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A peptidoglycan recognition protein involved in immune recognition and immune defenses in *Ruditapes philippinarum*

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ABSTRACT

Peptidoglycan recognition proteins (PGRPs) are important pattern recognition receptors in the innate immune system of invertebrates. In the study, a short PGRP (designed as RpPGRP) was identified and characterized from the manila clam *Ruditapes philippinarum*. The open reading frame of RpPGRP encoded a polypeptide of 249-amino acids with a calculated molecular mass of 27.2 kDa and an isoelectric point of 6.62. Multiple alignments and phylogenetic analysis strongly suggested that RpPGRP was a new member of the PGRP superfamily. In non-stimulated clams, RpPGRP exhibited different tissue expression pattern, and highly expressed in hepatopancreas and hemocytes. Expression of RpPGRP transcripts was significantly up-regulated in hemocytes of clams post *Vibrio anguillarum* or *Micrococcus luteus* challenge. The recombinant RpPGRP (rRpPGRP) exhibited high affinity to PGN, LPS and zymosan in a concentration-dependent manner. With a broad spectrum of bacterial binding activities, rRpPGRP exhibited strong agglutination activity to *Escherichia coli*, *Vibrio splendidus*, *V. anguillarum* and *M. luteus*. Furthermore, rRpPGRP exhibited Zn²⁺-dependent amidase activity and catalyzed the degradation of insoluble PGN. Especially, rRpPGRP exhibited significant antibacterial activity against *E. coli* and *M. luteus*. Moreover, the biofilm formation of *E. coli* could be inhibited after rRpPGRP incubation in the presence of Zn²⁺. This inhibitory effect of rRpPGRP might attribute to its amide bactericidal activity. Taken together, rRpPGRP played important roles in PGRP-mediated immune defense mechanisms, especially by recognizing antigens and eliminating bacteria.

1. Introduction

Immune recognition is one of the key steps of immune responses, and its essence is the specific recognition between pattern recognition receptors (PRRs) of host and the highly conserved structures referring of the pathogen associated molecular patterns (PAMPs) from various pathogens [1,2]. To date, numerous PRRs have been reported in invertebrates, such as peptidoglycan recognition proteins (PGRPs), C1q domain-containing (C1qDC) proteins, fibrinogen-related proteins (FREPs), galectins (GALEs), Gram-negative binding proteins (GNBPs), thioester containing proteins (TEPs), Toll-like receptors (TLRs), lipopolysaccharide, β -1, 3-glucan binding proteins (LGBPs), scavenger receptors (SRs) and NOD-like receptors (NLRs) [3–5].

PGRP was firstly identified from hemolymph and cuticle of silkworm *Bombyx mori*, which possessed affinity for PGN and the ability to trigger the prophenoloxidase cascade [6]. Subsequently, PGRPs have been identified in other invertebrates [7–9], and most of the invertebrate PGRP exhibit the ability of N-acetylmuramoyl-L-alanine amidases (NAMLAA) to cleave the lactylamide bond between muramic acid in bacterial PGN [10,11]. According to the predicted structures and molecular weight, identified PGRPs could be divided into three classes: short extracellular PGRPs (PGRP-S), intermediate PGRPs (PGRP-I), and long PGRPs (PGRP-L) [12]. The diversity of PGRPs in both category and structure suggests their multi-functions in innate immunity, such as functioning as PRRs to recognize and bind bacterial PGN [13,14], exhibiting bactericidal activity [15,16], and functioning

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as opsonins to induce agglutination or phagocytosis [17,18].

Ruditapes philippinarum is an economic species widely spread over many countries. Recent mass mortalities in manila clams have been attributed to pathogen invasion and environmental deterioration. Therefore, it is urgently necessary to characterize immune-related molecules for diseases control and the healthy management of clam aquaculture. Presently, studies on PGRP of *R. philippinarum* regarding antigen recognition and immune responses were limited, and the mechanism through which *R. philippinarum* defended the invasion of external pathogens remained unclear. In the present study, a short-type PGRP was identified from clam *R. philippinarum* (designed as RpPGRP), and its PAMPs binding ability, agglutination activity towards different microbes, amidase activity and the inhibitory activities against pathogenic microorganism were also investigated to better understand the immune responses of clams against pathogens invasion.

2. Materials and methods

2.1. Clams and bacterial challenge

Adult manila clams *R. philippinarum* with average shell length of 30 mm were collected from a local farm and maintained in aerated seawater at 20–22 °C for a week before processing. The clams were randomly distributed into nine tanks with 50 L capacity, each containing 50 clams. Three tanks served as the control, while the other six tanks were immersed with *V. anguillarum* or *M. luteus* at a final concentration of 1×10^7 CFU/mL, respectively. Hemolymphs of 6 individuals were randomly sampled from each treatment at 0, 6, 12, 24, 48 and 72 h post bacterial challenge. Meanwhile, five 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 RpPGRP transcript.

2.2. RNA extraction, cDNA synthesis and gene cloning

Total RNA was extracted from hemocytes, mantle, gills, hepatopancreas and adductor muscle using TRIzol reagent (Invitrogen, USA), and cDNA synthesis was performed according to M-MLV RT Usage information (Promega, USA). The reaction of reverse transcriptions was incubated at 42 °C for 1 h, and terminated by heating at 95 °C for 5 min.

A short PGRP was identified through large scale EST sequencing of the cDNA library constructed from manila clam [19]. Nested-PCR was performed with P1, P2 as forward primers (Table 1) and oligo (d_T) as reverse primer to amplify the 3' end of RpPGRP. The full-length cDNA of RpPGRP was 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

Table 1
Primers used in the present study.

Primer	Sequence (5'-3')	Sequence information
P1	CTGGGTCTGATGTCCCTTGC	3' RACE primer
P2	AAAGTGAATGTTCCGGCTAGAGTA	3' RACE primer
P3	AGTGAATGTTCCGGCTAGAGTAAGGG	Real-time PCR
P4	ATCACTCCATCCTCGACTGTCCATA	Real-time PCR
P5	CGCACTTCTCAGCCATCAT	β -actin primer
P6	GCAGCCGTCTCCATTTCTTGTTT	β -actin primer
P7	TCTGATGTCCCTTGCACTTCAG	Recombinant primer
P8	TCATCCTGGACATTCTGTGTTA	Recombinant primer
d_T	GGCCAGCGTCTGACTAGTACT ₁₇	Oligo (d_T)-adaptor

Simple Modular Architecture Research Tool version 4.0 [20]. 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.

2.4. The spatial and temporal expression patterns of RpPGRP mRNA

The spatial and temporal expression profiles of RpPGRP mRNA were performed on a 7500 Fast Real Time PCR system (Applied Biosystems, USA). Gene-specific primers (P3 and P4, Table 1) and β -actin primers (P5 and P6, Table 1) were used to amplify the fragments of RpPGRP and internal control, respectively. The purity of amplification products was evaluated by dissociation curve analysis. The $2^{-\Delta\Delta CT}$ method was used to analyze the relative expression level of RpPGRP [21]. All data were given in terms of relative mRNA expressed as mean \pm S.D. (N = 6). Statistical analysis were 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 RpPGRP

pEASY-blunt E1 expression kit (Transgen Biotech, Beijing, China) were used to express the recombinant protein RpPGRP according to the instructions. Firstly, the fragment encoding mature peptide of RpPGRP was amplified with a pair of specific primers (P7 and P8), and cloned into pEASY-blunt E1 vector. The recombinant plasmid was then expressed into *Escherichia coli* BL21 (DE3), and examined by 15% SDS-PAGE. The recombinant RpPGRP (rRpPGRP) was purified with a Ni²⁺ chelating Sepharose column, and then dialyzed against gradient urea-TBS glycerol buffer (10% glycerol, 2 mmol/L reduced glutathione, 0.2 mmol/L oxidized glutathione, 50 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 8.0) as described previously [22]. The concentration of renatured proteins were then measured by BCA method [23].

2.6. Preparation of antibodies and western blotting analysis

6-week-old mice were immunized with rRpPGRP to prepare polyclonal antibody. The mice were intraperitoneally injected with 100 μ g rRpPGRP with complete Freund's adjuvant (Sigma, USA) each, and then inoculated with 100 μ g rRpPGRP with incomplete Freund's adjuvant (Sigma, USA) two weeks later after the first immunization. The third and fourth injections were given by tail vein with 100 μ g rRpPGRP at a one-week interval. Seven days after the fourth injection the mice were sacrificed to collect immunized serum [24].

The samples containing rRpPGRP were electrophoretically transferred onto nitrocellulose membrane at 300 mA for 1 h after SDS-PAGE. The blotted membrane was blocked with 3% BSA in PBST (PBS containing 0.05% Tween-20) at 37 °C for 1 h, and incubated with 1:1000 diluted polyclonal antibody against rRpPGRP for 1 h at 37 °C, followed by three consecutive 5 min washed with PBST. Goat-anti-mouse IgG-alkaline phosphatase conjugate (Southern Biotech, USA) as secondary antibody was incubated with the membrane at 37 °C for 1 h. After washed in PBST for three times, the membrane was developed with NBT/BCIP visualization solution for 5 min. Pre-immunized serum was used instead of immunized serum as negative control.

2.7. PAMPs binding assay of rRpPGRP

Enzyme linked immunosorbent assay (ELISA) was performed to examine the PAMPs binding ability of rRpPGRP as described previously [25]. The 96-well microtiter plate coated overnight at 4 °C with LPS, PGN, glucan and zymosan (20 μ g/well, in 50 mM carbonate-bicarbonate buffer) was blocked with 3% BSA in PBS at 37 °C for 1 h, and then washed with PBST for three times. Several concentrations of rRpPGRP

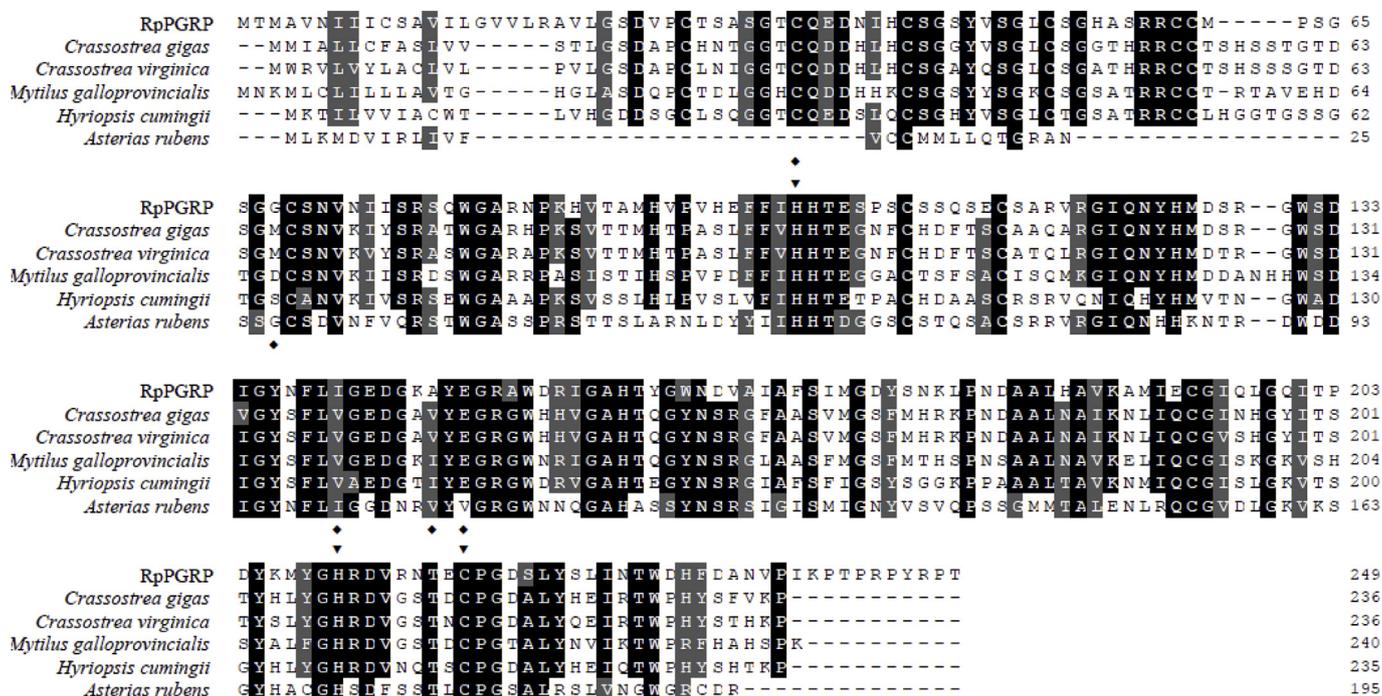


Fig. 1. Multiple alignments of RpPGRP with PGRPs in other animals. The protein sequences were listed as followed: *Crassostrea gigas* (XP_011422762), *Crassostrea virginica* (XP_022289085), *Mytilus galloprovincialis* (AJQ21541), *Hyriopsis cumingii* (AGU62945), *Asterias rubens* (ABB04459). Conserved amino acid residues were shaded in dark, and similar amino acids were shaded in gray. Zn²⁺ binding sites and amidase catalytic sites were marked with triangle (▼) and rhombus (◆), respectively.

were added in the presence of 5 mM CaCl₂ and 0.1 mg/mL BSA and incubated at 18 °C for 3 h. Following a further washing, 100 μL of 1:1000 diluted polyclonal antibody against rRpPGRP was added into the plate and incubated at 37 °C for 1 h. After three washes, these wells were then incubated in a goat-anti-mouse IgG-alkaline phosphatase conjugated with secondary antibody (Southern Biotech, USA) 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. Pre-immunized serum was used as negative control instead of immunized serum. Each experiment was performed in triplicate.

2.8. Bacterial agglutination assay

The bacterial agglutination assay was analyzed according to Yang et al. with some modification [14]. Briefly, *V. splendidus*, *V. anguillarum*, *E. coli* and *M. luteus* were labeled with crystal violet staining solution (Beyotime, Beijing, China), and re-suspended with TBS-Ca buffer (50 mmol/L Tris-HCl, 50 mmol/L NaCl, 10 mmol/L CaCl₂, pH 7.5) at 2.0 × 10⁷ cells/mL. 25 μL of rRpPGRP solution was added to 10 μL bacteria suspension [26]. After incubation at room temperature for 45 min, the samples were observed under a light microscope. The bacteria in TBS-Ca buffer without rRpPGRP were used as control.

2.9. Antimicrobial activity assay

E. coli and *M. luteus* were grown in Luria-Bertani broth and harvested at the logarithmic phase of growth. The bacterial cells were washed three times with sodium phosphate buffer (pH 7.4) and diluted to 1.0 × 10⁷ CFU/mL. The bacterial suspension (100 μL) was added to an equal volume of rRpPGRP (at a final concentration of 100 μg/mL) with 10 mM ZnCl₂ in TBS or not, respectively. Meanwhile cell suspensions without rRpPGRP were prepared as a negative control. Each sample was incubated with aeration at 220 rpm and the OD at 600 nm

(OD₆₀₀) was measured every 1 h.

2.10. Cell motility assay

Cell motility was examined on the soft-agar plates (0.3% agar). After *V. splendidus*, *V. anguillarum*, *E. coli* and *M. luteus* were cultured at the stationary phase, the bacteria mixed with rRpPGRP (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.

2.11. Biofilm formation

Attached biofilm formation was assayed in 96-well polystyrene plates (Corning Costar, USA) with crystal violet staining [27]. Briefly, *E. coli* MG1655 was incubated with rRpPGRP (final concentration of 1 μg/mL or 10 μg/mL) in the presence of Zn²⁺ for 8 h. To remove growth effects, we normalized biofilm formation by dividing total biofilm by the maximal bacterial growth as measured by turbidity at 620 nm. Ten replicate wells were repeated from two cultures independently.

2.12. Amidase activity assay

The relative enzymatic activity of rRpPGRP toward PGN was analyzed according to the method of Mellroth with modifications [16]. Shortly, insoluble PGN (from *S. aureus*, 1 mg/mL) was incubated with rRpPGRP (50 μg/mL) in HEPES buffer (20 mM, pH 7.2, 150 mM NaCl), HEPES-ZnCl₂ buffer (20 mM, pH 7.2, 150 mM NaCl, 10 mM ZnCl₂) and HEPES-ZnCl₂-EDTA buffer (20 mM, pH 7.2, 150 mM NaCl, 10 mM ZnCl₂, 10 mM EDTA). PGN (1 mg/mL) in HEPES buffer without rRpPGRP addition was used as control. The optical density (OD) at 540 nm was recorded every 15 min during a 120 min period.

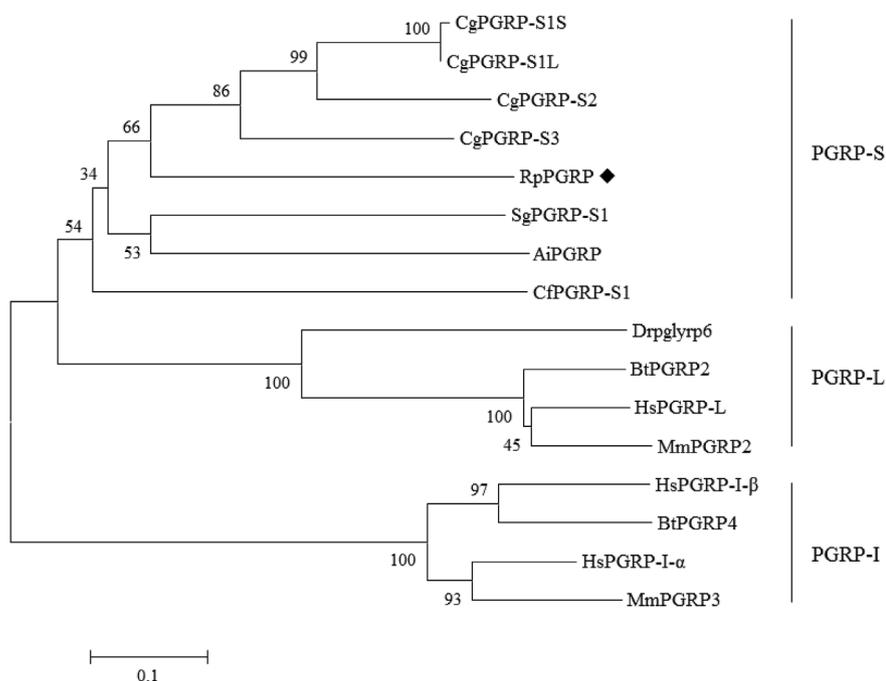


Fig. 2. A phylogenetic tree constructed by the neighbor-joining method using the Mega 4.1 program. The protein sequences used for the phylogenetic tree were listed as followed: CgPGRP-S1S (*C. gigas*, BAG31896), CgPGRP-S1L (*C. gigas*, BAG31897), CgPGRP-S2 (*C. gigas*, BAG31898), CgPGRP-S3 (*C. gigas*, BAG31899), SgPGRP-S1 (*Solen grandis*, JN642118), AiPGRP (*Argopecten irradians*, AAR92030), CfPGRP-S1 (*Chlamys farreri*, AAY53765), Drpglyrp6 (*Danio rerio*, ABE01406), BtPGRP2 (*Bos taurus*, XP_588006), BtPGRP4 (*B. taurus*, XP_874055), MmPGRP2 (*M. musculus*, NP_067294), MmPGRP3 (*M. musculus*, NP_997130), HsPGRP-L (*Homo sapiens*, AAL05629), HsPGRP-I- β (*H. sapiens*, Q96LB8) and HsPGRP-I- α (*H. sapiens*, NP_443123).

3. Results

3.1. cDNA cloning and sequence analysis of RpPGRP

The full-length cDNA of RpPGRP was deposited in GenBank database under the accession no. MH559337. The open reading frame (ORF) was of 750 bp encoding a polypeptide of 249 amino acids with an isoelectric point of 6.62 and predicted molecular weight of 27.2 kDa (Fig. S1). A typical signal peptide of 24 amino acid residues was identified in the N-terminus of RpPGRP by SignalP software. Blast analysis showed that RpPGRP exhibited high sequence identities with PGRPs from *Solen grandis* (44% identity, AEW43446) and *Crassostrea gigas* (55% identity, EKC26199) (Fig. 1). In RpPGRP, three amino acids (H¹⁰¹, H²¹⁰ and C²¹⁸) of the Zn²⁺ binding sites and five amino acids (H¹⁰¹, Y¹³⁶, H²¹⁰, T²¹⁶ and C²¹⁸) of amidase catalytic sites were well conserved (Fig. 1). A phylogenetic tree was constructed using neighbor-joining method with 1000 bootstrap test based on the multiple alignments of RpPGRP and PGRPs from other animals. In the phylogenetic tree, manila clam PGRP was clustered together with mollusks short-type PGRPs, such as *C. gigas* and *Crassostrea virginica* (Fig. 2).

3.2. The spatial and temporal mRNA expression profile of RpPGRP

The mRNA transcripts of RpPGRP were found to be ubiquitously expressed in all tissues detected. As revealed in Fig. 3, RpPGRP mRNA was dominantly expressed in hepatopancreas and hemocytes, moderately expressed in gills and mantle, and marginally expressed in muscle.

The temporal expression of RpPGRP mRNA in hemocytes was monitored after the manila clams were stimulated by *V. anguillarum* and *M. luteus*, respectively. After *V. anguillarum* challenge, the expression of RpPGRP transcripts was significantly up-regulated (6.46-fold, $P < 0.01$) at 6 h post-injection compared with the control group. After that, the expression level decreased drastically at 12 h ($P < 0.01$), 24 h ($P < 0.01$) and 48 h ($P < 0.01$). As time progressed, the expression level recovered to the original level at 72 h (Fig. 4A). As concerned to *M. luteus* stimulation, more acute up-regulation of RpPGRP transcripts was observed at 3 h (15.7-fold, $P < 0.01$), 6 h (4.2 fold, $P < 0.05$), 12 h (10.3-fold, $P < 0.01$) and 24 h (5.3-fold, $P < 0.05$) post challenge. After a sudden decrease at 48 h ($P < 0.05$), the expression level was recovered to the original level at 72 h compared with

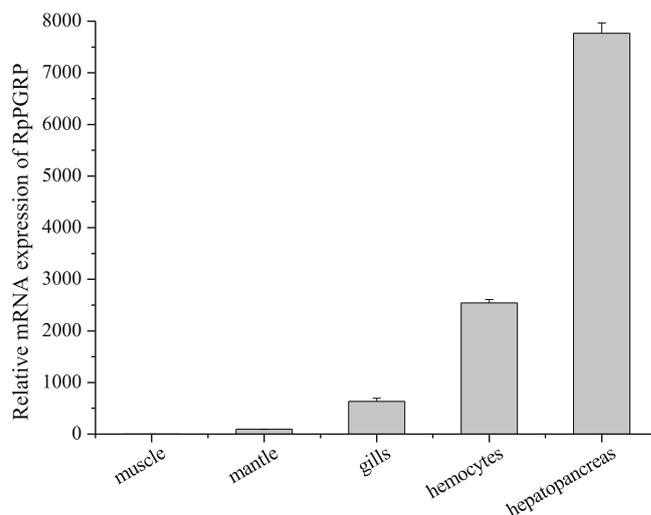


Fig. 3. The expression level of RpPGRP mRNA in different tissues. Transcript levels in mantle, gills, hemocytes, and hepatopancreas were normalized to that of muscle. Vertical bars represented the mean \pm S.D. (N = 6).

the control group (Fig. 4B).

3.3. Preparation of rRpPGRP and western blotting analysis

The whole cell lysate of *E. coli* BL21 (DE3) with pEASY-E1-RpPGRP was separated by SDS-PAGE after IPTG induction. A distinct band with molecular weight of about 25 kDa for RpPGRP was revealed, which was consistent with their predicted molecular mass (Fig. 5, lanes 2). The purified protein of rRpPGRP was revealed after purification by a Ni²⁺ chelating Sepharose column (Fig. 5, lane 3) and the concentration of purified rRpPGRP was 308 $\mu\text{g mL}^{-1}$ as determined by BCA method.

Western blotting was performed to identify the specificity of the antibody against rRpPGRP, and a clear reaction band representing rRpPGRP was immuno-stained, indicating the antibody could specifically recognize rRpPGRP (Fig. 5, lane 4). No reaction band was visible in negative control (data not show).

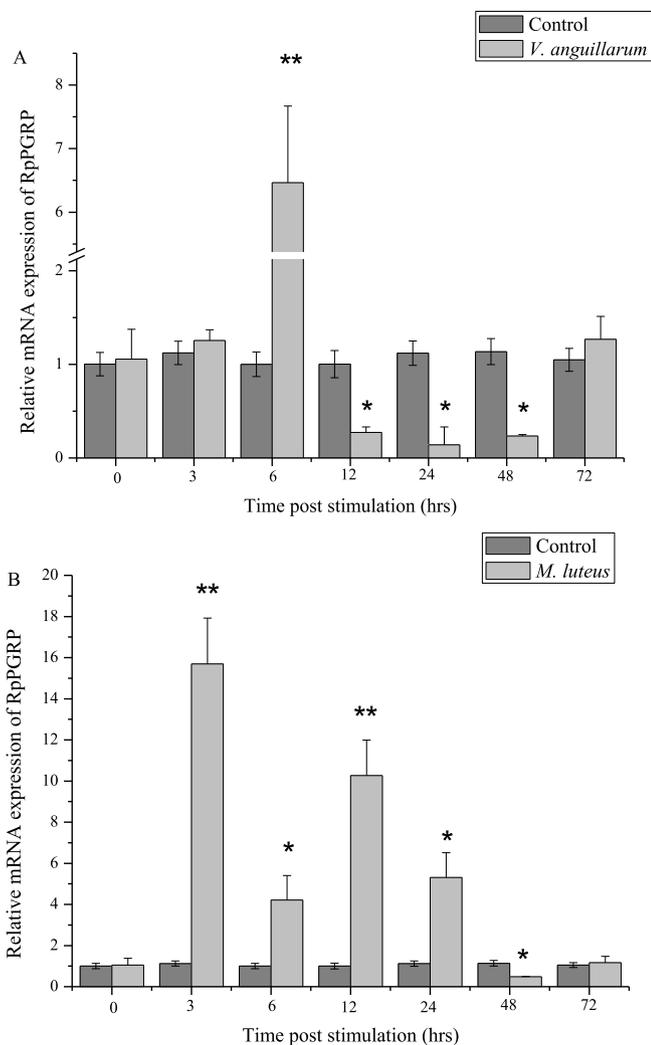


Fig. 4. Temporal expression of RpPGRP mRNA in hemocytes after *V. anguillarum* (A) and *M. luteus* (B) challenge. The values were shown as mean ± S.D. (N = 6) (*: P < 0.05, **: P < 0.01).

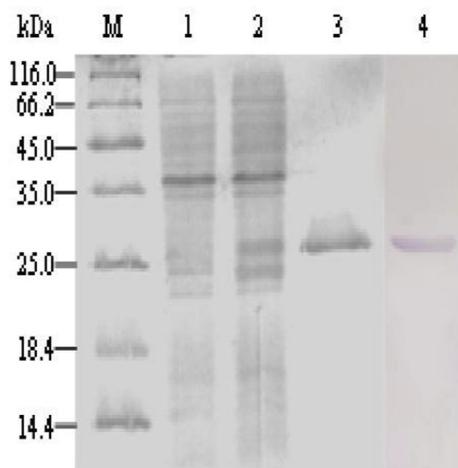


Fig. 5. SDS-PAGE and western-blot analysis of rRpPGRP. Lane M: Marker; lane 1: rRpPGRP in *E. coli* without induction; lane 2: induced rRpPGRP; lane 3: purified rRpPGRP; lane 4: immunostained with polyclonal antibody against rRpPGRP.

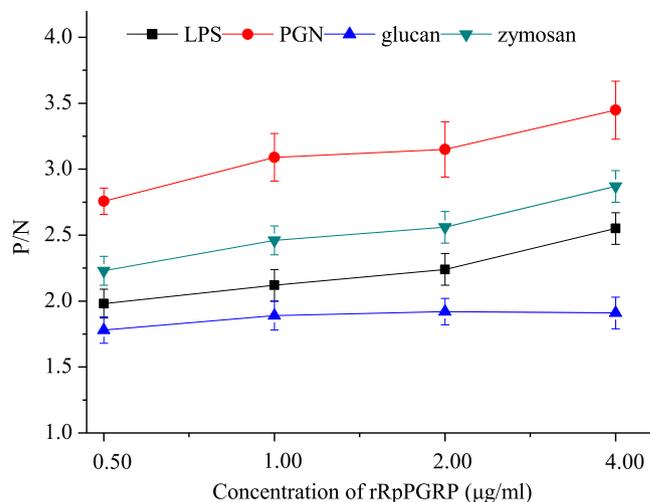


Fig. 6. PAMP binding ability of rRpPGRP. Plates were coated with LPS, PGN, glucan and zymosan, which were washed and incubated with several concentrations of rRpPGRP and PBS (data not shown) in the presence of 0.1 mg/mL BSA at 18 °C for 3 h. After incubated with polyclonal antiserum, the interaction was detected with goat-anti-mouse Ig-alkaline phosphatase conjugate at 405 nm. Samples with (sample – blank)/(negative – blank) > 2.1 were considered positive. Results were representative of average three such experiments.

3.4. PAMPs binding and bacterial agglutination activities of rRpPGRP

ELISA was performed to detect the binding activity of rRpPGRP towards LPS, PGN, glucan and zymosan. A dose-dependent binding activity of rRpPGRP towards LPS, PGN and zymosan was observed *in vitro*. However, no binding activity of rRpPGRP to glucan was detected even if at high concentration (Fig. 6).

Gram-negative bacteria (*V. anguillarum*, *V. splendidus*, *E. coli*) and Gram-positive bacteria (*M. luteus*) labeled by crystal violet were used to test agglutination activities of rRpPGRP. As shown in Fig. 7, all tested bacteria could be significantly agglutinated by rRpPGRP. No agglutination was observed in control groups (Fig. 7). Cell motility was examined as described previously on low-salt, soft-agar plates (1% tryptone, 0.25% NaCl and 0.3% agar), where the motility of all tested bacteria were limited after incubated with rRpPGRP (Fig. 8).

3.5. Amidase and antibacterial activity assay

Antimicrobial activities of rRpPGRP against Gram-negative bacteria *E. coli* and Gram-positive bacteria *M. luteus* were investigated. The rRpPGRP could strongly suppress the growth of *E. coli* (Fig. 9A) and *M. luteus* (Fig. 9B) in the presence of Zn²⁺, respectively. However, the inhibition effect of rRpPGRP on the growth of these two microbes was remarkably weakened in the absence of Zn²⁺ (Fig. 9A and B). Meanwhile, the incubation of rRpPGRP resulted in less biofilm dispersal of *E. coli* MG1655 on polystyrene surfaces, suggesting that rRpPGRP could dramatically reduce the biofilm formation in a concentration-dependent manner (Fig. 10).

Relative amidase activity of rRpPGRP towards PGN was measured by recording the decrease of OD at 540 nm. In the presence of ZnCl₂, rRpPGRP showed high degrading activity towards PGN. The OD was dropped from 0.268 to 0.142 within 120 min in the ZnCl₂ group. rRpPGRP in the non-Zn²⁺ group and Zn²⁺-EDTA group showed much lower degrading activity towards PGN compared with the ZnCl₂ group (Fig. 11). These results indicated amidase activity of rRpPGRP was zinc-dependent.

4. Discussion

There are accumulative evidences supporting the significant roles of

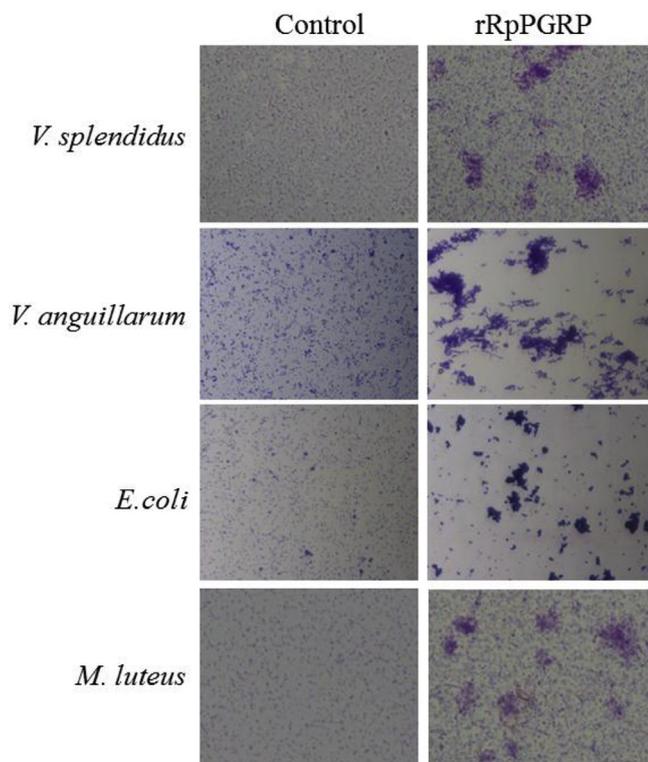


Fig. 7. Agglutination of bacteria by rRpPGRP. Agglutinations towards crystal violet-stained *E. coli*, *V. anguillarum*, *V. splendidus* and *M. luteus* were incubated with rRpPGRP, and then incubated at room temperature for 45 min and cells were then observed under light microscopy. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

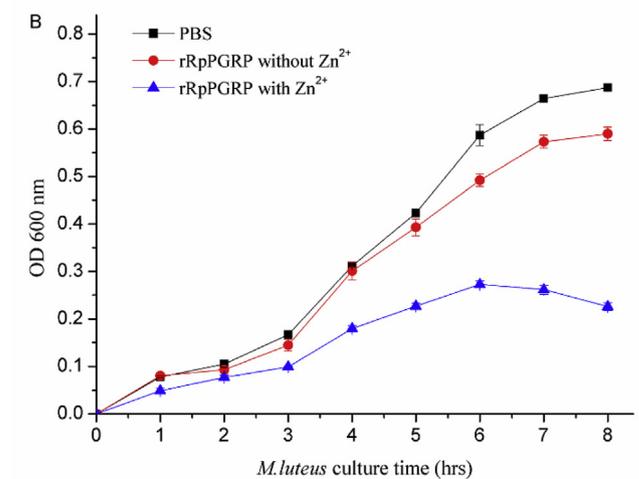
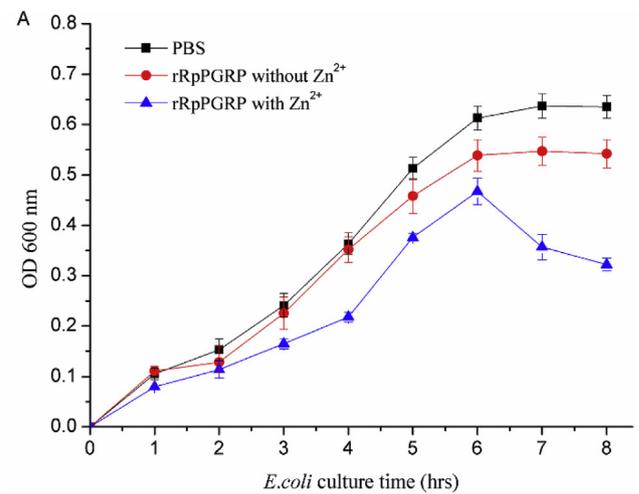


Fig. 9. Antibacterial activity of rRpPGRP. Growth suppressive tests of rRpPGRP against *E. coli* (A) and *M. luteus* (B) were measured at OD₆₀₀ every 1 h after starting the cultures. The data were the average ± S.D. of three independent cultures.

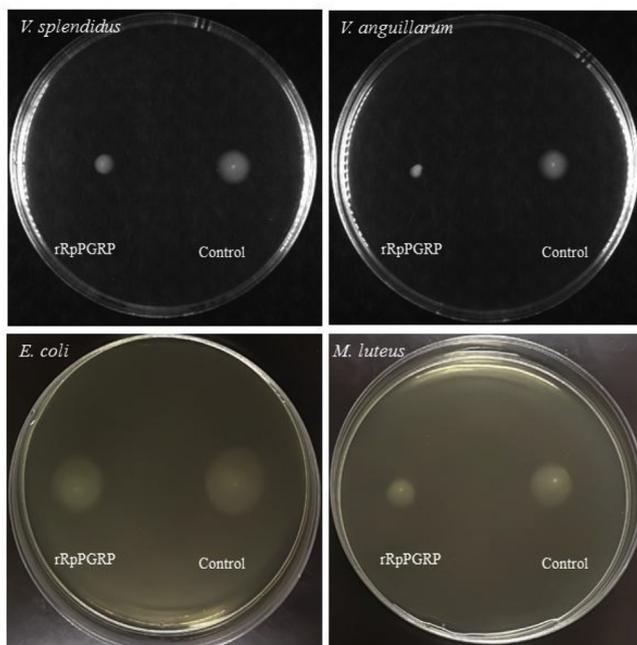


Fig. 8. Swimming motility of Gram-negative (*V. splendidus*, *V. anguillarum*, *E. coli*) and Gram-positive bacteria (*M. luteus*) treated with rRpPGRP. Motility halos were quantified after 16 h using at least three plates for each condition.

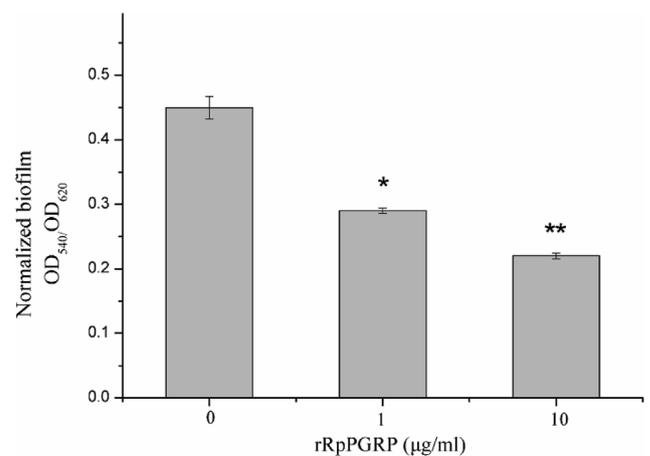


Fig. 10. Biofilm formation of *E. coli* MG1655 treated with rRpPGRP. Normalized biofilm formation (total biofilm/growth) was tested in 96-well polystyrene plates after treated with rRpPGRP. Data were the average of 10 replicate wells from two independent cultures. The values were shown as mean ± S.D.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

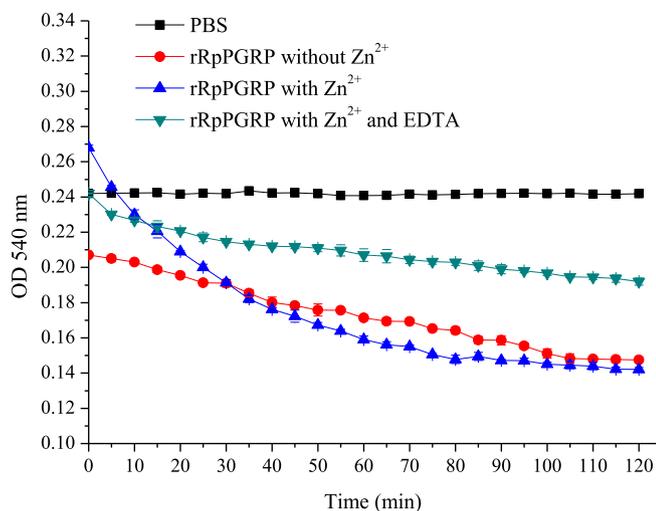


Fig. 11. Amidase activity of rRpPGRP. rRpPGRP was incubated with peptidoglycan, and the optical density (OD) was recorded at 540 nm every 15 min during a 120 min period. The enzyme activity was recorded as a decrease in optical density.

PGRPs in regulating the diverse immune responses, such as pathogen recognition [6,28,29], activation of proPO system [30], induction of antimicrobial peptide [31] and degradation of PGN [10,17,32]. In the present study, a short PGRP was identified from *R. philippinarum*, and the transcriptional responses to pathogen infection, the antibacterial activity and opsonic activity were also investigated.

Multiple alignments revealed that RpPGRP shared highly similarities with other PGRPs and kept a close evolutionary relationship with PGRPs from other mollusks. With the conserved structural characteristics such as PGRP domain, conserved cysteines, and Zn²⁺-binding sites, RpPGRP was suggested as a typical short PGRP with amidase activities.

Unveiling the mRNA distribution pattern of PGRPs in different tissues would be beneficial for dissecting their potential functions. Most PGRPs transcripts were widely expressed in immune organs, such as hemolymph [33] and gills [7]. The ubiquitous distribution of RpPGRP transcripts suggested its important roles in immune defense against pathogenic microorganism. Notably, RpPGRP transcripts were dominantly expressed in the tissues of hepatopancreas and hemocytes. The tissue expression patterns suggested that RpPGRP probably served as an effective peptide involving in the innate immune responses [7]. In the present study, the expression of RpPGRP transcripts could be induced by Gram-positive and Gram-negative bacteria challenge. Similarly, the expressions of PGRPs transcripts in hemocytes were significantly up-regulated after LPS or PGN challenge in razor clams [7]. These results suggest that RpPGRP was an inducible acute-phase protein that might be involved in the immune recognition [7,13,33].

Most members of PGRP superfamily could recognize and bind PAMPs [12,34]. Similar to PGRP from *Chlamys farreri* [13,34], rRpPGRP also display immuno-recognition activity towards PGN and LPS *in vitro*. Taking these results into account, rRpPGRP could possibly recognize both Gram-negative and Gram-positive bacteria, and then trigger the corresponding downstream reactions. These results indicated RpPGRP might serve as a multi-specific PRR involved in the immune recognition toward different invading microbes.

PGRPs usually have amidase activity to degrade PGN by hydrolyzing the amide bond that linked the peptide units to the muramic acid residues of the glycan strands [11,15], which will prevent excessive activation of the immune system by bacteria [16]. In the present study, Zn²⁺ binding sites and amidase catalytic sites were both well conserved in RpPGRP. Notably, significant degradation of PGN was detected after incubated with rRpPGRP in the presence of Zn²⁺, consistent with the

amidase activities of scallop [13] and razor clam [35].

Bacterial agglutination plays a role in infection control by concentrating pathogens or preventing their propagation in the host [36]. In this study, rRpPGRP exhibited obvious agglutination against Gram-positive and Gram-negative bacteria (e.g. *E. coli* and *V. anguillarum*) *in vitro*. Similar result was also supported by Masao Iizuka et al., where CgPGRP-S1S from Pacific oyster exhibited agglutination activity against *E. coli* cells [37]. The agglutination or antibacterial activities of rRpPGRP were also supported by swimming motility assay, which perhaps was performed by the bind between rRpPGRP and PAMPs [13]. These results indicated rRpPGRP could serve as a significant opsonin against invading bacteria.

Bactericidal activity of PGRPs was also performed as anti-bacterial proteins [13]. For instance, some PGRPs could target the bacteria cell wall without hydrolyzing PGN or permeability the cytoplasmic membrane [38,39]. Similar to other bactericidal PGRPs, rRpPGRP could strongly inhibit the growth of both Gram-negative bacteria *E. coli* and Gram-positive bacteria *M. luteus* in the presence of Zn²⁺. The result was consistent with that in *Drosophila* [11] and zebrafish [32], which supported that PGRPs could enzymatically degrade PGN on the cell wall and then serve as a scavenger participating in bacterial lysis. Notably, the biofilm formation of *E. coli* was significantly depressed by rRpPGRP even at low concentrations. Likewise, the biofilm formation was decreased by the defense peptide LL-37 at the very low and physiologically meaningful concentrations far below those required to kill or inhibit bacterial growth [40].

In conclusion, we describe the identification of RpPGRP and that its expression is influenced by invading pathogens. In addition, it is confirmed that the RpPGRP promotes the agglutination of pathogens, mediated antigen recognition and perform antibacterial activities against microbial pathogens. This study provides a fundamental basis for understanding the functional characteristics of RpPGRP and its role in the host immune defense mechanism of manila clam.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fsi.2019.03.017>.

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