Full length article

Scavenger receptor class B type I (SR-BI) in *Ruditapes philippinarum*: A versatile receptor with multiple functions

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ABSTRACT

In the present study, a scavenger receptor class B type I (designed as RpSR-BI) was cloned and characterized from manila clam *Ruditapes philippinarum*. The full-length cDNA of RpSR-BI was of 2000 bp, containing an open reading frame (ORF) of 1515 bp. Multiple alignments and phylogenetic analysis strongly suggested that RpSR-BI was a member of the scavenger receptors family. The mRNA transcript of RpSR-BI was constitutively expressed in all tested tissues, and mainly expressed in hepatopancreas and hemocytes. Generally, *Vibrio anguillarum* or *Micrococcus luteus* challenge induced the expression of RpSR-BI transcripts in hemocytes of manila clams. Recombinant protein of RpSR-BI (rRpSR-BI) could bind lipopolysaccharides, peptidoglycan and glucan, but not chitin *in vitro*. Coinciding with the PAMPs binding assay, a broad agglutination spectrum was displayed by rRpSR-BI including Gram-positive bacteria and Gram-negative bacteria. Moreover, rRpSR-BI could enhance the phagocytosis and chemotaxis of hemocytes. These results showed that RpSR-BI functioned as a pattern recognition receptor (PRR) with distinct recognition spectrum, and also as an opsonin involved in the innate immune response of *R. philippinarum*.

1. Introduction

Scavenger receptors have been demonstrated to play a crucial role in immune defense by serving as pattern recognition receptors (PRRs). In animal kingdom, scavenger receptors family comprise of nine classes of protein, such as eight classes (A, B, D, E, F, G, H and I) from mammal, and one class (C) from invertebrates [1–3]. Interestingly, more than fourteen different characteristic domains, such as SR, Collagen, CD36, LAMP, EGF, and so on were found in scavenger receptors. The combinations and permutations of these domains give rise to a considerable diversity of structure among classes [2], which contributes to a potential multi-function of this protein family.

Scavenger receptors, as pattern recognition proteins (PRPs), play important roles in discriminating self and non-self by recognizing constitutive and conserved pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) [4,5].

Scavenger receptors were originally found in apoptotic cells for binding lipids and proteins [4,6]. Other functions, such as immune recognition, lipid transport, cellular adhesion, and even taste sensing were proved in vertebrates sequentially [2,7,8]. In invertebrates, scavenger receptors and their functions have also been reported [9–11]. For example, class C scavenger receptor from fruit fly could bind both Gram-negative and Gram-positive bacteria. Class B scavenger receptor CD36 from shrimp and several ungrouped SRCR domain-containing proteins from starfish and scallop, were also found to recognize PAMPs or microbes, and exhibited obvious activities of agglutinating bacteria, promoting phagocytosis, or enhancing expression of antimicrobial peptides [9–11].

Manila clam *Ruditapes philippinarum* is an economic species widely spread over many countries. In recent years, mass mortalities in manila clams have been attributed to pathogen invasion and environmental deterioration. Due to lack of acquired immune system, mollusks rely solely on their innate immune system to defend against the invasion of

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microbial pathogens. To date, knowledge on the function of scavenger receptors in mollusks is still limited. In the present study, a scavenger receptor class B, type I (designed as RpSR-BI) was identified from the clam *Ruditapes philippinarum*, and the potential functions in immune responses were also investigated.

2. Materials and methods

2.1. Clams and bacterial challenge

Adult 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 every day.

Bacterial challenge experiment was performed according to our previous study [12]. Briefly, 300 clams were randomly divided into three treatments and kept in nine aerated tanks (50 L). Three tanks served as the control, while the other six groups were immersed with *Vibrio anguillarum* and *Micrococcus luteus* at a final concentration of 1×10^7 CFU mL^{-1}, respectively. These bacteria were cultured, harvested, 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, 48 and 72 h post bacterial challenge. During the bacterial challenge experiment, the seawater was not changed.

2.2. Gene cloning and sequence analysis of RpSR-BI

A SR-BI-like EST was identified through large scale EST sequencing of the constructed cDNA library [13]. Forward primers P1, P2 were used to clone the full-length cDNA of RpSR-BI with primer oligo (d$_T$) as the reverse primer (Table 1). The nucleotide sequence and deduced amino acid sequence was analyzed by using the BLAST algorithm and the Expert Protein Analysis System. The protein domain was predicted with the simple modular architecture research tool version 4.0 [14]. A neighbor-joining phylogenetic tree of scavenger receptor was constructed by MEGA 4.1 with 1000 bootstrap replicates.

2.3. Quantification analysis of RpSR-BI mRNA expression

The tissue distribution of RpSR-BI transcripts was analyzed in an Applied Biosystem 7500 Real-time PCR System. The RNA extraction, cDNA synthesis and quantitative PCR analysis were performed according to previously described [12]. Briefly, total RNAs from hemocytes, mantle, gills, hepatopancreas and adductor muscle were extracted from six individuals using Trizol reagent (Invitrogen, USA). After reverse transcription, a pair of gene-specific primers (P3 and P4, Table 1) was designed to detect the mRNA expression of RpSR-BI. β-actin primers (P5 and P6, Table 1) were used to amplify a 121 bp fragment as an internal control.

In addition, the mRNA expression of RpSR-BI in hemocytes after *V.*

Table 1
Primers used in the present study.

Primer	Sequence (5′-3′)	Sequence information
P1	TTTGCTACAATAAATGGCACAGAAG	3′ RACE primer
P2	TGGTTGTCCACTCAAGGGTCTAAT	3′ RACE primer
P3	ATAGAATTACTGAATGGCGGAACG	Real-time PCR
P4	GGCACCAAGGAAATAGGGCTGT	Real-time PCR
P5	CGCACTTCCTCACGCCATCAT	*β-actin* primer
P6	GCAGCCGTCTCCATTTCTTGTTC	*β-actin* primer
P7	ATGACTATCCGATCGAAGCCACTACG	Recombinant primer
P8	TCATCTTGGTCGGACCCGCCA	Recombinant primer
d$_T$	GGCCACGCGTCGACTAGTACT$_{17}$	Oligo (d$_T$)-adaptor

anguillarum and *M. luteus* challenge was also determined, respectively. At the end of each PCR, dissociation curve analysis of amplification products was performed to confirm the purity of PCR product. The $2^{-\triangle\triangle CT}$ method [15] was used to analyze the expression levels of RpSR-BI. All data were given in terms of relative mRNA expressed as mean ± S.D. (N = 6). Statistical analysis was performed by one-way analysis of variance (one-way ANOVA) with a Duncan test using SPSS 16.0 software, and *P* values less than 0.05 were considered statistically significant.

2.4. Recombinant expression of RpSR-BI

The fragment encoding 449 amino-acid was amplified by two specific primers (P7 and P8, Table 1). Then the PCR products were cloned into pEASY-E1 simple vector (Transgen Biotech, Beijing, China), and transformed into *E. coli* BL21 (DE3) competent cells. The positive transformant was induced by 1 mM IPTG for 4 h, and then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The recombinant RpSR-BI (rRpSR-BI) was purified by a Ni^{2+} chelating sepharose column, and refolded in gradient urea-TBS glycerol buffer. The concentration of refolded protein was measured by BCA method [16].

2.5. Preparation of antibody and western blot analysis

6-week-old mice were injected with the renatured protein rRpSR-BI in complete Freund's adjuvant (Sigma, USA) by intraperitoneal injection. Two weeks later, the rRpSR-BI in incomplete Freund's adjuvant (Sigma, USA) was injected intraperitoneally again. 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 [17,18].

SDS-PAGE was performed for western blot analysis, and the samples were electrophoretically transferred onto a 0.45 μm 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*-rRpSR-BI 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 stopped by washing the membrane with distilled water. Pre-immune serum was used as negative control.

2.6. PAMPs binding assay

The PAMPs binding activities of rRpSR-BI were determined by ELISA assay [19]. The microtiter plates were coated with 20 μg of 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 μL/well) at 37 °C for 1 h and washed three times with PBST, the plates were incubated with various concentrations of rRpSR-BI at 37 °C for 1 h, respectively. The plates were washed for three times and incubated with diluted polyclonal antibody against rRpSR-BI (1:1000 diluted in PBS) at 37 °C for 1 h. Afterwards, the plates were washed again and 100 μL of goat-*anti*-mouse Ig-alkaline phosphatase conjugate (1:3000 diluted in PBS, Southern Biotech, USA) was added and incubated at 37 °C for 1 h. After the last washing, pNPP substrate solution was added and incubated at room temperature in dark. The absorbance was measured at 405 nm, and the wells with 100 μL of carbonate-bicarbonate buffer were used as blank. Each experiment was carried out in triplicate. Samples with P (sample) − B (blank)/N (negative) − B (blank) > 2.1 were considered positive.
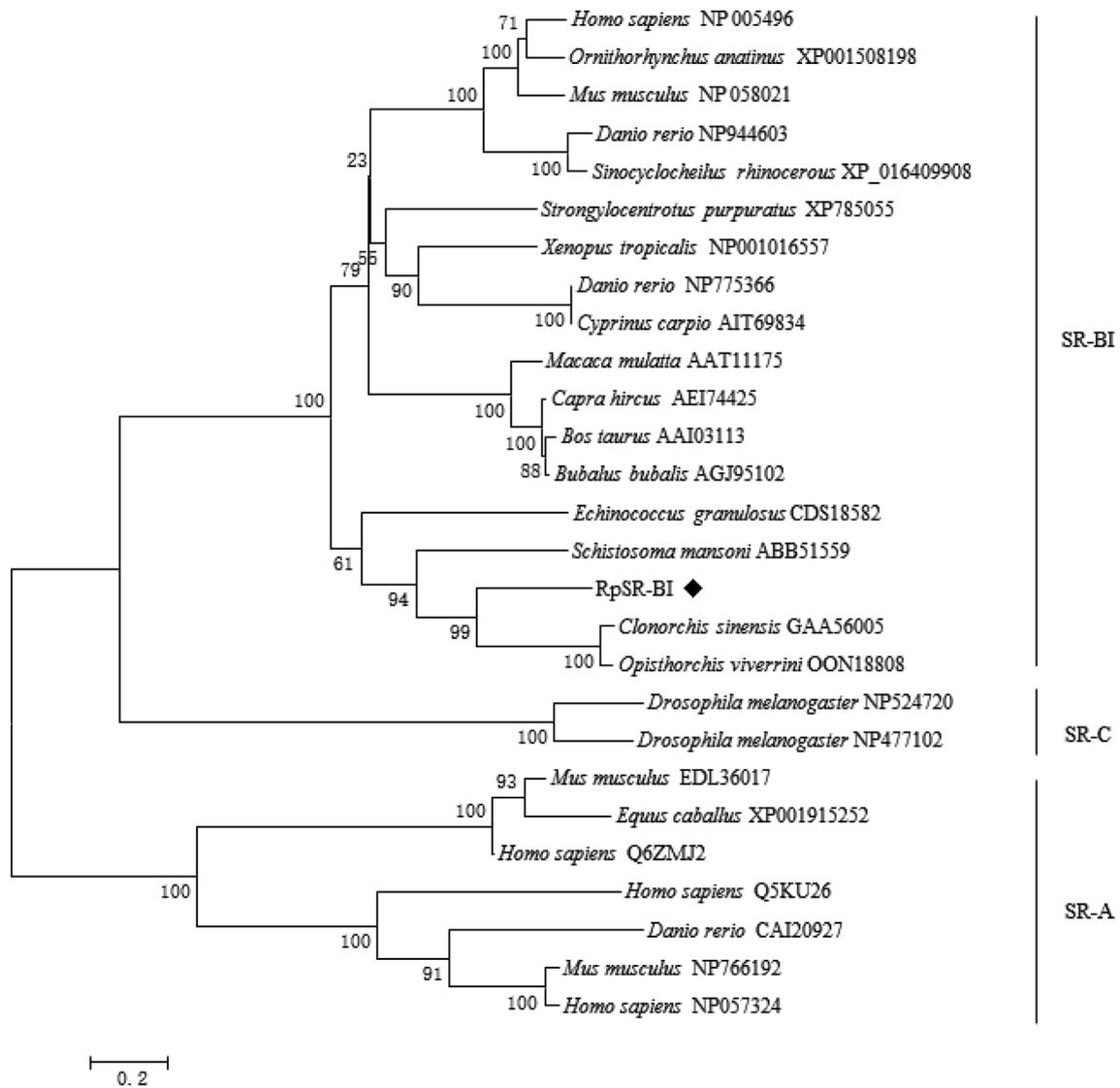
Fig. 1. A phylogenetic tree constructed by the neighbor-joining method using the Mega 4.1 program based on the multiple sequence alignments.
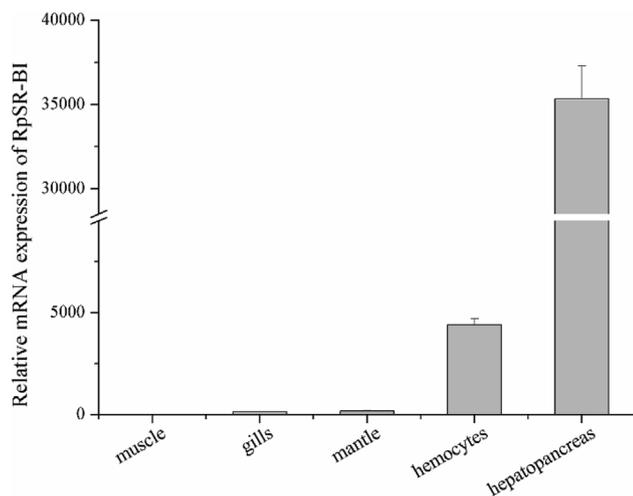

Fig. 2. Expression level of RpSR-BI mRNA in different tissues. Transcript level in mantle, gills, hemocytes, and hepatopancreas are normalized to that of muscle. Vertical bars represent the mean ± S.D. (N = 6).

2.7. Microbe agglutination

The microbial agglutination assay was determined against Gram-positive bacteria (*Staphyloccocus aureus*) and Gram-negative bacteria (*V. anguillarum, Escherichia coli, Vibrio alginolyticus, Vibrio splendidus* and *Vibrio harveyi*) according to the previous report [20]. Briefly, the exponential bacteria were harvested and stained by crystal violet. The labeled microbes were then suspended in TBS-Ca^{2+} buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM $CaCl_2$, pH 7.5). 10 μL of microbe suspension was incubated with rRpSR-BI solution (final concentration of 1 μM) at room temperature for 1 h. 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).

2.8. Phagocytosis assay

Hemocytes from manila clams 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 re-suspended in TBS buffer (Tris-HCl 50 mM; $CaCl_2$ 5 mM) and incubated with 1 μM rRpSR-BI at 18 °C for 30 min. After that, 5 μL of 3% fluorescent microspheres was added into each hemocytes suspension and
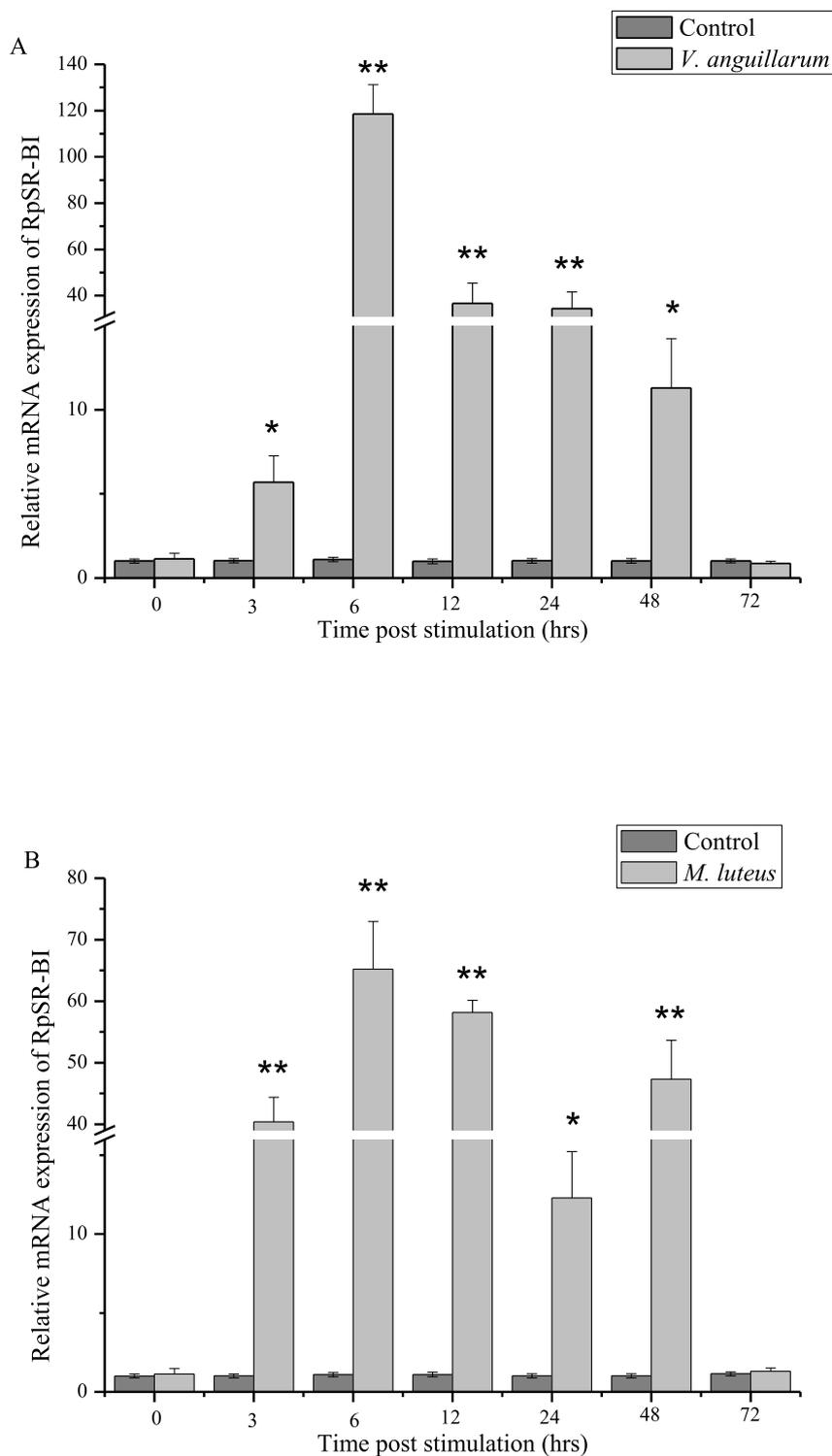
Fig. 3. Temporal expression of RpSR-BI mRNA in hemocytes after *V. anguillarum* (A) and *M. luteus* (B) challenge. The values are shown as mean ± S.D. (N = 6) (*: $P < 0.05$, **: $P < 0.01$).

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.

2.9. Chemotaxis assay

Chemotactic properties of the rRpSR-BI 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 [21]. Shortly, 250 μL of hemolymph from individual clam was added to the upper compartment, and 500 μL of rRpSR-BI or Tris-HCl (pH 8.0) were located in the lower compartment, respectively. After 4 h of incubation in the dark, cells in the lower compartment were recovered, centrifuged and then counted using an Accuri C6 flow cytometer (BD) with BD CFlow® software.
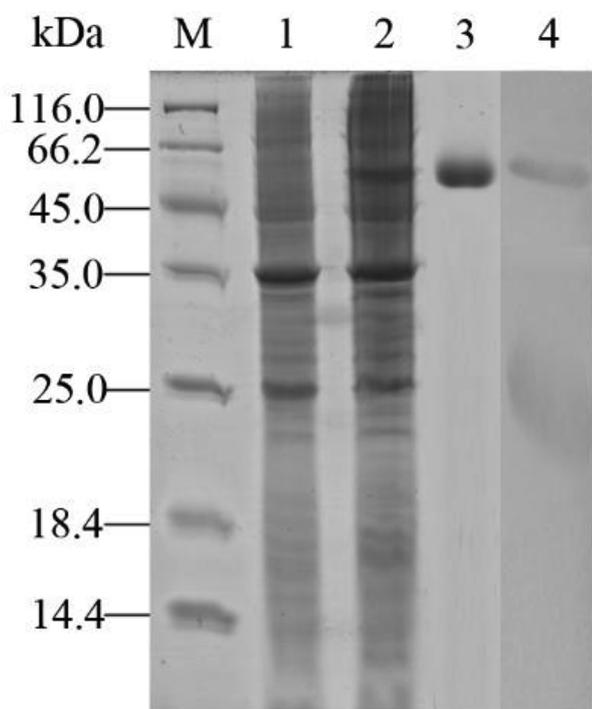
Fig. 4. SDS-PAGE and western-blot analysis of rRpSR-BI. M: protein marker; lane 1: negative control for rRpSR-BI (without induction); lane 2: induced expression of rRpSR-BI; lane 3: purified rRpSR-BI; lane 4: western blot of rRpSR-BI.
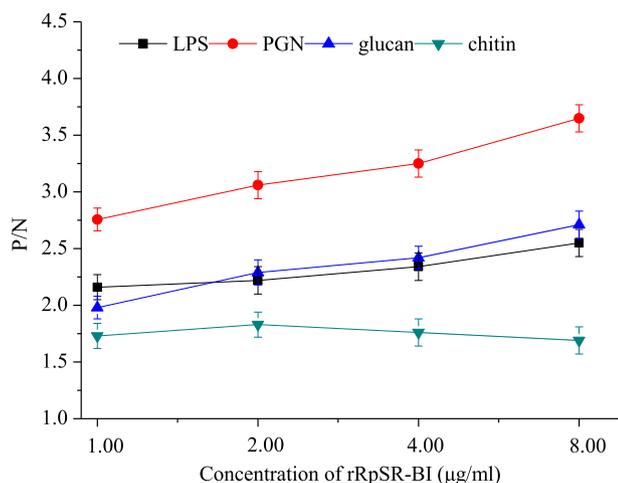

Fig. 5. rRpSR-BI binding activities towards PAMPs determined by ELISA analysis. The interaction is detected with goat–anti-rat Ig-alkaline phosphatase conjugate at 405 nm. Samples with P/N > 2.1 are considered positive. Results are representative of average of three independent experiments.

3. Results

3.1. Characteristics of the full-length cDNA of RpSR-BI

The complete cDNA sequence of RpSR-BI was deposited in GenBank database under the accession no. MH559336. The open reading frame of RpSR-BI was of 1515 bp, encoding a polypeptide of 504 amino acid residues. The mature peptide of RpSR-BI had a calculated molecular mass of 56.6 kDa and a pI of 6.84. RpSR-BI exhibited relatively high identities with scavenger receptors from other mollusks. For example, RpSR-BI shared 54% and 38% similarity with scavenger receptor from *Opisthorchis viverrini* (OON18808) and *Schistosoma japonicum*

(AAW26454), respectively. The phylogenetic analysis showed that SRs were split into three groups, including SR-BI, SR-A and SR-C clades, and RpSR-BI belongs to SR-BI superfamily (Fig. 1).

3.2. Tissue distribution and temporal expression profiles after bacterial challenge

The distribution of RpSR-BI transcripts in various tissues of unchallenged clams was analyzed by qRT-PCR. RpSR-BI transcripts were predominantly expressed in hepatopancreas and hemocytes, moderately in mantle and gills, and marginally expressed in muscle (Fig. 2).

The temporal expression profiles of RpSR-BI transcripts in hemocytes after bacterial challenge were shown in Fig. 3. After *V. anguillarum* challenge, the mRNA expression of RpSR-BI was significantly up-regulated and reached the maximum value (118.6-fold, $P < 0.01$) at 6 h. After that, the expression level decreased gradually, and recovered to the original level at 72 h post challenge (Fig. 3A). As concerned to *M. lutues* challenge, the expression of RpSR-BI transcripts was significantly up-regulated to 40.4-fold ($P < 0.01$), 65.2-fold ($P < 0.01$) and 58.2-fold ($P < 0.01$) of control group at 3 h, 6 h and 12 h, respectively. After a sudden decrease at 24 h, the expression of RpSR-BI transcripts was up-regulated to 47.3-fold ($P < 0.01$) compared with control group at 48 h (Fig. 3B).

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

The purified protein of rRpSR-BI was analyzed on 15% SDS-PAGE with an apparent 57 kDa band (Fig. 4, line 3), which was in accordance with the predicted molecular weight. Western blotting was performed to identify the specificity of the antibody against rRpSR-BI, and a clear reaction band representing rRpSR-BI was immuno-stained, indicating the antibody could specifically recognize rRpSR-BI (Fig. 4, line 4). Negative bands were not observed in the study (data not shown).

3.4. PAMPs binding assay

rRpSR-BI possessed binding activities to LPS, PGN and glucan, but not chitin (Fig. 5). The binding abilities of rRpSR-BI were dose-dependent. In addition, Several Gram-positive bacteria and Gram-negative bacteria were selected to test the microbe agglutination activity of rRpSR-BI. As revealed in Fig. 6, rRpSR-BI showed high agglutinate activities to *S. aureus*, *E. coli*, *V. anguillarum*, *V. alginolyticus*, *V. splendidus* and *V. harveyi* (Fig. 6).

3.5. Phagocytosis and chemotaxis assay

Phagocytosis of the hemocytes could be enhanced by rRpSR-BI. The phagocytic ability of hemocytes was 25.9% in the rRpSR-BI treatment group compared with that of only 15.0% in the control group (Fig. 7). In addition, the rRpSR-BI elicited a chemotactic response from hemocytes. Importantly, the chemotactic response in clams induced by rRpSR-BI increased 1.45-fold (5 μg/mL), 1.68-fold (10 μg/mL) compared with the migration in control solution (Fig. 8).

4. Discussion

Scavenger receptors play a crucial role in immune defense by serving as PRRs. To date, several scavenger receptors have been reported in mollusks, but the knowledge on the functions of scavenger receptor is still limited in mollusks. In the present study, a scavenger receptor was identified from manila clams, and the PAMPs recognition and binding activities, and opsonic activities were also investigated.

Like other mollusk SRs [23], RpSR-BI exhibited a wide range of tissues distribution with different levels in healthy manila clam, and mostly expressed in the hepatopancreas and hemocytes, which were
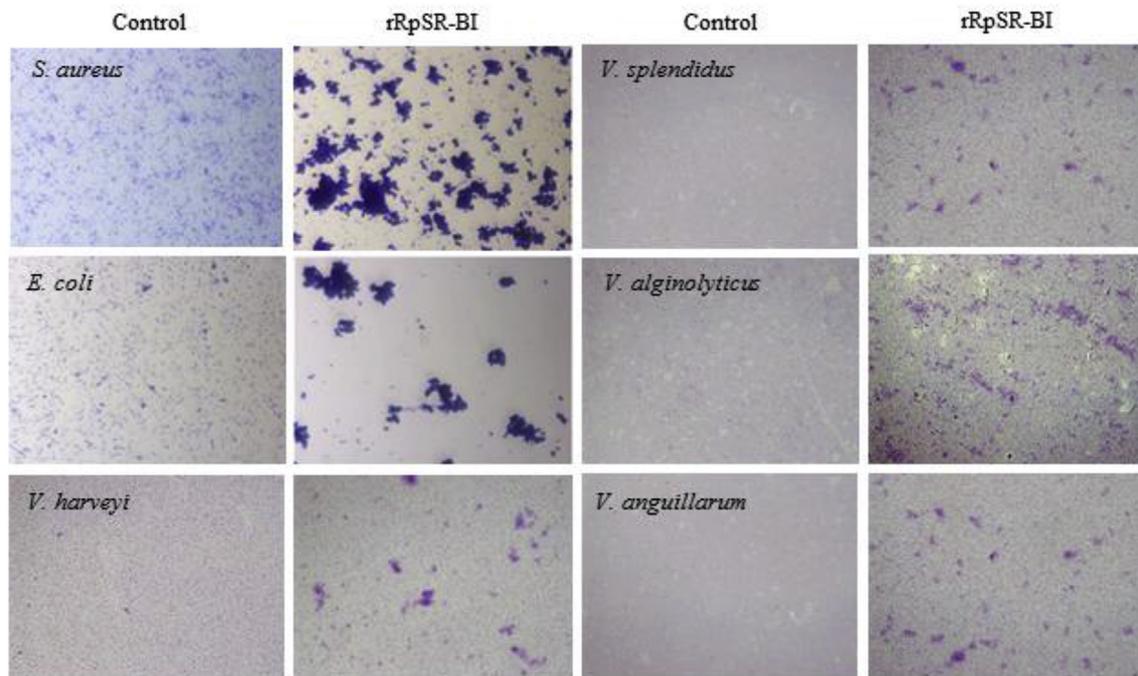
Fig. 6. The agglutination of Gram-positive bacterium (*Staphyloccocus aureus*) and Gram-negative bacteria (*Escherichia coli*, *Vibrio anguillarum*, *Vibrio alginolyticus*, *Vibrio splendidus* and *Vibrio harveyi*) induced by rRpSR-BI.
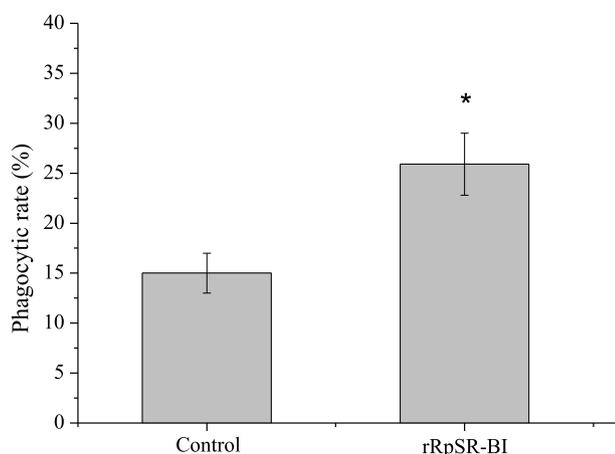

Fig. 7. Phagocytosis of hemocytes enhanced by rRpSR-BI. Phagocytosis is analyzed using an Accuri C6 flow cytometer (BD) with BD CFlow® software. The values are shown as mean ± S.D. (N = 3) (*: $P < 0.05$, **: $P < 0.01$).

considered as the main tissues involved in the innate immune defense of mollusk. Previous studies have demonstrated that SRs participated in the host immune responses to pathogenic challenge. For example, the mRNA expression of scavenger receptor could be induced by *V. anguillarum* and *S. aureus* in shrimp [11]. Likewise, the expression of scavenger receptors (OoSR-B, OoSR-I, OoSR-3, OoSR-4 and OoSR-5) transcripts was up-regulated in hemocytes of *O. ocellatus* challenged by *Listonella anguillarum* or *M. luteus*, respectively [22]. In the present study, RpSR-BI transcripts were significantly up-regulated in hemocytes after *V. anguillarum* or *M. luteus* stimulation, indicating the involvement of RpSR-BI in immune responses against invading pathogens. However, at 24 h after *M. lutues* challenge, expression of RpSR-BI transcripts decreased to 12.30-fold compared with the control group, which was perhaps influenced by variance of hemocytes amount.

Non-self recognition is the first step of immune responses against pathogens. PRPs were secreted to identify the conserved PAMPs of pathogens, which were responsible for recognizing and defending pathogenic microbes [24], thus can activate the signal pathways to synthesize immune effector proteins. Several scavenger receptors were reported for their specific binding to PAMPs in invertebrates [11,22]. For example, both OoSR-B and OoSR-I from *O. ocellatus*, which respectively belonged to class B and I scavenger receptor, could serve as PRRs to bind a broad range of PAMPs. Notably, OoSR-B was membrane receptor and located on the hemocytes membrane, while OoSR-I was secreted soluble receptor. Despite differences in their structure and localization, both of them play an important role in immune recognition [22]. In the present study, rRpSR-BI could bind several ligands, such as LPS, PGN and glucan, which were important components of Gram-negative bacteria, Gram-positive bacteria and fungi, respectively. The PAMPs-binding ability of rRpSR-BI supported that scavenger receptor perhaps took part in immune responses against bacteria and fungi, and they could serve as PRRs in the PAMPs recognition.

In the microbial agglutination assay, almost all the tested microorganisms could be agglutinated by rRpSR-BI. Similar results were also supported in shrimp [11]. The recombinant shrimp SR agglutinated bacteria in a calcium dependent manner, which could bind LPS and lipoteichoic acid [11]. These results suggest that the agglutination may be associated with its broad PAMPs recognition spectrum. The broad microbial agglutinating abilities manifested that RpSR-BI participated in immune responses against pathogenic microbes.

It is important to understand the opsonization activity of SRs on hemocytes. In the present study, rRpSR-BI could enhance the phagocytic activity of hemocytes significantly. Similar results were also reported in the kuruma shrimp, *Marsupenaeus japonicas* [25]. MjSRC could recruit and bind to β-arrestin2, and then initiate the phagocytosis of WSSV by hemocytes in a clathrin-dependent manner [25]. The phagocytic activity was supposed to restrict viral infection in invertebrates effectively. Meanwhile, the chemotactic abilities of clam hemocytes could also be prompted by rRpSR-BI. Usually, chemokines tend equally to arrest cells and to make them move, in the process of positioning target cells with spatiotemporal precision. The accumulated hemocytes carry out diverse functions including directly eliminating invading bacteria and production of cytokine [26], then causing the death of invading pathogens.
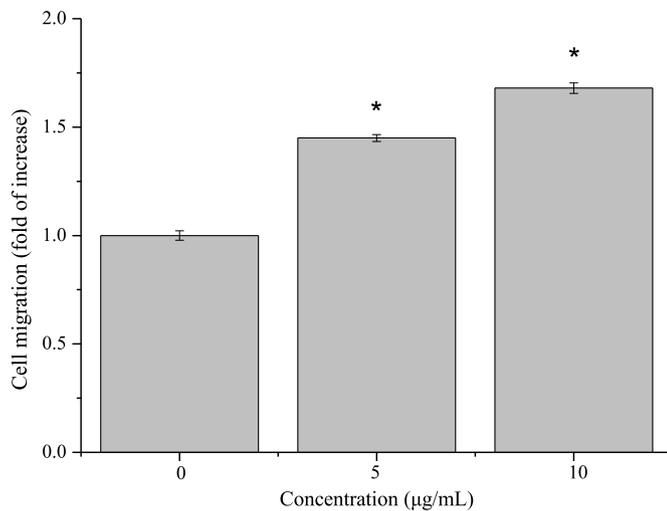
Fig. 8. Chemotactic activity of rRpSR-BI. Hemocytes from individual clams are seeded in the upper chamber and rRpSR-BI in the lower chamber. PBS (0.01 M) is used as the blank control. Cells are counted using an Accuri C6 flow cytometer (BD) with BD CFlow® software. The values are shown as mean ± S.D. (N = 6) (*: $P < 0.05$, **: $P < 0.01$).

Acknowledgments

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