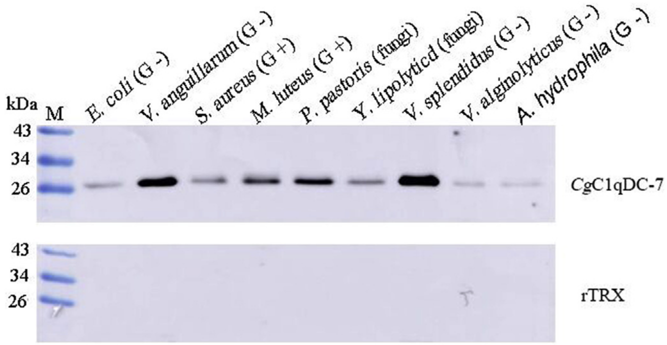

Fig. 6. The bacteria binding activity of r*Cg*C1qDC-7 revealed by Western blot. Western blot analysis of r*Cg*C1qDC-7 and rTrx's binding activity towards different microbes using anti-r*Cg*C1qDC-7 antibody.

found in a diverse range of proteins in both vertebrates and invertebrates [33]. In particular, this family is incredibly expanded in oyster and 321 C1qDC sequences are annotated in the genome of *C. gigas*, indicating the complexity and diversity of the immune recognition process in marine mollusc. A better understanding of the structure and function of C1qDCs from oyster would provide further insights into the functional and evolutionary research of this protein family.

In the present study, a novel C1qDC protein, named *Cg*C1qDC-7, was characterized from Pacific oyster *C. gigas*. Its ORF was of 555 bp, encoding a putative polypeptide of 185 amino acids with a predicted molecular weight of 24.88 kDa and isoelectric point (PI) of 6.64. SMART analysis revealed that there was a signal peptide (residues 1–25) and a C1q domain (residues 49–181) in *Cg*C1qDC-7. *Cg*C1qDC-7 shared high sequence identity with C1qDCs from molluscan species. In the phylogenetic tree, *Cg*C1qDC-7 was firstly clustered with C1qDC from *C. virginica*, and then gathered with C1qDC from other invertebrates such as *M. galloprovincialis*, *M. yessoensis,* and *P. canaliculate*. These evidences suggested that *Cg*C1qDC-7 belonged to C1qDC superfamily, and the C1q domain might endow *Cg*C1qDC-7 with the capability to recognize and bind specific substrates. Based on its sequence homology, molecular architecture characteristics and similarity in domain structure, *Cg*C1qDC-7 could be considered as a new member of C1qDC protein family. Since there was no collagen region in the N-terminal, *Cg*C1qDC-7 should be a member of gC1q proteins, which was in consistence with the previous reports that most of characterized invertebrate C1qDC proteins were gC1q proteins. For example, in Mediterranean mussel *M. galloprovincialis*, 167 of the 168 C1qDC gene models were gC1q proteins [14]. Some invertebrate gC1q proteins have been demonstrated to be involved in immune response and function as pathogen recognition molecule, such as *Cf*C1qDC, *Ai*C1qDC-1, *Mg*C1q and *Cg*C1qDC [16,17,19,20,34], which implied that *Cg*C1qDC-7 could function as PRR in immune response.

To investigate the potential function of *Cg*C1qDC-7 in immune response, its expression pattern in different tissues was examined. The mRNA transcript of *Cg*C1qDC-7 was ubiquitously detected in seven tissues including adductor muscle, hepatopancreas, labial palp, gonad, mantle, gill, and hemocytes. The constitutive expression in various tissues contributed an opportunity for *Cg*C1qDC-7 to interact with many kinds of PAMPs. The significantly higher expression levels were observed in hemocyte and gill, which favored that hypothesis that *Cg*C1qDC-7 was a novel PRR and responsible for the immune recognition. Gill is the organ responsible for seizing oxygen and alga from the seawater, which makes it the first protective barrier in the recognition of external pathogens [35,36]. The significantly higher expression level of *Cg*C1qDC-7 in gill indicated that it might play important roles in immune recognition. Many PRRs such as *Mg*C1q-8 [14] and *Cg*DM9CP-2 (DM9 containing protein from oyster *C. gigas*) [37] were also found to be highly expressed in gill, which was in consistent with the findings in the present study. In addition, bivalves process an open circulatory system, which forms a symbiont of hemocytes and pathogens. In order to maintain homeostasis, hemocytes of bivalve have evolved to be the most important immune cells, which are vital for pathogen recognition, prevention, and clearance. Other members of the C1qDC superfamily in bivalves, such as *Cg*C1qDC-1, *Cg*C1qDC-2 [19,20] and *Mg*C1qs [14], have also been identified from hemocytes, and they are responsible for the clearance of Gram-negative bacteria. These results suggested that *Cg*C1qDC-7 might act as a PRR during the innate immune response in oyster.

C1qDC relies on its gC1q domain to recognize a variety of self and non-self ligands, including a broad range of PAMPs of bacteria, yeast, virus, and parasite [8,38]. In order to explore the binding specificity of *Cg*C1qDC-7, four common PAMPs were selected for the PAMPs binding assay, and r*Cg*C1qDC-7 was found to directly bind all the tested PAMPs, including LPS from Gram-negative bacteria *E. coli*, PGN from Gram-positive bacteria, Man from fungi, and virus analogues Poly (I:C). The binding spectrum of *Cg*C1qDC-7 was similar to that of C1qDCs from other molluscan species. For example, *Cf*C1qDC and *Ai*C1qDC-2 from scallops could bind LPS, PGN, β-glucan, and Poly (I:C) [16,39]. *Cg*C1qDC-1-5 from oyster all exhibited higher binding affinity to LPS [19,20]. These results demonstrated that *Cg*C1qDC-7 was a novel PRR with a wide binding spectrum towards bacteria, fungi and virus, and it could play an important role not only in antibacterial immunity, but also in antivirus immunity. Since the C1qDCs superfamily was incredibly expanded in oyster, it could be inferred that C1qDCs might play central roles in the immune recognition of oyster by recognizing all kinds of invading pathogens.

The microorganism binding activity of *Cg*C1qDC-7 towards different microbes was examined to further explore its binding specificity. The prokaryotic expressed r*Cg*C1qDC-7 displayed strong binding affinity towards Gram-negative bacteria *V. splendidus* and *V. anguillarum*, and affinity towards Gram-positive bacteria and fungi. These results were consistent with the binding affinity of r*Cg*C1qDC-7 towards PAMPs. The frequent outbreaks of bacterial diseases have caused high mortalities of cultured mollusc and resulted in huge economic losses. Vibriosis, caused by *V. splendidus* and *V. anguillarum,* is one of the most serious bacterial diseases resulting in high mortalities in aquaculture animals, including turbot, scallop, clam, and oyster [40–44]. The present results suggested that *Cg*C1qDC-7 exhibited binding affinity towards *Vibrios* and might be critical for immune response against vibriosis.

In conclusion, a novel C1qDC (*Cg*C1qDC-7) with a globular C1q domain was identified from Pacific oyster *C. gigas*. *Cg*C1qDC-7 was highly expressed in immune recognition-related tissues such as gill and hemocytes, and exhibited high binding affinity towards *Vibrios*. r*Cg*C1qDC-7 showed high binding selectiveness to LPS and PGN, and low affinity to MAN and Poly (I:C). These results demonstrated that *Cg*C1qDC-7 was a novel PRR involved in the immune defense with a broad recognition spectrum.

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