



## Full length article

# Immune recognition, antimicrobial and opsonic activities mediated by a sialic acid binding lectin from *Ruditapes philippinarum*



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## ABSTRACT

In the present study, a sialic acid-binding lectin was identified and characterized from Manila clam *Ruditapes philippinarum* (designed as RpSABL-1). Multiple alignments strongly suggested that RpSABL-1 was a new member of the sialic acid-binding lectin family. In non-stimulated clams, RpSABL-1 transcripts were constitutively expressed in all five tested tissues, especially in hepatopancreas. After *Vibrio anguillarum* challenge, the expression of RpSABL-1 mRNA was significantly up-regulated at 6 h ( $P < 0.05$ ), 12 h ( $P < 0.01$ ) and 24 h ( $P < 0.01$ ). Recombinant RpSABL-1 protein (rRpSABL-1) displayed apparent binding activities towards lipopolysaccharides (LPS) and peptidoglycan (PGN), but not to glucan or chitin *in vitro*. Coinciding with the PAMPs binding assay, rRpSABL-1 exhibited obvious agglutination activities against Gram-positive bacterium *Staphylococcus aureus*, Gram-negative bacteria *Escherichia coli*, *V. anguillarum* and *Vibrio harveyi*. Meanwhile, rRpSABL-1 showed anti-bacterial activities against *E. coli*, and biofilm formation of *E. coli* could also be inhibited after incubated with rRpSABL-1. Moreover, the encapsulation, phagocytosis and chemotactic ability of hemocytes could be enhanced by rRpSABL-1. All these results suggested that RpSABL-1 could function as a pattern recognition receptor with versatile functions in the innate immune responses of *R. philippinarum*.

## 1. Introduction

Lectins are important immune molecules involved in the recognition or binding of terminal sugars in bacteria, and thus trigger effective immune responses against pathogens [1]. They could be broadly divided into more than ten groups based on their structures and functions in vertebrates and invertebrates. The best-known ones are c-type, s-type, i-type, p-type lectins, pentraxins and discoidins [2]. I-type lectins are initially introduced by Powell and Varki [3,4], which could bind carbohydrate ligands through their immunoglobulin superfamily domains.

Sialic acid-binding lectins (SABLs) are members of i-type lectins, and show high affinity to *N*-acetyl or *N*-glycolyl carbohydrates, such as *N*-acetylneuraminic acid (NeuNAc), *N*-acetyl-D-galactosamine (GalNAc), *N*-acetyl-D-glucosamine (GlcNAc) and *N*-acetyl-D-mannosamine (ManNAc) [5]. In vertebrates, SABLs are named as sialic acid-binding immunoglobulin-like lectins (Siglecs) encoding an N-terminal V-set Ig-like domain [6]. To date, many Siglecs (e.g. sialoadhesin, myelin-associated glycoprotein, CD22 and CD33-related Siglecs) have been

identified from human, and highly expressed on the important immune cells, such as B cells, conventional dendritic cells (cDCs), macrophages and natural killer cells (NKs) [7]. They exhibit high affinity to sialic acid and other ligands [7,8], and play vital functions in the induction of apoptosis, inhibition of cellular proliferation, inhibition of cellular activation, induction of pro-inflammatory cytokine secretion, and in the case of Siglec-H on plasmacytoid DCs, suppression of interferon- $\alpha$  (IFN $\alpha$ ) production [9–11]. In invertebrates, many SABLs have been identified and characterized from marine shellfish [12–14], and their functions in innate immunity have been reported, such as neutralization and clearance of pathogens [1], immune recognition [12], opsonization [13] and bacterial agglutination [14]. For example, a SABL from *Limulus polyphemus* was successfully commercially available as a cytochemical probe for detection, and isolation of sialic acid in a variety of systems [15]. In *Cepaea hortensis*, the SABL could agglutinate human erythrocytes, as well as interact with bacteria containing type-specific polysaccharides [16].

*Ruditapes philippinarum* is an economic species widely spread over many countries. The recent mass mortality of Manila clams has been

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attributed to pathogen invasion and environmental deterioration [17]. Therefore, it is urgently necessary to characterize immune-related molecules for disease control and the healthy management of clam aquaculture. To date, several SABLs or Clq domain containing proteins have been reported in mollusks, such as *Solen grandis* [18], *Mytilus galloprovincialis* [19] and *Crassostrea hongkongensis* [20]. In the present study, a SABL (designed as RpSABL-1) was identified from the clam *R. philippinarum*, and the spatiotemporal expression profiles, bactericidal activities as well as opsonic activities were also investigated to better understand the immune recognition of clams against pathogens.

## 2. Materials and methods

### 2.1. Clams and bacterial challenge

Healthy Manila clams (shell length of 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 processing. The clams were fed with an algae mixture of *Isochrysis galbana* and *Phaeodactylum tricornutum*, and the seawater was totally renewed daily.

Bacterial challenge experiment was performed according to our previous description [21]. Briefly, 300 clams were randomly divided into two treatments and kept in six aerated tanks (50 L). Three tanks served as the control, while the other three tanks were immersed with *V. anguillarum* at a final concentration of  $1 \times 10^7$  CFU/mL. The *V. anguillarum* was cultured in 2216E medium at 28 °C for 24 h, harvested by centrifuging at 4000 g, 25 °C for 10 min, washed with filter-sterilized sea water (FSSW) for three times, and re-suspended in FSSW. The hemocytes of six individuals were randomly sampled at 0, 6, 12, 24 and 48 h post bacterial challenge.

### 2.2. Gene cloning and sequence analysis of RpSABL-1

A RpSABL-1 EST was identified through large scale EST sequencing of the constructed cDNA library [22]. Forward primers P1, P2 were used to clone the full-length cDNA of RpSABL-1 with oligo ( $d_T$ ) as the reverse primer (Table 1). The nucleotide and deduced amino acid sequences were analyzed using the BLAST algorithm and the Expert Protein Analysis System. The protein domains were predicted with the simple modular architecture research tool version 4.0 [23]. Multiple alignments were performed with the ClustalW Multiple Alignment program and Multiple Alignment Show program.

### 2.3. Quantification analysis of RpSABL-1 mRNA expression

The tissue distribution of RpSABL-1 transcripts was analyzed with the Applied Biosystem 7500 Real-time PCR System. The RNA extraction, cDNA synthesis and quantitative PCR analysis were performed according to the method described previously [21]. Briefly, about 50 mg tissues from six healthy adult clams were sampled, including mantle, gills, hepatopancreas and adductor muscle. Hemolymph (about 0.2 mL per individual) was collected using a 1 mL syringe equipped

**Table 1**  
Primers used in the present study.

Primer	Sequence (5'-3')	Sequence information
P1	TTTGTTTCTGGTAGCCGAGGTA	3' RACE primer
P2	ACTTTGTAGCAITTCAGCAGGTC	3' RACE primer
P3	CAGGTGCAACGACAAATCTCAA	Real-time PCR
P4	GGAATAAACACCAGCAACAGGA	Real-time PCR
P5	CGCAGCTCCTCAGCCATCAT	$\beta$ -actin primer
P6	GCAGCCGTCTCCATTCTTGTTTC	$\beta$ -actin primer
P7	GCAAATATAGGGGACGTTG	Recombinant primer
P8	TCATTATTGTTACAGTTGGAAGAA	Recombinant primer
$d_T$	GGCCACGGCTCGACTAGTACT <sub>17</sub>	Oligo ( $d_T$ )-adaptor

with a 22G needle from the pericardial cavity, and then centrifuged at 500 g, 4 °C for 10 min to collect the hemocytes. The total RNAs from these collected tissues were extracted from six individuals using Trizol reagent (Invitrogen, USA). The first-strand synthesis was carried out based on Promega M-MLV RT Usage information using the DNase I (Promega)-treated total RNA as template and oligo ( $d_T$ )-adaptor primer (Table 1). After reverse transcription, two pairs of gene-specific primers (P3 and P4, Table 1) were designed to detect the mRNA expression of RpSABL-1.  $\beta$ -actin primers (P5 and P6, Table 1) were used to amplify a 121 bp fragment as an internal control. In addition, the mRNA expressions of RpSABL-1 in hemocytes of clams after *V. anguillarum* challenge were also determined. At the end of each PCR, dissociation curve analysis of amplification products was performed to confirm the purity of PCR product. The  $2^{-\Delta\Delta CT}$  method [24] was used to analyze the expression levels of RpSABL-1. All data were given in terms of relative mRNA expressed as mean  $\pm$  S.D. (N = 6). Statistical analysis was performed by one-way analysis of variance (one-way ANOVA) with the Duncan test using SPSS 16.0 software, and *P* values less than 0.05 were considered statistically significant.

### 2.4. Recombinant expression of RpSABL-1

Two specific primers (P7 and P8) were used to amplify the fragment encoding the mature peptide of RpSABL-1. The product was cloned into pEASY-E1 simple vector, and then transformed into *E. coli* BL21 (DE3). To examine the expression of the foreign gene/fragment in recombinant *E. coli*, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to test the positive transformants after IPTG induction for 4 h. The recombinant protein of RpSABL-1 was purified by a Ni<sup>2+</sup> chelating sepharose column and refolded in gradient urea-TBS glycerol buffer. The concentration of refolded protein was measured by BCA method [25].

### 2.5. Preparation of antibody and western blotting analysis

The renatured proteins were dissolved in ddH<sub>2</sub>O for preparation of antibodies and stored at –80 °C before use. After breeding for several days, 6 weeks old mice were injected with the renatured protein rRpSABL-1 in complete Freund's adjuvant (Sigma, USA) by intraperitoneal injection. Two weeks later, intraperitoneal injection was performed with rRpSABL-1 in incomplete Freund's adjuvant (Sigma, USA). The next two injections were immunized at tail at a one-week interval. The mice were sacrificed to collect immunized serum 4 days after the last immunization.

Samples of the recombinant protein were separated by SDS-PAGE. After electrophoresis, proteins were 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-rRpSABL-1 serum (1:1000 diluted in PBS). 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-indolylphosphate (BCIP, Sigma, USA) for 5 min, and stopped by washing the membrane with distilled water. Pre-immune serum was used as negative control.

### 2.6. PAMPs binding assay

The pathogen-associated molecular pattern (PAMP) binding activities of rRpSABL-1 were performed by ELISA assay [26,27]. The microtiter plates were coated with 20  $\mu$ g lipopolysaccharides (LPS), peptidoglycan (PGN), glucan or chitin (Sigma, USA) in carbonate bicarbonate buffer (pH 9.8) overnight at 4 °C, respectively. After blocked with 3% BSA in PBS (200  $\mu$ L/well), the plates were incubated

with various concentrations (1, 2, 4 and 8  $\mu\text{g}/\text{mL}$ ) of rRpSABL-1 at 37 °C for 1 h. The plates were washed three times and incubated with 100  $\mu\text{L}$  polyclonal anti-rRpSABL-1 antibody (1:1000 diluted in PBS) at 37 °C for 1 h. Then the plates were washed again and 100  $\mu\text{L}$  of goat-anti-mouse Ig-alkaline phosphatase conjugate (1:5000 diluted in PBS, Southern Biotech, USA) was added and incubated at 37 °C for 1 h. The wells were washed three times, and 100  $\mu\text{L}$  of 0.1% (w/v) p-nitrophenyl phosphate (pNPP, Sigma, USA) in 50 mM carbonate bicarbonate buffer (pH 9.8) containing 0.5 mM  $\text{MgCl}_2$  was added to each well, and then incubated at room temperature in dark for 30 min. The reaction was stopped by 2 M NaOH and the absorbance was measured at  $\text{OD}_{405}$ . The PBS buffer was used as blank with pre-immune and rTrx as negative control. Each experiment was carried out in triplicate. Samples with P (sample) – B (blank)/N (negative) – B (blank) > 2.1 were considered positive.

## 2.7. Microbe agglutination assay

The microbial agglutination assay was determined against Gram-positive bacterium (*Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*, *V. anguillarum* and *Vibrio harveyi*) according to the previous report [28]. Briefly, the exponential bacteria were harvested and stained by crystal violet, and then suspended in TBS buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.5). 10  $\mu\text{L}$  of microbe suspension was incubated with 25  $\mu\text{L}$  of rRpSABL-1 solution (final concentration of 1  $\mu\text{g}/\text{mL}$ ) at room temperature for 1 h, respectively. Microbes dissolved in TBS buffer were selected as the control. Thereafter, 10  $\mu\text{L}$  of the mixture was mounted onto a glass slide and observed under a light microscopy (BX51, Olympus, Japan).

## 2.8. Antimicrobial activity

Bactericidal activity of rRpSABL-1 against *E. coli* was evaluated by a liquid microdilution assay. Briefly, *E. coli* was incubated in Luria-Bertani broth, and 100  $\mu\text{L}$  of cell suspension ( $\text{OD}_{600} = 0.01$ ) was added to equal volume of rRpSABL-1 (final concentration of 1  $\mu\text{g}/\text{mL}$ ). Both PBS and rTrx was used as negative controls with ampicillin as a positive control. Each sample was incubated at 37 °C and the  $\text{OD}_{600}$  was measured at hourly intervals.

## 2.9. Biofilm formation

Attached biofilm formation was assayed by crystal violet staining in 96-well polystyrene plates (Corning Costar, USA). Briefly, *E. coli* MG1655 ( $\text{OD}_{600} = 0.05$ ) was incubated with rRpSABL-1 (final concentration of 0.01  $\mu\text{g}/\text{mL}$  or 0.1  $\mu\text{g}/\text{mL}$ ) for 8 h. Then the supernatant was poured out, and the plates were washed three times with distilled water. After the plates were dried, 300  $\mu\text{L}$  of crystal violet solution (0.1%, completely dissolved in distilled water) was added into each well and incubated for 20 min at room temperature. Then the staining solution was poured out, and the plates were washed three times with distilled water. 300  $\mu\text{L}$  of 95% ethanol was added into each well and soaked for 5 min. The total biofilm was measured by turbidity at 540 nm. To remove growth effects, we normalized biofilm formation by dividing total biofilm of the maximal growth bacteria at  $\text{OD}_{620}$ . Ten replicate wells were repeated from two cultures independently.

## 2.10. In vitro encapsulation assay

In vitro encapsulation assay was performed according to the previous study [28]. Shortly, Ni-NTA agarose beads (Qiagen, Germany) were equilibrated in TBS buffer containing 10 mM  $\text{CaCl}_2$ , and then incubated with rRpSABL-1 (final concentration of 1.0  $\mu\text{g}/\text{mL}$ ) at 4 °C overnight. Protein-coated beads were washed with TBS for three times, and suspended in TBS. The hemolymph was withdrawn and diluted in equal volume of anticoagulant buffer (50 mM Tris-HCl, 2% glucose, 2% NaCl, 20 mM EDTA, pH 7.4). After the hemocytes were settled down,

1  $\mu\text{L}$  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). Each treatment had three duplications.

## 2.11. Phagocytosis assay

Hemocytes from Manila clams were collected with equal volume of pre-chilled anticoagulant (50 mM Tris-HCl, 2% glucose, 2% NaCl, 20 mM EDTA, pH 7.4). After harvested by centrifugation, hemocytes were re-suspended in TBS buffer (50 mM Tris-HCl, 5 mM  $\text{CaCl}_2$ ) and incubated with 1  $\mu\text{M}$  rRpSABL-1 at 18 °C for 30 min, respectively. After incubated at 18 °C for 30 min, 5  $\mu\text{L}$  3% fluorescent microsphere was added into each hemocytes suspension. The mixture was incubated for 1 h at room temperature, and phagocytosis was then analyzed using an Accuri C6 flow cytometer (BD) with BD CFlow<sup>®</sup> software. Differences were considered significant at  $P < 0.05$  and marked by an asterisk.

## 2.12. Chemotaxis assay

Chemotactic properties of the rRpSABL-1 were determined using PET cell culture inserts of 8.0 mm pore size in 24-well plates (Becton & Dickinson, Franklin Lakes, NJ, USA) [29]. Shortly, 250  $\mu\text{L}$  of hemolymph was added to the upper compartment, and 500  $\mu\text{L}$  of rRpSABL-1 (0.1 and 1.0  $\mu\text{g}/\text{mL}$ ) or Tris-HCl (pH 8.0) were located in the lower compartment, respectively. After 4 h of incubation in the dark at 15 °C, cells in the lower compartment were recovered, centrifuged and then counted using an Accuri C6 flow cytometer (BD) with BD CFlow<sup>®</sup> software.

## 3. Results

### 3.1. Homologous analysis of RpSABL-1

The complete cDNA sequence of RpSABL-1 was deposited in GenBank database under the accession no. MH107143. RpSABL-1 exhibited relatively high similarities with SABLs (or C1q domain-containing protein) from other mollusks. For example, RpSABL-1 shared 59% similarity with SABL from *Aplysia californica* (XP\_005112559) (Fig. 1). Eight invariant amino acid residues in human C1q domain-containing protein were totally conserved in RpSABL-1 (Fig. 1).

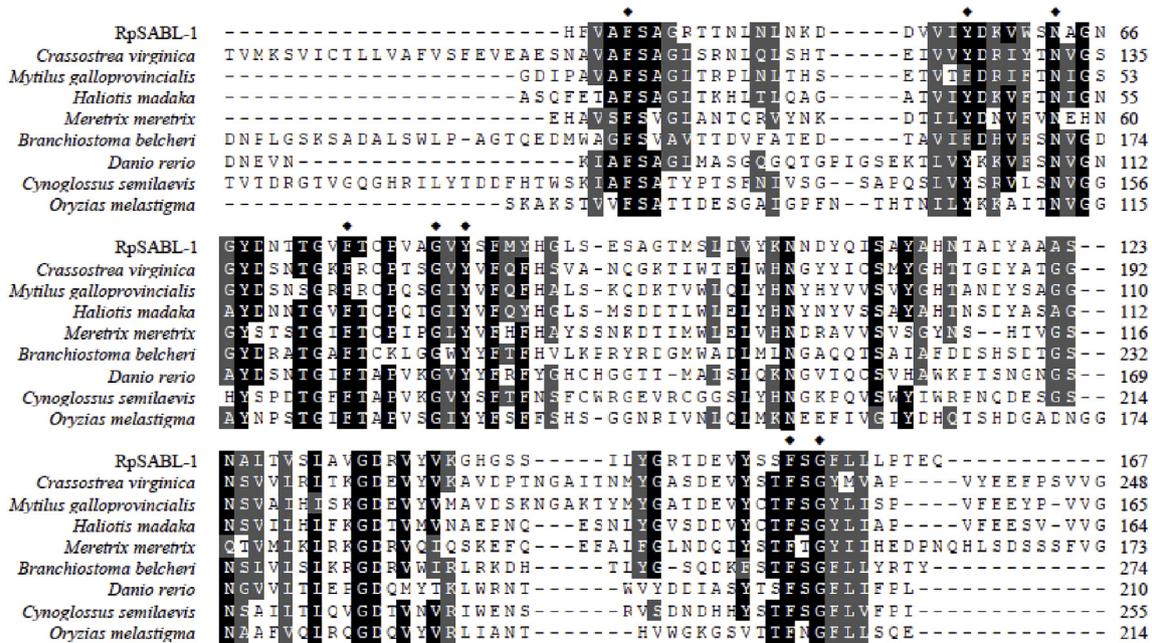
### 3.2. Tissue distribution and temporal expression profiles after *V. anguillarum* challenge

The distribution of RpSABL-1 transcripts in various tissues of unchallenged clams was analyzed by qRT-PCR. The RpSABL-1 mRNA transcripts were predominantly expressed in hepatopancreas, moderately expressed in gills and hemocytes, and marginally expressed in mantle and muscle (Fig. 2). The expression levels in hepatopancreas, gills and hemocytes were extremely significantly higher ( $P < 0.01$ ) than that in muscle (Fig. 2).

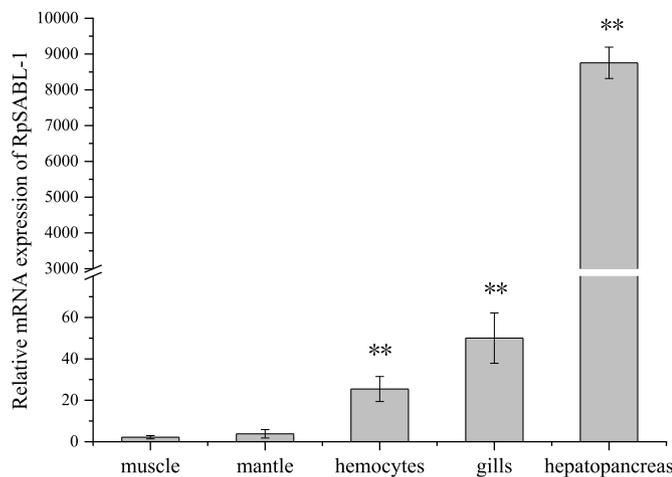
The temporal expression profiles of RpSABL-1 transcripts in hemocytes after bacterial challenge were shown in Fig. 3. After *V. anguillarum* challenge, the mRNA expression of RpSABL-1 was significantly up-regulated at 6 h (3.8-fold,  $P < 0.05$ ) and reached to the maximum value at 24 h (36.1-fold,  $P < 0.01$ ). After that, the expression level of RpSABL-1 transcripts decreased at 48 h post challenge.

### 3.3. Purification, refolding of the recombinant protein and western blotting analysis

The purified rRpSABL-1 was analyzed on 15% SDS-PAGE with an apparent 19 kDa band visualized (Fig. 4, line 3), which was in accordance with the predicted molecular weight. Antibody was prepared using the purified protein, and then western blotting was performed to



**Fig. 1.** Multiple alignments of RpSABL-1 with SABLs from other animals, including *Crassostrea virginica* (XP\_022323368); *Mytilus galloprovincialis* (CBX41690); *Haliotis madaka* (ALU63755); *Meretrix meretrix* (ADX99230); *Branchiostoma belcheri* (XP\_019644839); *Danio rerio* (AAI58180); *Cynoglossus semilaevis* (AIY69145); *Oryzias melastigma* (AEA51018). Conserved amino acid residues were shaded in dark, and similar amino acids were shaded in gray. Eight invariant amino acid residues were labeled by rhombus (◆).



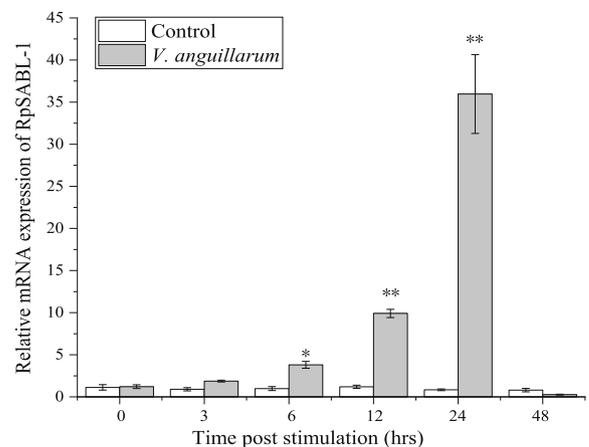
**Fig. 2.** Tissue distribution of RpSABL-1 mRNA detected by qRT-PCR. Transcript level in mantle, gills, hemocytes, and hepatopancreas was normalized to that of muscle. Vertical bars represented the mean  $\pm$  S.D. (N = 6) (\*:  $P < 0.05$ , \*\*:  $P < 0.01$ ).

identify its specificity. A clear reaction band was observed, supporting that the antibody could react with rRpSABL-1 specifically (Fig. 4, line 4). No bands were observed in the negative control of the present study (data not shown).

### 3.4. PAMPs binding and microbial agglutination assay

rRpSABL-1 displayed binding activity towards LPS and PGN, but not to glucan or chitin (Fig. 5A). The binding abilities of rRpSABL-1 were dose-dependent towards PAMPs. No binding activity towards any ligands was observed for the control group (Fig. 5B).

Several Gram-positive and Gram-negative bacteria were selected to test the microbe agglutination activity of rRpSABL-1. After crystal violet staining, agglutination could be directly observed under light microscopy after incubated with rRpSABL-1. As revealed in Fig. 6,



**Fig. 3.** Temporal expression of RpSABL-1 mRNA in hemocytes of clams after *V. anguillarum* challenge. The values were shown as mean  $\pm$  S.D. (N = 6) (\*:  $P < 0.05$ , \*\*:  $P < 0.01$ ).

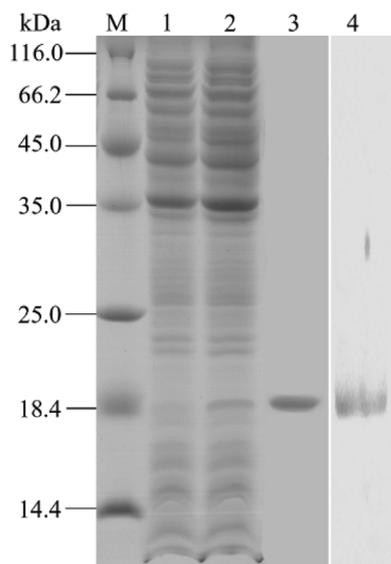
rRpSABL-1 showed high agglutinative activities towards *V. anguillarum*, *E. coli*, *V. harveyi* and *S. aureus*.

### 3.5. Antibacterial assay

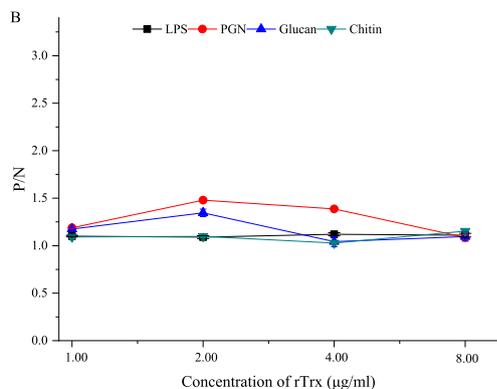
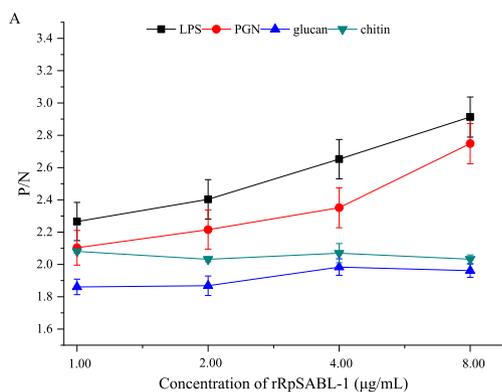
As illustrated in Fig. 7, rRpSABL-1 and ampicillin showed inhibitory effect on the growth curves of *E. coli*. Compared with ampicillin, rRpSABL-1 also suppressed the growth of *E. coli* during the exponential phase (2–6 h) and the stationary phase (6–8 h). Meanwhile, the incubation of RpSABL-1 resulted in less biofilm dispersal of *E. coli* MG1655 on polystyrene surfaces, suggesting that RpSABL-1 could dramatically reduce the biofilm formation in a concentration-dependent manner (Fig. 8).

### 3.6. In vitro encapsulation assay

rRpSABL-1-coated agarose beads were used to investigate the

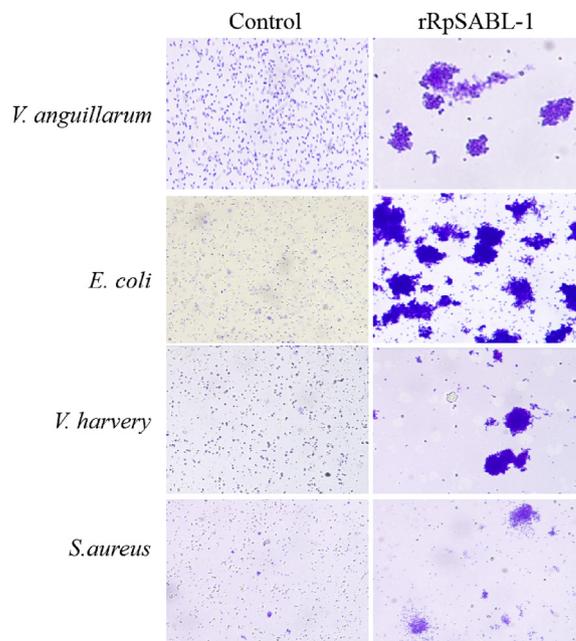


**Fig. 4.** SDS-PAGE and western blotting analysis of rRpSABL-1. M: protein marker; lane 1: negative control for rRpSABL-1 (without induction); lane 2: induced rRpSABL-1; lane 3: purified rRpSABL-1; lane 4: western blotting of rRpSABL-1.

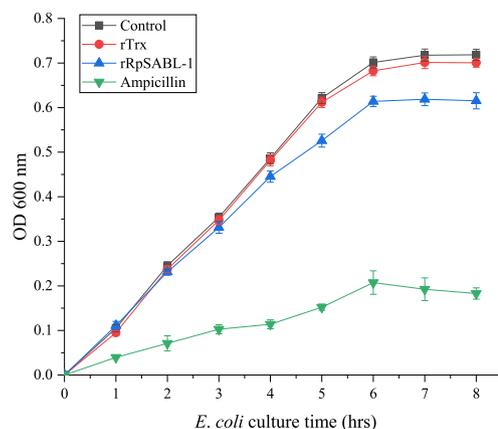


**Fig. 5.** PAMPs binding ability of rRpSABL-1. The interaction was detected with goat-anti-rat Ig-alkaline phosphatase conjugate at 405 nm. Samples with P/N > 2.1 were considered positive. Results were representative of the mean of three replicates ± S.D.

encapsulation ability of hemocytes towards non-self. After incubated with rRpSABL-1, beads were encapsulated by hemocytes from Manila clams compared with those in control group. After blocked by



**Fig. 6.** The agglutination of Gram-positive bacterium (*S. aureus*) and Gram-negative bacteria (*E. coli*, *V. anguillarum* and *V. harveyi*) induced by rRpSABL-1.



**Fig. 7.** Antibacterial activity of rRpSABL-1. Growth suppressive tests of rRpSABL-1 against *E. coli* were measured at OD<sub>600</sub> every 1 h after starting the cultures. The data were the average of three independent cultures.

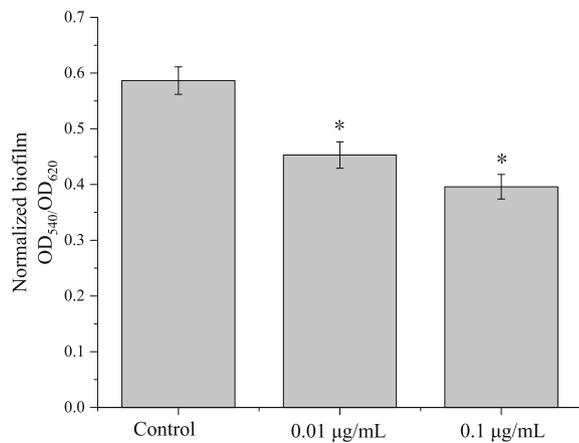
antibodies, only a few protein-coated beads were encapsulated (Fig. 9), suggesting that rRpSABL-1 could mediate the encapsulation ability of hemocytes.

### 3.7. Phagocytosis assay and chemotaxis assay

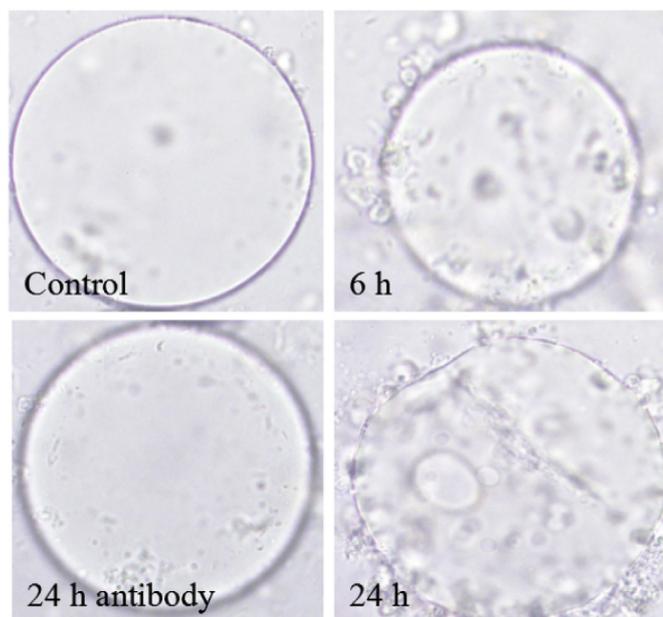
As revealed in Fig. 10, phagocytosis of the hemocytes could be noticeably enhanced by rRpSABL-1. The phagocytosis rate was calculated to increase approximately 11.9% after the incubation of rRpSABL-1 (Fig. 10). In addition, rRpSABL-1 elicited a chemotactic response from hemocytes of clams. It was found that chemotactic hemocytes induced by rRpSABL-1 (final concentration of 1.0 µg/mL) was 1.37-fold increase ( $P < 0.05$ ) compared with the migration in the control solution (Fig. 11).

## 4. Discussion

Lectins are important pattern-recognition receptors (PRRs), which are capable of binding terminal sugars specifically, such as N-acetyl or



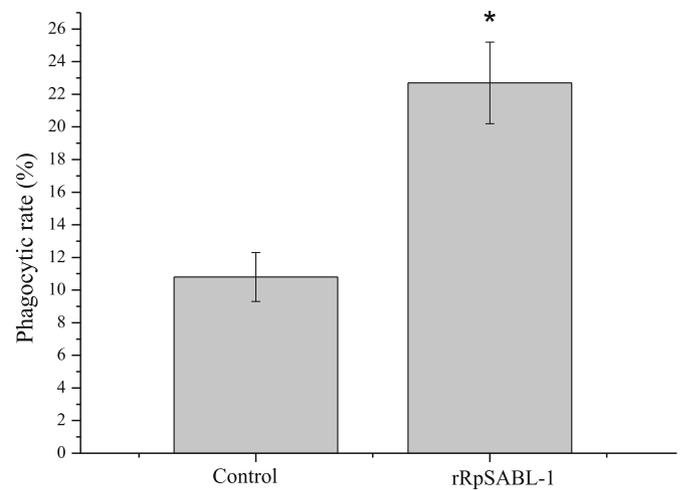
**Fig. 8.** Biofilm formation of *E. coli* MG1655 treated with rRpSABL-1. Normalized biofilm formation (total biofilm/growth) was tested in 96-well polystyrene plates after treated with rRpSABL-1. Ampicillin was used as positive control. Data were the average of 10 replicate wells from two independent cultures. The values were shown as mean  $\pm$  S.D. (N = 10) (\*:  $P < 0.05$ , \*\*:  $P < 0.01$ ).



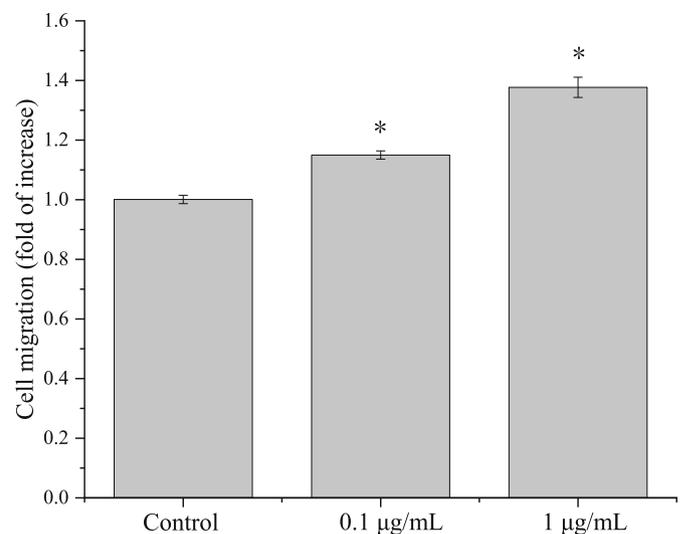
**Fig. 9.** Hemocytic encapsulation enhanced by rRpSABL-1. Nickel agarose beads were coated with rRpSABL-1 and anti-rRpSABL-1 antibodies. The negative control was prepared with nickel agarose beads coated with TBS. The protein-coated beads were observed under microscopy at 6 and 24 h after incubation.

N-glycolyl carbohydrates, glycoproteins and glycolipids [15]. To date, some I-type lectins and their functions have been reported in mollusks. However, the knowledge on the functions of I-type lectins in Manila clam is still in its infancy. In the present study, a SABL was identified, and the PAMPs recognition, microbe agglutination and enhancement of opsonization were also investigated.

The tissue specific expression pattern of RpSABL-1 was performed for a better understanding the potential functions in immune responses. In healthy clams, RpSABL-1 transcripts were mostly expressed in hepatopancreas, which was an evolutionary forerunner in the integration of immunity and metabolism. However, the transcripts of VpSABL, previously detected in Manila clam, was dominantly expressed in mantle [14]. The differences in tissue distribution suggested that they are involved in different immune responses by exerting specific immune functions. After *V. anguillarum* challenge, the RpSABL-1 transcripts



**Fig. 10.** Phagocytosis of hemocytes enhanced by rRpSABL-1. Phagocytosis was analyzed using an Accuri C6 flow cytometer (BD) with BD CFlow<sup>®</sup> software. The values were shown as mean  $\pm$  S.D. (N = 3) (\*:  $P < 0.05$ , \*\*:  $P < 0.01$ ).



**Fig. 11.** Chemotactic activity of hemocytes enhanced by rRpSABL-1. The number of migrating cells from upper chambers to the lower chambers was counted using an Accuri C6 flow cytometer (BD), relative to hemocytes migrating to lower chambers containing Tris-HCl (pH 8.0). The values were shown as mean  $\pm$  S.D. (N = 6) (\*:  $P < 0.05$ , \*\*:  $P < 0.01$ ).

were significant up-regulated in hemocytes. Similarly, the expression of SABLs transcripts was also induced by Gram-negative bacteria in several marine shellfish, such as clam [14], oyster [20] and scallop [12,31]. These results suggest that RpSABL-1 was an inducible acute-phase protein involved in the immune responses of manila clams.

PRRs were secreted to identify the conserved PAMPs of pathogens [32], thus could activate the signaling pathways to synthesize immune effectors. Although SABLs were known for their specific binding ability to sialic acids, other non-self ligands could also be recognized by them [33]. In the present study, rRpSABL-1 could bind several ligands, such as LPS and PGN, which were important components of Gram-negative and Gram-positive bacteria, respectively. The PAMPs-binding ability of rRpSABL-1 supported that RpSABL-1 might serve as PRRs in the recognition of PAMPs. In microbial agglutination and antibacterial assay, the tested microorganisms could also be agglutinated or suppressed by rRpSABL-1. Unlike some c-type lectins, RpSABL-1 was short of Ca<sup>2+</sup> binding motifs (e.g. Gln-Pro-Asn or Gln-Pro-Asp et al.) [34,35], then the microbial agglutination assay was performed without the addition of Ca<sup>2+</sup>. Meanwhile, rRpSABL-1 exhibited obvious agglutination

activities against the tested bacteria without  $\text{Ca}^{2+}$ . The results supported that  $\text{Ca}^{2+}$  was not essential for the agglutination activity of rRpSABL-1. The antibacterial ability of rRpSABL-1 was perhaps contributed to the limitation of biofilm formation in *E. coli*. In human, defense peptide LL-37 could decrease the biofilm formation of *E. coli* at the very low and physiologically meaningful concentrations far below those required to kill or inhibit bacterial growth [36]. Similar results were also supported by some immune molecules identified from invertebrates, such as defensin from *R. philippinarum* [37], gomesin from *Acanthoscurria gomesiana* [38], and  $\beta$ -thymosin from *Paracentrotus lividus* [39]. These results suggested that RpSABL-1 perhaps participated in the immune recognition and responses against pathogenic microbes.

Oposonic ability of hemocytes could be prompted by the coordination from both cellular and humoral effectors in innate immunity [40,41]. For example, capsule formation involves cooperation between one or more classes of hemocytes and is likely mediated by cytokines and adhesion molecules [40]. In the present study, the chemotactic and encapsulation abilities of hemocytes could be prompted by rRpSABL-1. 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 directly eliminating invading bacteria and production of cytokine [42]. Meanwhile, rRpSABL-1 could enhance the phagocytic activity of hemocytes significantly. Similar results were also reported in the scallop *Chlamys farreri* [43] and razor clam *S. grandis* [28]. These results together indicated that RpSABL-1 has a vital cytokine function on both cellular and humoral immunity.

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