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Two c-type lectins from *Venerupis philippinarum*: Possible roles in immune recognition and opsonization

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ABSTRACT

In the study, two c-type lectins were identified and characterized from the manila clam *Venerupis philippinarum* (designed as VpClec-1 and VpClec-2, respectively). Multiple alignments and phylogenetic analysis strongly suggested that they were new members of the c-type lectin superfamily. In normal tissue of clams, both VpClec-1 and VpClec-2 transcripts were highly expressed in the tissue of hepatopancreas. After *Vibrio anguillarum* challenge, the temporal expression of both VpClec-1 and VpClec-2 transcripts was up-regulated in the hemocytes of manila clams. The recombinant protein VpClec-1 (rVpClec-1) showed obvious binding activities to lipopolysaccharide (LPS), peptidoglycan (PGN), glucan and zymosan *in vitro*, while the recombinant protein VpClec-2 (rVpClec-2) could only bind LPS, glucan and zymosan. Coinciding with the PAMPs binding assay, both rVpClec-1 and rVpClec-2 displayed broad agglutination and antibacterial activities towards *Vibrio harveyi*, *Vibrio splendidus*, *Vibrio anguillarum*, *Enterobacter cloacae* and *Aeromonas hydrophila*. Moreover, the phagocytosis and encapsulation ability of hemocytes could be significantly enhanced by rVpClec-1 and rVpClec-2. Notably, the rVpClec-1 but not rVpClec-2 elicited a chemotactic response from hemocytes. All the results showed that VpClec-1 and VpClec-2 functioned as pattern recognition receptors (PRRs) with distinct recognition spectrum, and involved in the innate immune responses of manila clams.

1. Introduction

Lectins are important immune molecules involved in non-self recognition, and initiate effective immune responses against microbial invaders [1]. Presently, lectins are ubiquitously found in both vertebrates and invertebrates, and can be broadly divided into more than ten groups based on their structures and functions, such as c-type, s-type, i-type, p-type lectins, pentraxins and discoidins [2]. Among them, c-type lectins (CTLs) usually contain carbohydrate-recognition domain (CRD) that forms a characteristic double-loop structure, disulfide-bond positions, and calcium-binding sites. They can mediate specific recognition and bind to oligosaccharides both in the extracellular matrix and on solid surfaces such as microorganisms [3].

As important pattern recognition receptors (PRRs), CTLs discriminate self and non-self by recognizing constitutive and conserved pathogen-associated molecular patterns (PAMPs) of pathogen [4]. The character contributes to their roles as cellular receptors for microbial carbohydrates or as soluble proteins existing in tissue fluids [5–7]. Usually, the CTLs contain one or more carbohydrate recognition domains (CRDs) of about 130 amino acid residues, in which calcium binding site-2 involve in carbohydrate binding activity [6]. Most dual-CRD c-type lectins comprise a QPD (Gln-Pro-Asp) motif and an EPN (Glu-Pro-Asn) motif which are specific for galactose and mannose binding, respectively [8–10]. In addition, some CTLs consist of a single CRD containing a QPD or an EPN motif or a mutated form of EPN. Only a few species are reported to have an EPS motif in single-CRD lectins, such as FcLec3 from *Fenneropenaeus chinensis* [11] and MjLecA from *Marsupenaeus japonicus* [12]. Generally, CTLs with different CRDs and motifs contributes to their roles in immune responses, such as inflammation, opsonization, cell-cell and cell to extracellular matrix

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interactions, fertilization and regeneration [13–15].

Venerupis philippinarum is an economic species widely spread over many countries. The recent mass mortality of manila clams has been attributed to pathogen invasion and environmental deterioration [16]. To date, many c-type lectins have been identified from marine invertebrates, such as *Sinonovacula constricta* [17], *Crassostrea gigas* [18], *Mizuhopecten yessoensis* [19] and *Saxidomus purpuratus* [20]. Their functions in innate immune system have also been reported, such as neutralization and clearance of pathogens [1], nodule formation [13], activation of prophenoloxidase [14], immune recognition [21], opsonization [22] and bacterial agglutination [23]. However, knowledge on the function of c-type lectins in manila clam is still limited. In the present study, two c-type lectins were identified from *V. philippinarum* (designed as VpClec-1 and VpClec-2), and the spatial and temporal expression profiles, PAMPs recognition patterns, antibacterial and opsonic activities were also investigated in the manila clam.

2. Materials and methods

2.1. Clams cultivation and bacterial challenge

Healthy Manila clams (shell length is 3.0–4.0 cm) were purchased from a local culture farm and acclimatized in the aerated seawater at 20–22 °C for 10 days before formal experiment. The clams were fed with an algae mixture of *Isochrysis galbana* and *Phaeodactylum tricornutum*, and the seawater was totally renewed daily.

The clams were randomly divided into six tanks with 50 L capacity, each containing 50 individuals. Three tanks of the clams served as the control group, while the other three tanks were immersed with *Vibrio anguillarum* at a final concentration of 1×10^7 CFU/mL. Hemolymphs of 6 individuals were randomly sampled from each treatment at 0, 6, 12, 24 and 48 h post bacterial challenge. Meanwhile, five kinds of tissues including hemocytes, mantle, gills, hepatopancreas and adductor muscle were dissected from 6 individuals of the control group to investigate the tissue-specific expression of VpClec-1 and VpClec-2 transcripts.

2.2. RNA extraction, cDNA synthesis and gene cloning

Total RNA was extracted from hemocytes, mantle, gills, hepatopancreas and adductor muscle in *V. philippinarum* using TRIzol reagent (Invitrogen, USA). The quality and concentration of total RNA were estimated using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA). Total RNA was treated with RQ1 RNase-Free DNase (Promega, USA) to remove DNA contamination. To synthesize cDNA by reverse transcription, 2 μg total RNA, 200 units M-MLV reverse transcriptase (Promega, USA) and 0.5 μM oligo (d_T) primer were reacted for 1 h at 42 °C in 25 μL reaction mixture.

Two short c-type lectins were identified through large scale EST sequencing of the cDNA library constructed from manila clam [24]. Nested-PCR was performed with P1, P2, P3, P4 as forward primers (Table 1) and oligo (d_T) as reverse primer to amplify the 3' end of VpClec-1 and VpClec-2, respectively. The procedure was listed as follows: the first cycle included an extended (5 min) denaturation period during which polymerase was added (hot-start PCR); 35 cycles of 94 °C for 50 s, 60 °C for 30s and 72 °C for 30 s; the last cycle had an extended elongation period of 72 °C for 10 min. The PCR products were gel-purified, cloned into the pMD19-T simple vector (TaKaRa, Japan) and sequenced in both directions with primers M13–47 and M13-RV. The full-length cDNA of VpClec-1 and VpClec-2 were obtained by overlapping the original EST sequence and the amplified fragments.

2.3. Bioinformatics analysis

The nucleotide sequence was analyzed using the BLAST algorithm, and the deduced amino acid sequence was analyzed with the Expert Protein Analysis System. The protein domains were predicted with the Simple Modular Architecture Research Tool version 4.0 [25]. Multiple alignments were performed with the ClustalW Multiple Alignment program and Multiple Alignment Show program. A phylogenetic tree was constructed by MEGA 4.1 software with the neighbor-joining (NJ) algorithm, and the reliability of branching was tested with 1000 bootstrap replicates.

Table 1
Primers used in the present study.

Primer	Sequence (5′-3′)	Sequence information
P1	GCTCGTGTTATTTCTTTGGTC	3′ RACE primer
P2	GGTATCTTTGCCAACCATCGC	3′ RACE primer
P3	CTTGCTGTAGCGATGGTTGGAT	3′ RACE primer
P4	GCACGGGTGTATTGTCAAAGTC	3′ RACE primer
P5	GATCGGACTGACGGACGAAAT	Real-time PCR
P6	GTTGGCAAAGATACCCGCACA	Real-time PCR
P7	GCGTGAAACTTTGGCGAAATGA	Real-time PCR
P8	GACAATACACCCGTGCAGCAGA	Real-time PCR
P9	GACTCTGGTGATGGTGTCACCCA	β-actin primer
P10	ATCTCCTTCTGCATTCTGTCGGC	β-actin primer
P11	GAATGCCCCAATGGATGGACAT	Recombinant primer
P12	TCATTACAATTCATCAGCCGACA	Recombinant primer
P13	AAAGCTTGCTGTAGCGATGGT	Recombinant primer
P14	TCATTAATACACTATTTTGCAAT	Recombinant primer
d_T	GGCCACGCGTCGACTAGTACT$_{17}$	Oligo (d_T)-adaptor

2.4. The spatial and temporal expression patterns of VpClec-1 and VpClec-2 mRNA

The spatial and temporal expression profiles of VpClec-1 and VpClec-2 mRNA were performed on a 7500 Fast Real Time PCR system (Applied Biosystems, USA). Gene-specific primers (P5 and P6 for VpClec-1, P7 and P8 for VpClec-2, Table 1) were used to amplify the fragments of VpClec-1, VpClec-2 respectively. β-actin (P9 and P10, Table 1) was used for reference gene, The cycling protocol was 1 cycle of 94 °C for 5 min; 40 cycles of 94 °C for 50 s, 60 °C for 60 s and 72 °C for 50 s followed by 1 cycle of 72 °C for 10 min. The purity of amplification products was evaluated by dissociation curve analysis. The $2^{-\triangle\triangle CT}$ method was used to analyze the relative expression level of VpClec-1 and VpClec-2 [26]. The relative mRNA expression were given in terms of mean ± S.D. (N = 6). Statistical analysis was performed by one-way analysis of variance (one-way ANOVA) followed by a Duncan test using SPSS 16.0 software, and *P* values less than 0.05 were considered statistically significant.

2.5. Recombinant expression of VpClec-1 and VpClec-2

Two specific primers (P11 and P12 for VpClec-1, P13 and P14 for VpClec-2, Table 1) were used to amplify the fragment encoding mature peptide of VpClec-1 and VpClec-2, respectively. The fragments were cloned into pEASY-E1 simple vector (Transgen Biotech, Beijing, China), and transformed into phage resistant chemically competent cells, respectively. The pEASY-E1-VpClec-1 and pEASY-E1-VpClec-2 plasmids were extracted and transformed into *E. coli* BL21 (DE3). Then SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to test the positive transformants after induced by 1 mM IPTG for 4 h. The recombinant proteins (rVpClec-1 and rVpClec-2) were purified by a Ni^{2+} chelating sepharose column, and refolded in gradient urea-TBS glycerol buffer. The concentration of refolded proteins was measured by BCA method [27].

2.6. Preparation of antibody and Western blot analysis

The renatured proteins were dissolved in ddH_2O for preparation of antibodies. After breeding for several days, 6 weeks old mice were

injected with the renatured protein rVpClec-1 and rVpClec-2 in complete Freund's adjuvant (Sigma, USA) by intraperitoneal injection, respectively. Two weeks later, the rVpClec-1 and rVpClec-2 in incomplete Freund's adjuvant (Sigma, USA) were intraperitoneally injected again. The next two injections were immunized at tail at a one-week interval. Four days after the last immunization, the mice were sacrificed to collect immunized serum [28,29].

For western blotting analysis, the recombinant protein was separated using 15% SDS-PAGE, and then the separated proteins were electrophoretically transferred onto a 0.45 mm nitrocellulose membrane at 300 mA for 1.5 h. The membrane was blocked with PBS containing 3% bovine serum albumin (BSA) at 37 °C for 1 h. After washed three times with PBS containing 0.05% Tween-20 (PBST), the membrane was incubated with anti-rVpClec-1 or anti-rVpClec-2 serum (1:1000 diluted in PBS), respectively. Then the membrane was washed with PBST for three times and incubated with goat-anti-mouse Ig-alkaline phosphatase conjugate (Southern Biotech, 1:5000 diluted in PBS) at 37 °C for 1 h. After washed for three times, the protein bands were stained with freshly prepared substrate solution containing nitroblue tetra-zolium (NBT, Sigma, USA) and 5-bromo-4-chloro-3-indolyphosphate (BCIP, Sigma, USA) for 5 min, and the membrane was washed with distilled water to stop the dyeing process. Pre-immune serum was used as negative control.

2.7. PAMPs binding assay

Columns of the 96-well assay plate were coated with lipopolysaccharide (LPS), peptidoglycan (PGN), glucan or chitin (Sigma-Aldrich, USA), and blocked with 3% BSA as previously described [30]. Then 100 μL of VpClec-1 and rVpClec-2 in TBS-Ca^{2+} buffer (50 mM Tris- HCl, 100 mM NaCl, 10 mM $CaCl_2$, pH 7.5) at different concentrations (1.0, 2.5, 5.0 and 10.0 μg/mL) were added to each column and incubated for 1 h at room temperature, respectively. The plates were incubated with 100 μL serums of rVpClec-1 and rVpClec-2 (1:1000 diluted in PBS) at 37 °C for 1 h, respectively. The wells were then incubated with rVpClecs antibody (1:5000) and goat-anti-mouse Ig-alkaline phosphatase conjugate (1:5000) (Southern Biotech, USA). Finally, pNPP substrate solution was added and incubated in the dark at room temperature. The absorbance was measured at 405 nm with a microplate reader (Tecan M200, Switzerland). The wells with 100 μL of carbonate-bicarbonate buffer were used as blanks. As the negative control, pre-immunized serum was used instead of immunized serum. Every experiment was performed in triplicate.

2.8. Microbe agglutination assay

The microbial agglutination assay was determined against Gram-positive bacteria *Staphyloccocus aureus* and Gram-negative bacteria (*Vibrio harveyi*, *Vibrio splendidus*, *Vibrio anguillarum*, *Enterobacter cloacae* and *Aeromonas hydrophila*) according to the method described previously [21]. Briefly, the exponential bacteria were harvested and stained by crystal violet. The labeled microbes were then suspended in TBS-Ca^{2+} buffer. Then 10 μL of microbe suspension was incubated with 25 μL of rVpClec-1 and rVpClec-2 solution (final concentration of 10 μg/mL) at room temperature for 1 h, respectively. Microbes dissolved in TBS buffer were selected as the control. Thereafter, 10 μL of the mixture was mounted onto a glass slide and observed by a light microscopy (BX51, Olympus, Japan).

2.9. Cell motility assay

Cell motility was examined on the soft-agar plates (0.3% agar). After Gram-positive bacterium (*S. aureus*) and Gram-negative bacteria (*V. harveyi*, *V. splendidus*, *V. anguillarum*, *E. cloacae* and *A. hydrophila*) were cultured at the stationary phase, the bacteria mixed with rVpClec-1 and rVpClec-2 in TBS-Ca^{2+} buffer (final concentration of 10 μg/mL) were located on the soft-agar plates. Motility halos were quantified after 16 h using at least three plates for each condition.

2.10. Phagocytosis assay

Hemocytes from Manila clam were collected with equal volume of pre-chilled anticoagulant (Tris-HCl 50 mM; glucose 2%, NaCl 2%; EDTA 20 mM; pH 7.4). After harvested by centrifugation, hemocytes were resuspended in TBS-Ca^{2+} buffer and incubated with rVpClec-1 and rVpClec-2 (final concentration of 1 or 10 μg/mL) at 18 °C for 30 min, respectively. 5 μl of 3% fluorescent microspheres was added into each hemocytes suspension and incubated for another 1 h at room temperature. Phagocytosis was analyzed using an Accuri C6 flow cytometer (BD) with BD CFlow® software. Differences were considered significant at $P < 0.05$ in *t*-test and marked by an asterisk.

2.11. Chemotaxis assay

Chemotactic properties of the rVpClec-1 and rVpClec-2 were determined using Costar Transwells with 6.5 mm diameter and 8 μm pores (Corning, NY) according to Pablo Balseiro et al. with some modification [31]. Shortly, 250 μL of hemolymph from individual clams was added to the upper compartment, and 500 μL of rVpClecs (1 or 10 μg/mL) or Tris-HCl (pH 8.0) in TBS-Ca^{2+} buffer were located in the lower compartment, respectively. After 4 h of incubation in the dark, cells in the lower compartment were centrifuged and then counted using an Accuri C6 flow cytometer (BD) with BD CFlow® software.

2.12. In vitro encapsulation assay

In vitro encapsulation assay was performed according to the previous study [21]. Briefly, Ni-NTA agarose beads (Qiagen, Germany) were equilibrated in TBS-Ca^{2+} buffer, and then incubated with rVpClec-1 and rVpClec-2 (the final concentration was 10 μg/mL), respectively. Protein-coated beads were washed with TBS for three times, and then suspended in TBS. The hemolymph was withdrawn and diluted in anticoagulant. After the hemocytes were settled down, 1 μL of the protein-coated agarose beads (120–150 beads) was added and incubated at 18 °C. Encapsulation of the beads was observed and counted after 6 h and 24 h by a light microscopy (BX51, Olympus, Japan). Every treatment had three duplications.

3. Results

3.1. cDNA cloning and sequence analysis of VpClec-1 and VpClec-2

The full-length cDNA of VpClec-1 and VpClec-2 were deposited in GenBank database under the accession no. MH107138 and MN150184, respectively. VpClec-1 and VpClec-2 exhibited relatively high similarities with c-type lectins from other mollusks. For example, VpClec-1 shared 39.01% similarity with c-type lectin from *Argopecten irradians* (ACS72239), while VpClec-2 was mostly homologous with lectin from *Crassostrea gigas* (XP_011419342, 40.62% similarity). These two VpClecs shared 25.31% similarity with each other (Fig. 1). The phylogenetic analysis showed that these sequences of c-type lectins were split into three groups, including mollusks, insects and vertebrate clades (Fig. 2). In the mollusks group, VpClec-1 was first clustered together with *Azumapecten farreri*. Meanwhile, VpClec-2 was clustered most closely with *Pomacea canaliculata*, then they formed a sister group with other mollusks.

3.2. The mRNA expression profile of VpClec-1 and VpClec-2 in different tissues and after bacterial stimulation

The mRNA transcripts of VpClec-1 and VpClec-2 were found to be ubiquitously expressed in all tissues detected. As revealed in Fig. 3, both

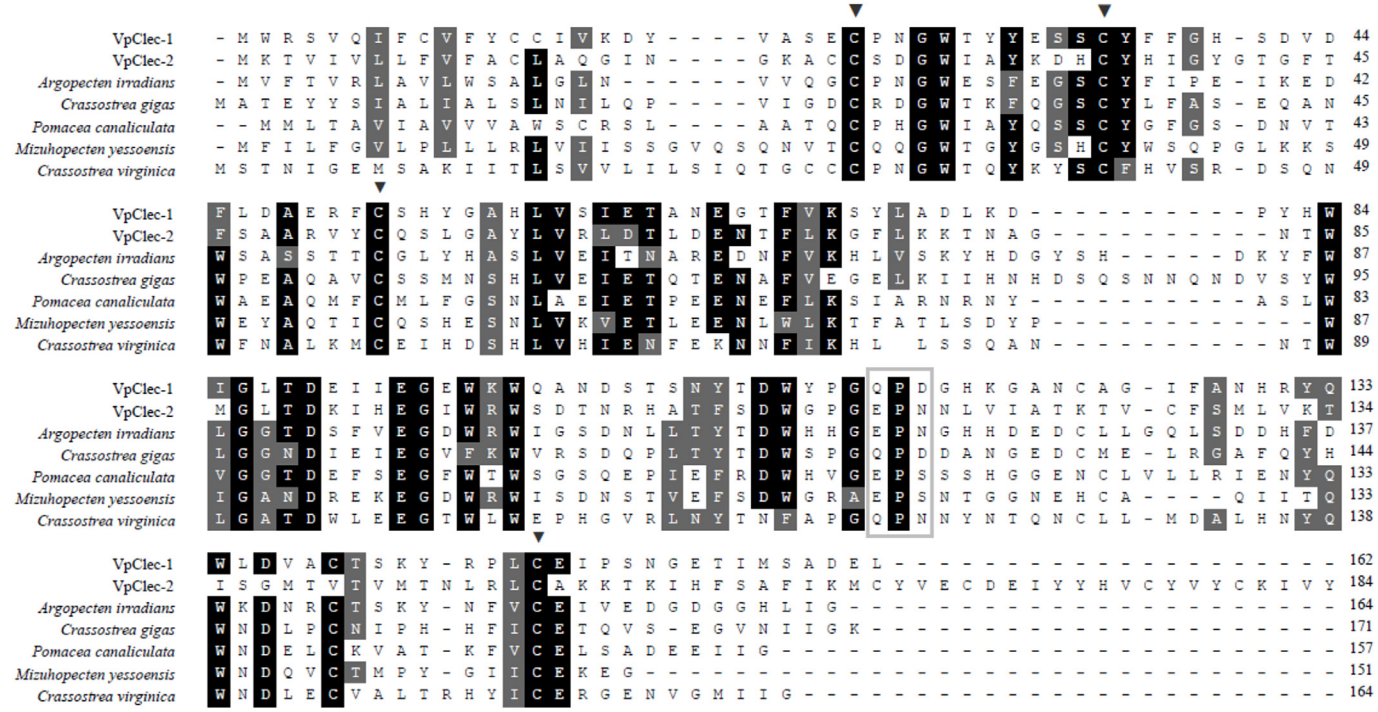

Fig. 1. Multiple alignments of VpClec-1 and VpClec-2 with other c-type lectins from *Mizuhopecten yessoensis* (XP_021339975), *Pomacea canaliculata* (XP_025097818), *Crassostrea virginica* (XP_022328732), *Argopecten irradians* (ACS72239), and *Crassostrea gigas* (XP_011420165). Conserved amino acid residues were shaded in dark, and similar amino acids were shaded in gray. Conserved cysteine residues involved in the formation of the CRD internal disulfide bridges were marked with ▼. The letters in box indicate the motif for determining ligand binding specificity.

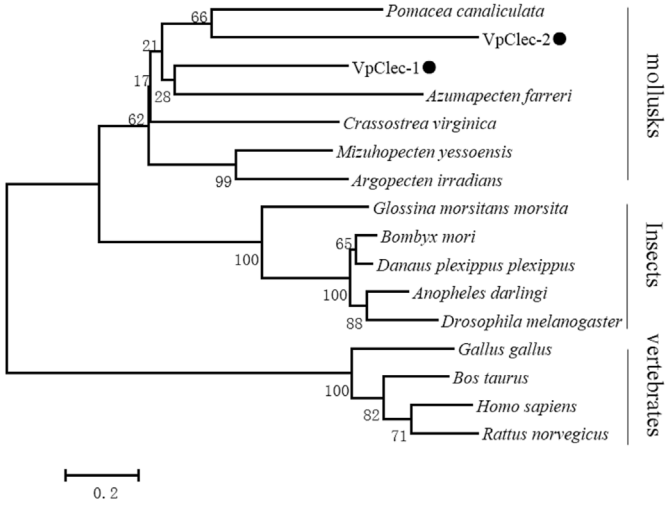

Fig. 2. A phylogenetic tree of c-type lectins constructed by the neighbor-joining method using the Mega 4.1 program. The protein sequences of c-type lectins used for the phylogenetic tree were listed as followed: *Homo sapiens* (AIL02126), *Rattus norvegicus* (AAA41551), *Bos Taurus* (AAC37310), *Gallus gallus* (CAA23711), *Azumapecten farreri* (ABB71672), *Mizuhopecten yessoensis* (XP_021340191), *Argopecten irradians* (ACS72239), *Pomacea canaliculata* (XP_025097818), *Crassostrea virginica* (XP_022295727), *Glossina morsitans morsitans* (ABD60991), *Drosophila melanogaster* (XP_002087961), *Anopheles darling* (ETN63566), *Bombyx mori* (NP_001040251) and *Danaus plexippus plexippus* (OWR42922).

VpClec-1 and VpClec-2 mRNA was dominantly expressed in hepatopancreas ($P < 0.01$), moderately in gills ($P < 0.01$) and hemocytes ($P < 0.01$), and marginally expressed in muscle. The temporal expression of VpClec-1 and VpClec-2 mRNA in hemocytes was monitored after the manila clams were stimulated by *V. anguillarum*. After bacterial challenge, the expression of VpClec-1 transcripts was significantly up-regulated (2.27-fold, $P < 0.05$) at 6 h post stimulation compared with the control group. After that, the expression level of VpClec-1 mRNA decrease remarkably at 12 h (0.56-fold, $P < 0.05$). As time progressed, the expression level increased to 81.70-fold ($P < 0.01$) of the control at 24 h, and then recovered to the original level at 48 h (Fig. 4). As concerned to VpClec-2, acute up-regulation of VpClec-2 transcripts was observed at 6 h (8.25-fold, $P < 0.01$), 12 h (16.7-fold, $P < 0.01$) and 24 h (24.2- fold, $P < 0.01$) post bacterial challenge. After that, the expression level of VpClec-2 transcripts returned to the original level at 48 h compared with the control group (Fig. 4).

3.3. Purification, refolding the recombinant proteins and western blotting analysis

The purified proteins of rVpClec-1 and rVpClec-2 were analyzed on 15% SDS-PAGE with an apparent 16 kDa and 19 kDa band visualized (Fig. 5A, line 3 and line 7), which was in accordance with the predicted molecular weight of 15.98 kDa and 18.74 kDa, respectively. The concentration of rVpClec-1 and rVpClec-2 was determined to be 319.2 and 337.3 μg/ml, respectively. Western blotting was performed to identify the specificity of the antibodies against rVpClec-1 and rVpClec-2. Clear reaction bands with high specificity were observed, which indicated that the antibodies could react with rVpClec-1 and rVpClec-2, respectively (Fig. 5A, line 4). Negative bands were not observed in the study (data not shown).

3.4. PAMPs binding, microbial agglutination and swimming motility assay

Both rVpClec-1 and rVpClec-2 possessed binding activity towards LPS, glucan and zymosan (Fig. 5B), and the binding abilities of rVpClec-1 and rVpClec-2 were dose-dependent. In addition, rVpClec-1 but not rVpClec-2 could also bind PGN *in vitro* directly. As revealed in Fig. 5C, both rVpClec-1 and rVpClec-2 showed strong agglutination activities towards *V. harveyi*, *V. splendidus*, *V. anguillarum*, *E. cloacae* and *A. hydrophila*, while no obvious agglutination activities against *S. aureus* was

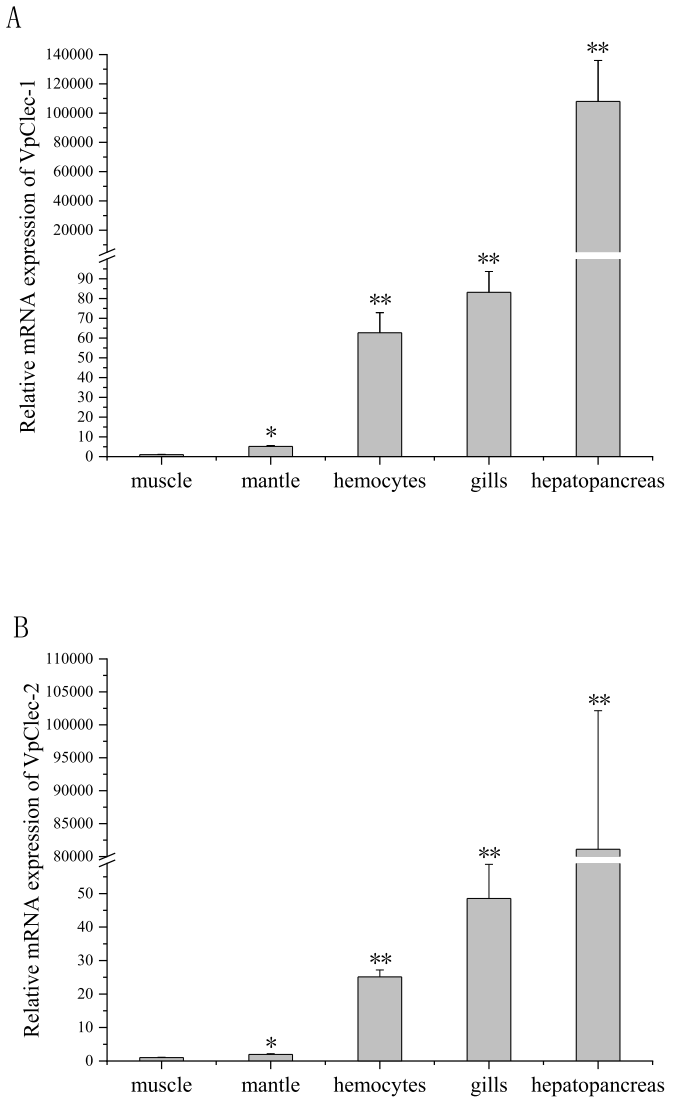

Fig. 3. VpClec-1 and VpClec-2 mRNA expression level in different tissues detected by qRT-PCR. Transcript levels in mantle, gills, hemocytes and hepatopancreas were normalized to that of muscle. Vertical bars represented the mean ± S.D. (N = 6) (*: $P < 0.05$, **: $P < 0.01$).

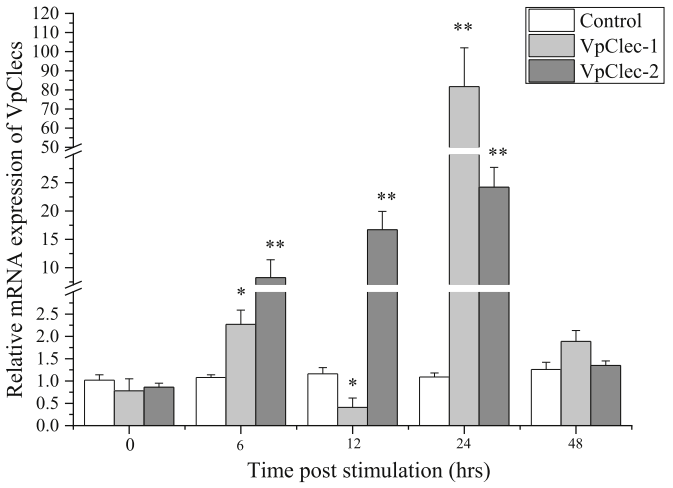

Fig. 4. Temporal expression of VpClec-1 and VpClec-2 mRNA in hemocytes after *V. anguillarum* challenge. The values were shown as mean ± S.D. (N = 6) (*: $P < 0.05$, **: $P < 0.01$).

observed in both recombinant proteins. Compared with rVpClec-2, rVpClec-1 showed higher antibacterial/agglutination activities against *V. anguillarum*, *E. cloacae* and *A. hydrophila*, but not *S. aureus* or *V. splendidus* (Fig. 5D).

3.5. Phagocytosis and chemotaxis assay

As revealed in Fig. 6A, phagocytosis of the hemocytes could be enhanced by both rVpClec-1 and rVpClec-2 incubation. The phagocytic ability of hemocytes was 23.1% (1 μg/mL, $P < 0.05$), 16.5% (10 μg/mL, $P > 0.05$) in the rVpClec-1 treatment compared with that of only 15.0% in the control group. For rVpClec-2, the phagocytic ability of hemocytes was 24.7% (1 μg/mL, $P < 0.05$) and 20.3% (10 μg/mL, $P < 0.05$). In addition, the chemotactic response in hemocytes induced by rVpClec-1 increased 1.32-fold (1 μg/mL, $P < 0.01$), 1.44-fold (10 μg/mL, $P < 0.01$) compared with the migration in control solution (Fig. 6B). However, no obvious chemotactic response from hemoctyes was elicited by rVpClec-1.

3.6. In vitro encapsulation assay

rVpClec-1 and rVpClec-2-coated agarose beads were used to investigate the encapsulation ability of hemocytes towards non-self. After incubated with 10 μg/mL recombinant proteins for 6 h, about 54% and 46% beads were encapsulated by hemocytes from manila clams in the rVpClec-1 and rVpClec-2 treated groups, respectively (Fig. 7A). After 24 h of incubation, more than 90% beads were encapsulated by hemocytes in both treatments. In the control group, only a few (< 5%) were encapsulated by hemocytes (Fig. 7B). After blocked by antibodies, the adhesion of hemocytes (7% for rVpClec-1, 8% for rVpClec-2) was blocked effectively (Fig. 7B).

4. Discussion

Lectins are important PRRs that bind specifically to the unique carbohydrate moieties on microbes [32–34]. Presently, many lectins have been identified and characterized in different species of marine mollusks [23,35]. However, the knowledge on the functions of c-type lectins in manila clam is still in its infancy. In the present study, two c-type lectins were identified from *V. philippinarum*, and their spatio-temporal expression profiles, the PAMPs recognition and binding activities, antibacterial activities and opsonic activities were also investigated.

Based on multiple alignments and phylogenetic analysis, VpClec-1 and VpClec-2 was highly homologous to *A. farreri* and *P. canaliculata*, respectively. Both of them clustered with invertebrates c-type lectins, indicating that they belonged to the c-type lectins family. In addition, the carbohydrate recognition domain (CRD) with Gln-Pro-Asp (QPD) motif was found in VpClec-1, while Glu-Pro-Asn (EPN) motif was found in VpClec-2. Usually, the EPN or QPD motif is important for combination with galactose and mannose, respectively [6]. However, not all CTLs are included the EPN or QPD motif. Mutated motifs are also detected in CRD, such as EPD, EPK, EPS, EPQ, QPG, QPS, QPN, QPT, and YPT, which do not affect the agglutinating activity or their specificity [36,37].

The tissue specific expression patterns were performed for a better understanding of the potential functions of VpClec-1 and VpClec-2 in immune responses. In the study, both VpClec-1 and VpClec-2 transcripts were mostly expressed in hepatopancreas, which were evolutionary forerunners in the integration of immunity and metabolism. In *Litopenaeus vannamei*, LvLT is expressed solely in the tissue of hepatopancreas [38]. However, CFLec-1 transcripts in *C. farreri* are highly expressed in gills [21]. The different distribution of CTLs transcripts perhaps contribute to its important roles in immune defense against pathogenic microorganism. Meanwhile, both VpClec-1 and VpClec-2 transcripts were also highly expressed in hemocytes. Usually,

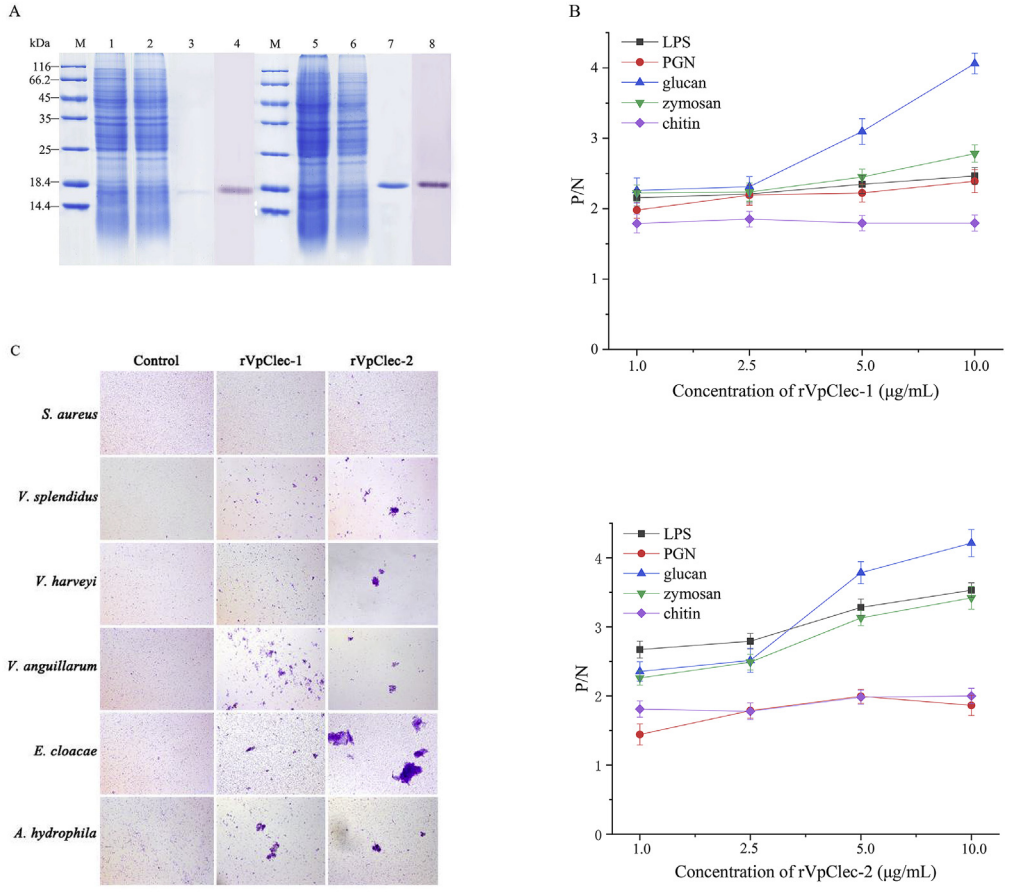

Fig. 5. Immune recognition and antibacterial activities of the recombinant VpClec-1 and VpClec-2. (A) SDS-PAGE analysis of the rVpClec-1 and rVpClec-2. Lane M: protein molecular standard; Lane 1 and 5: bacterial lysate after induction with IPTG; lane 2 and 6: un-induced bacterial lysate; lane 3 and 7: purified rVpClec-1 and rVpClec-2; lane 4 and 8: Western blot analysis of rVpClec-1 and rVpClec-2. (B) PAMPs binding analysis of rVpClec-1 and rVpClec-2. The interaction was detected with goat–anti-rat Ig-alkaline phosphatase conjugate at 405 nm. Samples with P/N > 2.1 were considered positive. Results were representative of average three such experiments. (C) The agglutination of Gram-positive bacterium (*Staphyloccocus aureus*) and Gram-negative bacteria (*Vibrio harveyi*, *Vibrio splendidus*, *Vibrio anguillarum*, *Enterobacter cloacae* and *Aeromonas hydrophila*) induced by rVpClec-1 and rVpClec-2. (D) Swimming motility of Gram-positive bacterium (*Staphyloccocus aureus*) and Gram-negative bacteria (*Vibrio harveyi*, *Vibrio splendidus*, *Vibrio anguillarum*, *Enterobacter cloacae* and *Aeromonas hydrophila*) treated with rVpClec-1 and rVpClec-2.

hemocytes play a central role in the mediation of immune capability via phagocytosis, encapsulation and nodule formation [39], and also participate in the processes of tissues/shell repair [40,41] and detoxication [42] in invertebrates. After *V. anguillarum* challenge, VpClec-1 and VpClec-2 transcripts were significantly up-regulated in hemocytes. Similar expression profiles of CTLs transcripts were also induced by Gram-negative bacteria in several marine shellfish, such as oyster [43], abalone [21,44]. These results suggested that both VpClec-1 and VpClec-2 were inducible acute-phase proteins involved in the immune responses of manila clams. However, at 12 h after *V. anguillarum* challenge, expression of VpClec-1 transcripts decreased to 0.56-fold compared with the control group, which was perhaps influenced by variance of hemocytes amount.

PRRs are secreted to identify the conserved PAMPs of pathogens,

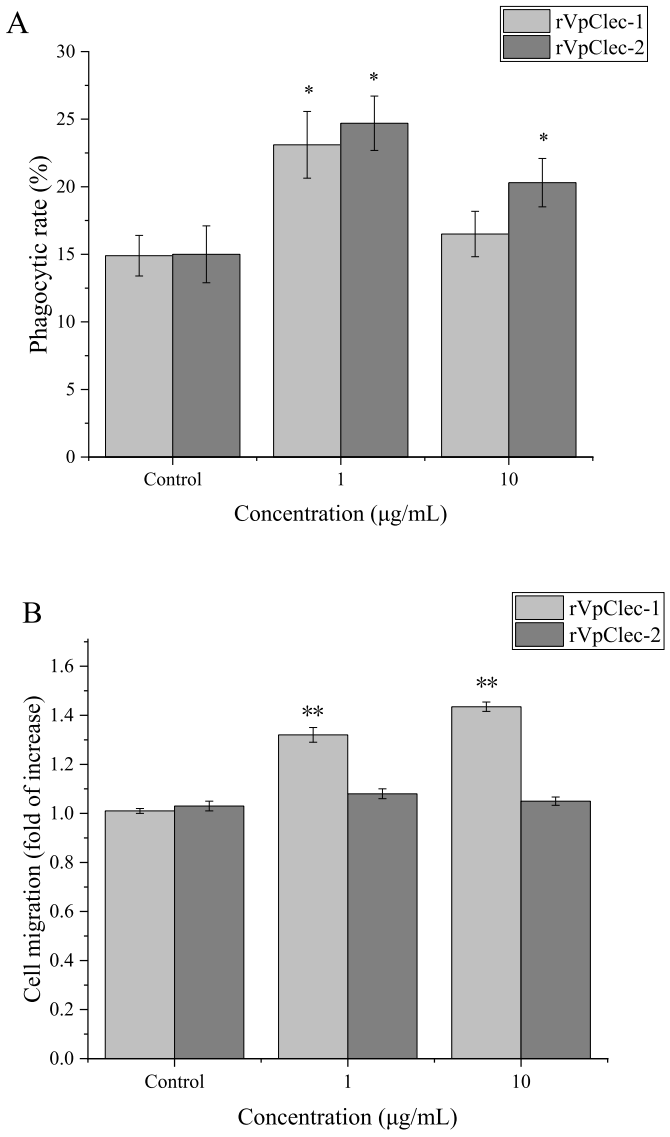

Fig. 6. Chemotactic (A) and phagocytosis (B) activity of rVpClec-1 and rVpClec-2. (A) Number of hemocytes were counted after incubated with rVpClecs. PBS (0.01 M) was used as the blank control. Cells were counted using an Accuri C6 flow cytometer (BD) with BD CFlow® software. The values were shown as mean ± S.D. (N = 6) (*: $P < 0.05$, **: $P < 0.01$). (B) Phagocytosis was analyzed using an Accuri C6 flow cytometer (BD) with BD CFlow® software. The values were shown as mean ± S.D. (N = 3) (*: $P < 0.05$, **: $P < 0.01$).

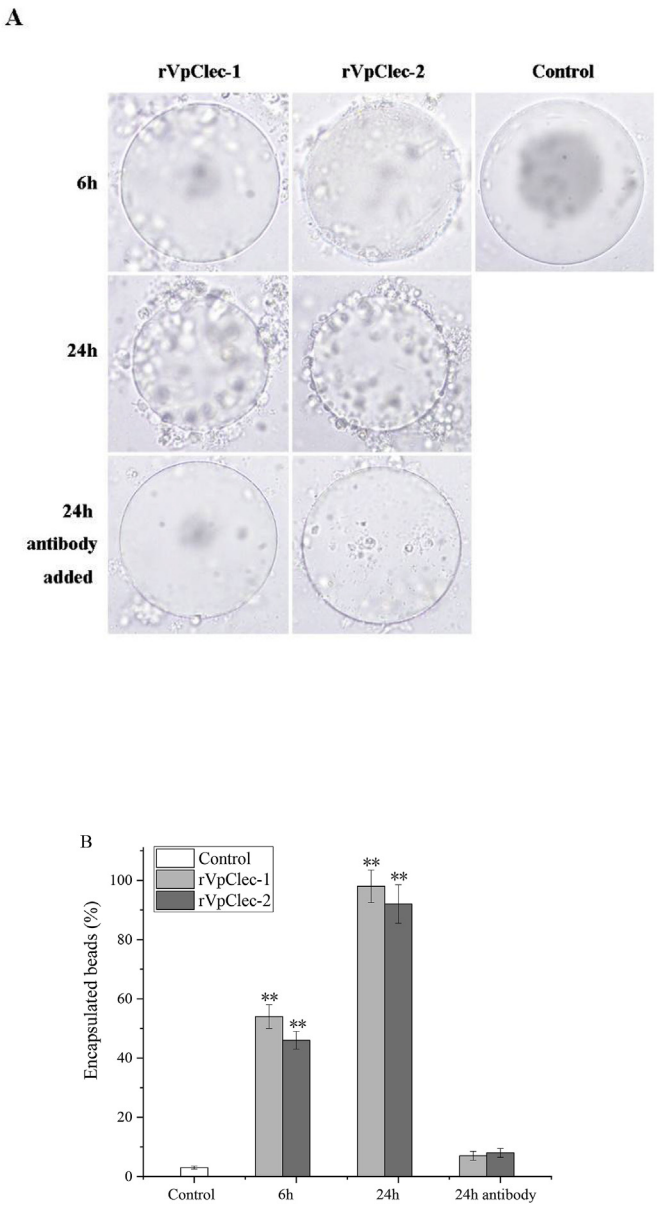

Fig. 7. The promotion of rVpClec-1 and rVpClec-2 on encapsulation of hemocytes in manila clam. (A) Nickel agarose beads were coated with rVpClec-1, rVpClec-2, anti-rVpClec-1 antibodies and anti-rVpClec-2 antibodies. The negative control was prepared with nickel agarose beads coated with TBS. The protein-coated beads were observed by microscopy at 6 and 24 h after incubation. (B) The ratio of beads encapsulated by hemocytes at 6 h and 24 h. The columns represented the mean of three individual counts. Extremely significant ($P < 0.01$) difference from control group was indicated with two asterisks.

which are responsible for recognizing and defending pathogenic microbes [45], thus can activate the signaling pathways to synthesize immune effectors. Although c-type lectins are known for their specific binding ability, other non-self ligands can also be recognized by them [46,47]. For example, SsCTL4 from black rockfish, MnCTLDcp1 from *Macrobrachium nipponense* and MrCTL from *Macrobrachium rosenbergii* showed high binding ability to LPS and PGN, which contributed to the non-self-recognition and clearance of invaders [48–50]. In the present study, both rVpClec-1 and rVpClec-2 could bind several ligands, such as LPS and glucan, which were important components of Gram-negative bacteria and fungi, respectively. Meanwhile, rVpClec-1, but not rVpClec-2, could bind PGN indicated that these two VpClecs might play different roles in non-self recognition and the downstream immune responses. Altogether, the PAMPs-binding ability of rVpClec-1 and rVpClec-2 supported that these lectins might take part in immune responses against bacteria and fungi, and they could serve as PRRs in the PAMPs recognition.

In invertebrates, many lectins are involved in carbohydrate-mediated pathogen recognition, self/non-self discrimination, and regulation of the immune responses [51]. Recombinant AjCTL-2 from *Apostichopus japonicas* endow its multifunction to recognize microbes with preferential binding of D-galactose [52]. In razor clam, ScCTL displayed strong binding affinity towards Gram-positive bacterium (*M. luteus*) and Gram-negative bacteria (*E. coli*, *V. parahaemolyticus*, *V. anguillarum* and *V. harveyi*) in the presence of Ca^{2+} [17]. In the present assay, almost all the tested Gram-negative microorganisms could be agglutinated by rVpClecs, which might be induced by their broad PAMPs recognition spectrum. However, although rVpClec-1 possessed obvious binding activity towards PGN, no agglutinating abilities against *S. aureus* was observed. The reason may lie in the secretion of aminoglycosides

modifying enzyme or repressor protein by *S. aureus* [53,54]. Notably, the motility of *S. aureus* can be limited by rVpClecs, especially rVpClec-1. This is a countermeasure of *S. aureus* for adapting unfavorable environment [46,47].

The opsonic activity of chemokines on hemocytes is essential in innate immunity. In the present study, both rVpClec-1 and rVpClec-2 could enhance the phagocytic activity of hemocytes significantly, especially induced by rVpClec-2. Similar results is also reported in the scallop *C. farreri* [55]. Notably, the chemotactic ability of hemocytes could be prompted by rVpClec-1 but not rVpClec-2, which may be influenced by their different functional structures. Usually, chemokines tend equally to arrest cells and to make them move, in the process of positioning target cells with spatiotemporal precision. After that, hemocytes accumulate in large numbers in the inflamed or injured tissues, which carry out diverse functions including direct elimination of invading bacteria and production of cytokine [56]. Meanwhile, the encapsulation ability of hemocytes could also be prompted by rVpClec-1 and rVpClec-2. The encapsulation response requires the coordination from cellular and humoral factors of the immune defense system [57,58]. For example, capsule formation involves cooperation between one or more classes of hemocytes and is likely mediated by cytokines and adhesion molecules [57]. Together, opsonization of hemocytes mediated by VpClec-1 and VpClec-2, such as phagocytosis and encapsulation, were verified in the immune defense of manila clam.

In conclusion, both rVpClec-1 and rVpClec-2 could recognize and bind several ligands, such as LPS, glucan and zymosan, supporting their vital roles in immune recognition. In addition, rVpClec-1 and rVpClec-2 promoted the agglutination of microbes and performed antibacterial activities against tested bacteria. Furthermore, enhancement of chemotaxis, phagocytosis and encapsulation of hemocytes were also demonstrated, revealing that both VpClec-1 and VpClec-2 could function as opsonins in immune responses of manila clams.

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