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Characterization of gene expression profiles and functional analysis of peptidoglycan recognition protein 2 from rock bream (*Oplegnathus fasciatus*)

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ABSTRACT

Peptidoglycan recognition protein 2 (PGRP2) is a Zn^{2+}-dependent peptidase that plays important roles in binding to microbial components of the cell membrane, inducing phagocytosis and antimicrobial activity. Rock bream (*Oplegnathus fasciatus*) PGRP2 (RbPGRP2) was identified in the intestine by next generation sequencing (NGS) analysis. The open reading frame (ORF) the RbPGRP2 cDNA (470 amino acid residues) contains a peptidoglycan recognition protein domain (residues 300 to 446). Alignment analysis revealed that RbPGRP2 shares 37.6–53.5% overall sequence identity with the PGRP2s of other species. Phylogenetic analysis revealed that RbPGRP2 clustered together with PGRP2s from teleosts. In healthy rock bream, RbPGRP2 was found to be ubiquitously expressed in all of the examined tissues, especially in the liver. RbPGRP2 expression was significantly upregulated in all of the examined tissues of rock bream after infection with *Edwardsiella piscicida*, *Streptococcus iniae* and red sea bream iridovirus (RSIV) compared with the control. Purified rRbPGRP2 interactions with bacteria and inhibited the growth of bacteria in the presence of Zn^{2+}. These results indicate that RbPGRP2 plays an important role in the innate immune response against bacterial infection.

1. Introduction

The innate immune system is the first line of defence against pathogens in most multicellular organisms. These employ pattern recognition receptors (PRRs) to recognize conserved microorganism structures or pathogen-associated molecular patterns (PAMPs) in order to detect pathogens. Upon PAMPs engagement, PRRs notify the host of the presence of the infection and then it activates the appropriate intracellular signalling pathways to trigger an antimicrobial response [1].

Peptidoglycan (PGN) is an essential component of the bacterial cell wall in almost all bacteria that contributes to its mechanical strength and the maintenance of a defined cell shape. It is intimately involved in the processes of cell growth and cell division [2,3]. PGN induces the release of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α), interleukin-1 (IL-1) and IL-6 in humans [4,5]. PGN consists of glycan strands connected by *N*-acetylglucosamine and *N*-acetylmuramic acid residues linked by β(1 → 4) glyosidic bonds [6].

Peptidoglycan recognition protein 2 (PGRP2), also called PGRP-L, PGLYRP2 and *N*-acetylmuramoyl-L-alanine amidase, is a Zn^{2+}-dependent peptidase that hydrolyses the link between *N*-acetylmuramoyl residues and L-alanine amino acid residues in PGN [7]. In mammals, PGRP2 is constitutively produced in the liver and secreted into the blood [8,9]. It has been shown that neutrophilic and eosinophilic granulocytes contain NAMLAA but not monocytes in humans [10]. The PGRP2 plasma level has been reported to be a biomarker that can be used to differentiate between septic and non-infected patients [11]. PGRP2 has also been identified in bacteria as well as in animal models [12–14]. In *Escherichia coli*, amidases have been shown to act in the cleavage of the septum shared by daughter cells to allow for cell separation [12]. In addition, the inactivation of the PGRP2 gene in *Vibrio anguillarum* resulted in a marked increase of oxidative stress and susceptibility to organic acids [14]. Thus, amidase activity plays an important role in the survival of the pathogen and the host.

To date, PGRP2 has been identified in many fish species including the large yellow croaker (*Pseudosciaena crocea*), red drum (*Sciaenops ocellatus*) and turbot (*Scophthalmus maximus* L.) [15–17], but little is

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known about its functions in fish. In the large yellow croaker, maternally derived PGRP2 mRNA is highly expressed in the unfertilized eggs but shows low expression throughout embryonic development and the yolk-sac larval stage [15]. Red drum PGRP2 has been shown to be able to recognize and bind to bacteria and inhibit their proliferation and infection [16].

The rock bream (*Oplegnathus fasciatus*) is one of the most popular and economically important fish species farmed in South Korea. In recent years, however, rock bream aquaculture has experienced an explosion of bacterial and viral diseases that have caused considerable economic loss in South Korea [18].

Here, we report the first identification and molecular characterization of PGRP2 in rock bream (RbPGRP2). RbPGRP2 mRNA expression was also analysed in various tissues after pathogen challenge. Furthermore, RbPGRP2 was produced as a recombinant protein (rRbPGRP2) using an *E. coli* expression system and we used it to analyse its immunological function. These results will contribute to the knowledge on PGRP2 immunological function in innate immunity.

2. Materials and methods

2.1. Sequence and phylogenetic analysis of RbPGRP2

An open reading frame (ORF) containing the sequence of RbPGRP2 was obtained from the intestines of a rock bream by next generation sequencing (NGS) analysis [19]. Sequencing was performed to confirm the integrity of the cDNA sequence. The nucleotide sequences and predicted amino acid sequences of RbPGRP2 were analysed using the program BLAST of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast). The protein structure was predicted by the Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/). Multiple alignments were performed with the DNAMAN program version 10 (Lynnon Biosoft, Canada). A phylogenetic tree was constructed with Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 using the neighbour-joining method. Support for each node was derived from 2000 bootstrap replicates.

2.2. Quantitative real-time PCR analysis of RbPGRP2

2.2.1. Experimental animals and challenge experiments

Healthy rock bream (weight: 140.4 ± 39.7 g, body length: 19.0 ± 1.8 cm) were obtained from a local market (Tongyeong, Republic of Korea) and maintained at 20–22 °C in aerated seawater until the experiment ended.

For the bacterial challenge experiment, *Streptococcus iniae* FP5228 and *Edwardsiella piscicida* FSW910410 were obtained from the Fish Pathology Division of the National Institute of Fisheries Science (Busan, Republic of Korea). For viral infection, iridovirus was isolated from diseased fish from a farm in the Republic of Korea.

2.2.2. Expression of RbPGRP2 in various tissues

Tissue samples of the trunk kidney, head kidney, gill, spleen, heart, liver, brain, stomach, intestine, skin, muscle and whole blood were obtained from three healthy rock bream individuals for tissue mRNA expression profile detection. The peripheral blood leukocytes (PBLs) and red blood cells (RBC) were separated by a density-gradient centrifugation method using a 53% Percoll (Sigma-Aldrich, USA) gradient on whole blood. All samples were stored immediately at −80 °C until total RNA isolation. Total RNA was extracted using TRIzol reagent (Invitrogen, USA), and cDNA synthesis was carried out using the PrimeScript™ 1st strand cDNA Synthesis Kit (TaKaRa, Japan) according to the manufacturer's instructions. The tissue expression profile of RbPGRP2 mRNA was assayed by quantitative real-time PCR (RT-qPCR) with a DICE Real-Time System Thermal Cycler (TaKaRa) using SYBR premix Ex Taq™ (TaKaRa) and specific primers (Table 1). The relative mRNA expression levels were calculated using the comparative Ct ($2^{-\Delta\Delta CT}$) method and normalized to elongation factor 1 alpha (EF-1α).

2.2.3. Expression of RbPGRP2 after treatment with the pathogens

Healthy rock breams were randomly divided into three groups and challenged with an intraperitoneal injection of pathogenic *S. iniae* (3×10^6 cells/fish), *E. piscicida* (2×10^6 cells/fish) or red seabream iridovirus (RSIV) (1.04×10^4 copies/fish), respectively. The animals were maintained in seawater at 23 ± 1 °C throughout the experiment. Tissues (whole kidney, gill, liver and spleen) were taken from the three fish at 1, 3, 6, 12, 24, 36 and 48 h post-injection (hpi). The control fish were injected with the same volume of phosphate buffered saline (PBS). Total RNA extraction, cDNA synthesis and RT-qPCR were performed as described above. All data are reported as the RbPGRP2 mRNA levels relative to that of the EF-1α gene mRNA and are expressed as the means ± SDs.

2.3. Recombinant protein of RbPGRP2 (rRbPGRP2) expression and purification

The open reading frame (ORF) of RbPGRP2 was amplified by PCR as above using specific primers optimized for oligomer design (Table 1). The PCR products were digested with *Xba* I and *Xho* I and ligated into the pET-22b(+) vector (Novagen, Germany) cut with the same restriction enzymes, which was then transformed into *E. coli* BL21 (DE3). For expression of rRbPGRP2, the cells were cultured in Luria Bertani (LB) broth containing ampicillin at 25 °C with shaking at 150 rpm, and grown until it reached an optical density of 1.8–2.0 at 600 nm (OD_{600}), when protein expression was induced using isopropyl-β-D-thiogalactoside (IPTG) at a final concentration of 0.5 mM at 25 °C. The induced cells were harvested and resuspended in denaturation buffer (50 mM Tris-HCl, 0.2 M NaCl, 5 mM EDTA and 2 M urea, pH 8.0), and sonicated for 30 min. After centrifugation, purification of His-tagged RbPGRP2 was performed on a Ni-NTA affinity chromatography column (QIAGEN, Germany) and finally washed with distilled water and equilibration buffer (50 mM Tris-HCl, 0.2 M NaCl and 10% glycerol, pH 7.4). For the elution of rRbPGRP2, the column was subjected to gradual mixing of the elution buffer (equilibration buffer containing 0.5 M imidazole). The purity of the rRbPGRP2 was analysed by 17% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The final protein concentration of rRbPGRP2 was determined by the Bradford assay.

2.4. Bacterial binding assay of rRbPGRP2

The binding activity of rRbPGRP2 to bacteria was tested via an enzyme linked immunoassay (ELISA) test. To determine the specificity towards rRbPGRP2 of the commercial His-tag antibody (Invitrogen), western blotting was performed before the experiments. The bacterial strains (*Streptococcus parauberis* PH0710, *S. iniae* and *E. piscicida*) were cultured in a brain heart infusion (BHI) medium until the OD_{600} reached 0.8 and they were resuspended in carbonate bicarbonate buffer (Sigma-Aldrich) at a concentration of 10^8 colony forming units (CFU)/mL. The ELISA plate was coated with bacteria by incubation at 4 °C overnight and it was blocked using 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) at 22 °C for 2 h. The plate was washed three times with TBST (TBS with 0.05% Tween 20) and different concentrations of rRbPGRP2 in the presence and absence of 10 μM $ZnCl_2$ were added to the plates. After incubation at 22 °C for 90 min, the His-tag antibody was diluted 1:1000 in TBS and incubated at 22 °C for 1 h, followed by incubation with alkaline phosphatase (AP)-conjugated anti-mouse IgG (Sigma-Aldrich) (1:2000) for 1 h. The results were measured by adding alkaline phosphatase, and the OD of each well was read at 405 nm on a Victor 3 microplate reader (PerkinElmer, USA).

2.5. Antibacterial activity assay

The bacteria (*S. iniae* and *E. piscicida*) were first cultured in BHI

Table 1
Primer sequences used in this study.

Usage	Primer name	Primer sequence (5′–3′)
RT-qPCR (control)	EF-1α (F)	CCCCTGCAGGACGTCTACAA
	EF-1α (R)	AACACGACCGACGGGTACA
RT-qPCR	RbPGRP2 (F)	CTCCTCAGGTCTTCACCCTC
	RbPGRP2 (R)	GAGACCTCCATCCCTAGAAC
Recombinant protein	rRbPGRP2 (F)	CTGTTCCACTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATAC
	rRbPGRP2 (R)	GATAAGGCTCTCGAGTTAGTGGTGGTGGTGGTGGTGACGTTTAACCTCACCGAAAT

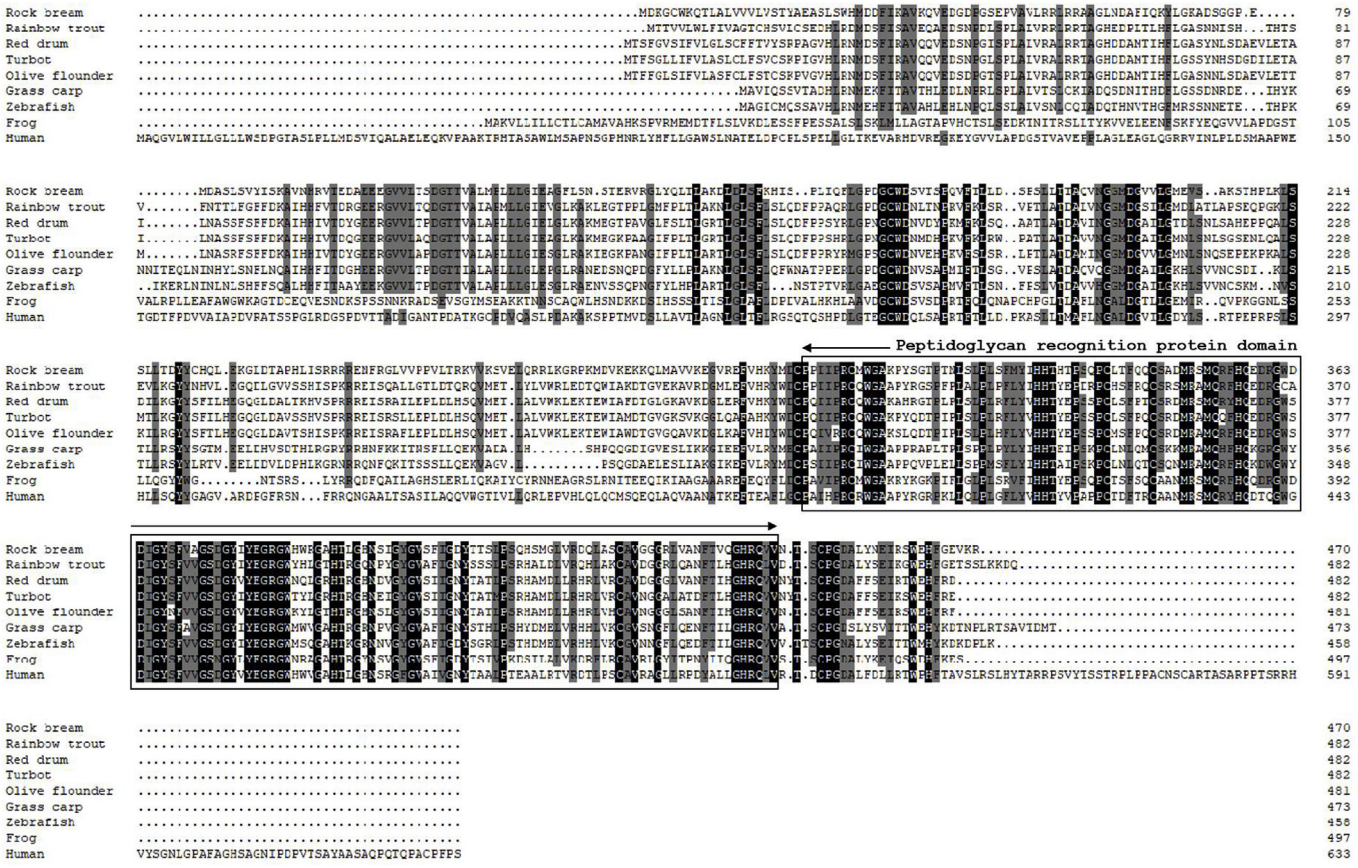

Fig. 1. Multiple alignments of amino acid sequences of RbPGRP2 from rock bream with other species were analysed using the DNAMAN 10.0 program and were based on the following sequence data: Turbot (ANO39625), red drum (ACJ13032), rainbow trout (AGF29404), olive flounder (GF29407), grass carp (ADD23340), zebrafish (DQ447202), frog (XP_012809166) and human (AAI44239). The peptidoglycan recognition protein domain is shown by arrows and box. Black boxes: identity = 100%; Grey boxes: 75% ≤ identity < 100%.

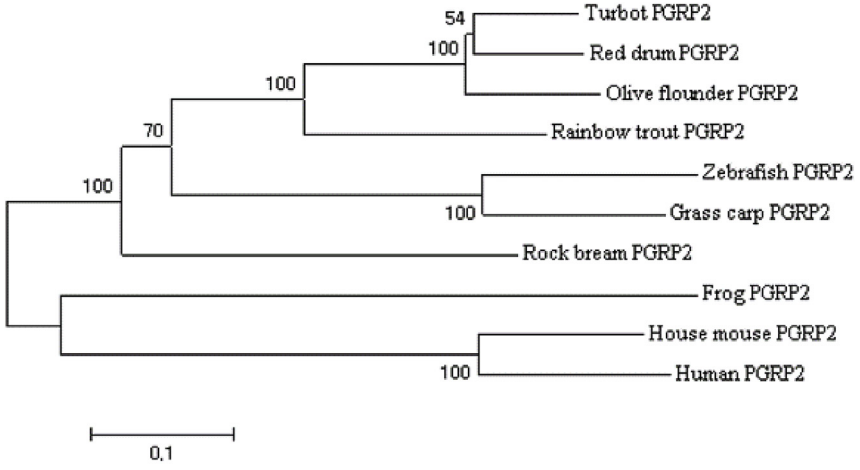

Fig. 2. A neighbour-joining tree of RbPGRP2 constructed using the MEGA 6.0 software and were based on the following sequence data: Turbot (ANO39625), red drum (ACJ13032), rainbow trout (AGF29404), olive flounder (GF29407), grass carp (ADD23340), zebrafish (DQ447202), frog (XP_012809166), house mouse (ABM92420) and human (AAI44239). The bootstrap confidence values shown at the nodes of the tree are based on 2000 bootstrap replicates. The scale bar is equal to 0.1 changes per amino acid position.

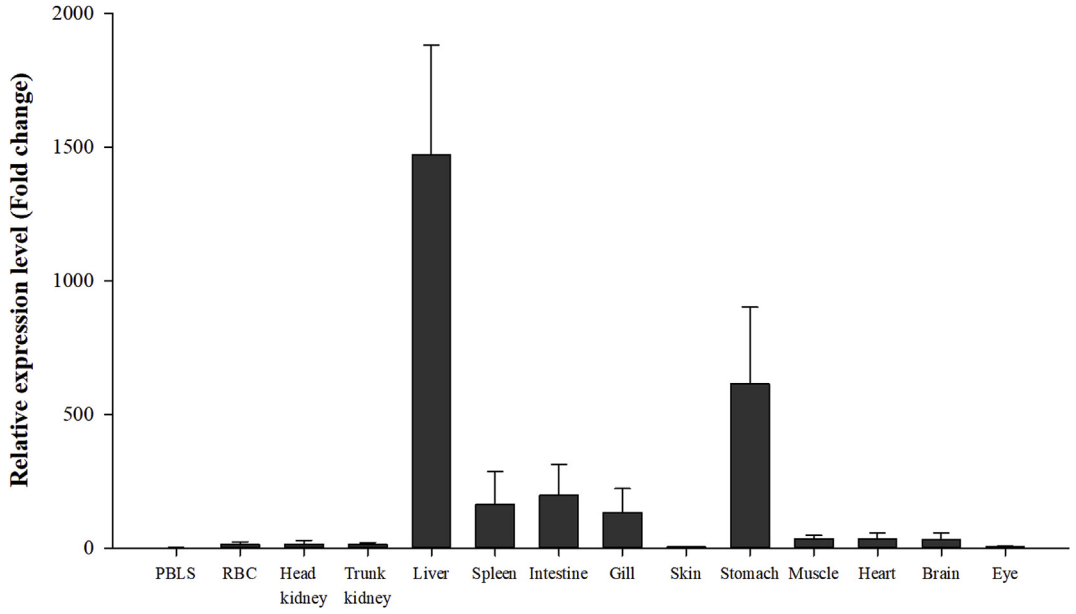

Fig. 3. Expression analysis of RbPGRP2 mRNA in different tissues from healthy rock breams using RT-qPCR. RbPGRP2 was quantified relative to that of the EF-1α gene. Expression levels were calibrated against the tissue that had the lowest expression level (PBLs).

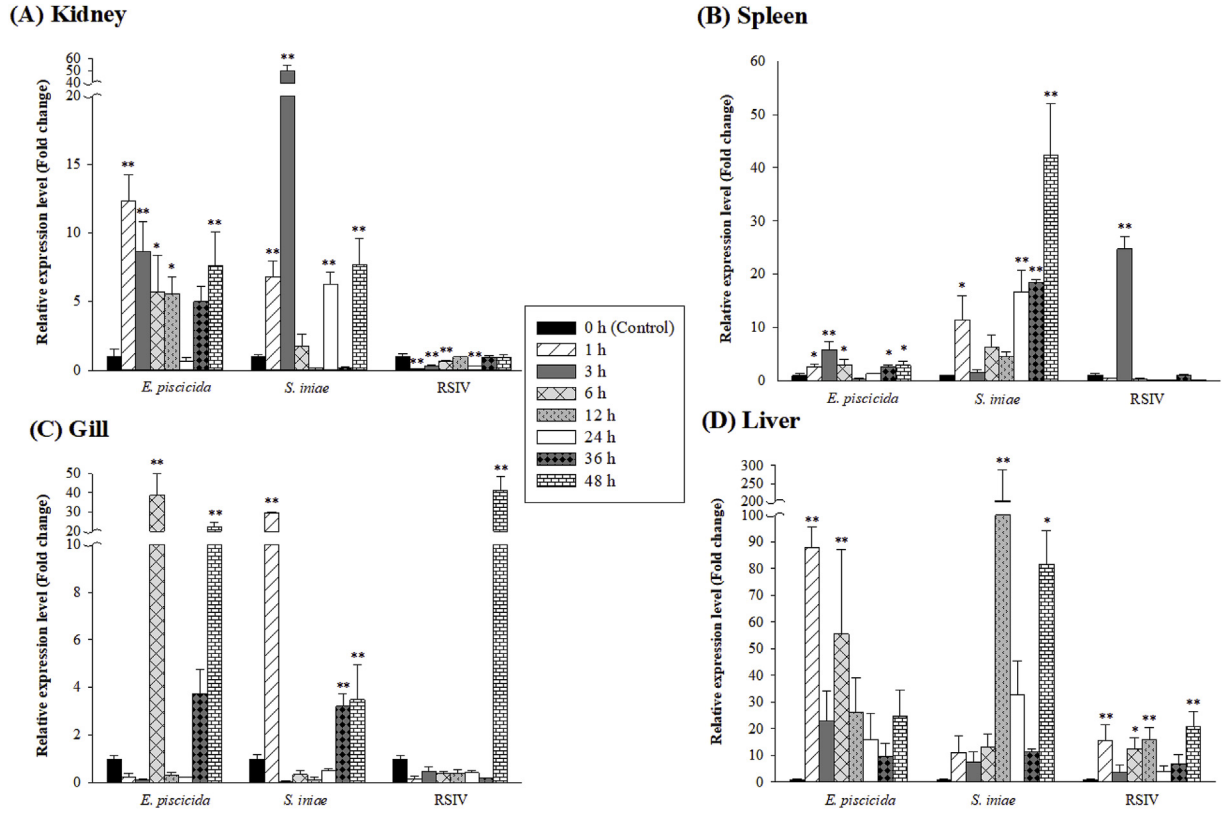

Fig. 4. Expression analysis of RbPGRP2 mRNA in the kidney, spleen, gill and liver of rock bream infected with *Edwardsiella piscicida*, *Streptococcus iniae* and red sea bream iridovirus (RSIV) using RT-qPCR. RbPGRP2 were quantified relative to that of the EF-1α gene. Gene expression and its significance are represented as the mean ± SD (N = 5). Asterisks indicate significant differences (*$P < 0.05$ and **$P < 0.01$) versus the control (0 h).

medium to an OD_{600} of 0.6–1.0 at 27 °C then diluted to 10^4 CFU/mL. One hundred microliters of bacterial inoculum was added to each well of a 96 well plate. Then, we added either 100 μL of rRbPGRP2 (50 μg/mL), BSA (50 μg/mL) or PBS in the presence and absence of 10 μM $ZnCl_2$. The plate was incubated at 27 °C for 1 or 2 h and aliquots of the dilutions were plated on BHI agar plates. Cell viability after rRbPGRP2 addition during growth was estimated by determining the CFU on the BHI agar plates.

2.6. Statistical analysis

The results were assessed using one-way analysis of variance (ANOVA) followed by Tukey's test (*P value < 0.05 and **P value < 0.01) using the SPSS software (ver. 19). All samples were

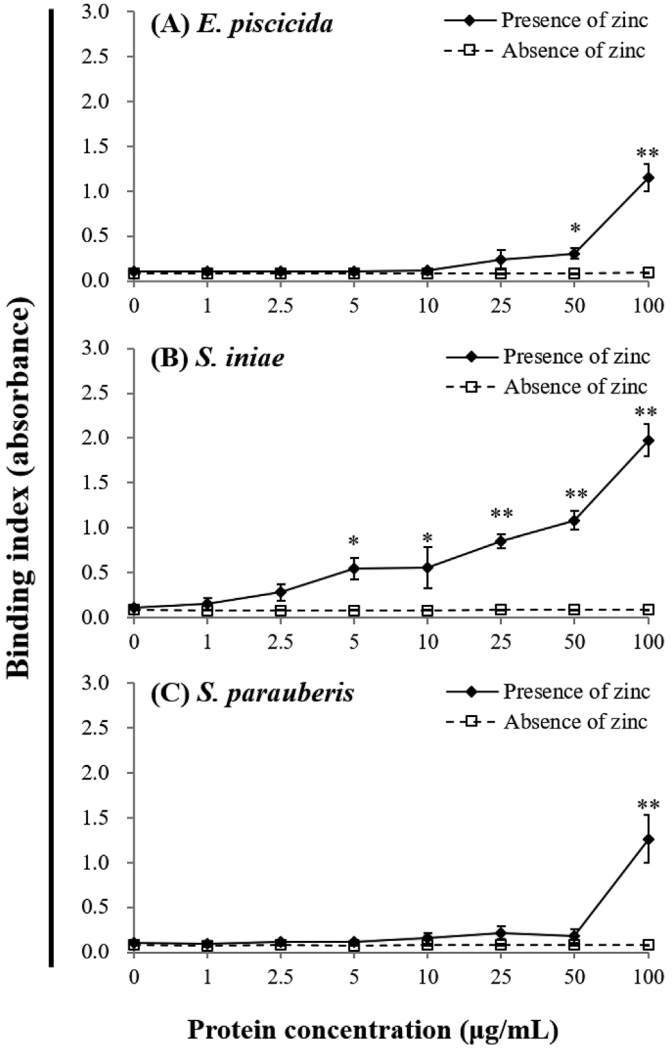

Fig. 5. ELISA analysis of the interaction between rRbPGRP2 and bacteria including (A) *Edwardsiella piscicida*, (B) *Streptococcus iniae* and (C) *Streptococcus parauberis*) in the presence and absence of Zn^{2+} ion. Bacteria were incubated with different concentrations of rRbPGRP2. Data are the mean of three independent assays and are shown as the mean ± SD. Asterisks indicate significant differences (*$P < 0.05$ and **$P < 0.01$) versus the control (0 μg/mL).

analysed in triplicate; the results are reported as the mean ± standard deviation (SD).

3. Results

3.1. Characterization of the RbPGRP2 sequence

RbPGRP2 (GenBank accession number: MH643948) has an ORF that encodes a protein of 470 amino acid residues with a calculated molecular mass of 52.1 kDa and a theoretical isoelectric point of 7.65. It was predicted to be a transmembrane protein with a PGN recognition protein domain (residues 300 to 446). RbPGRP2 shares 37.6–53.5% overall sequence identity with the PGRP2 of other species (Fig. 1).

A phylogenetic tree was further constructed based on the deduced amino acid sequences of RbPGRP2 of other species, and it showed that RbPGRP2 clustered together with PGRP2s of teleosts. RbPGRP2 shares the highest protein sequence similarity (53.5%) with rainbow trout PGRP2 and they are most closely linked in the phylogenetic tree (Fig. 2).

3.2. Expression profiles of the RbPGRP2 gene

To identify tissue-specific expression patterns, RT-qPCR was used to determine RbPGRP2 mRNA expression patterns in each tissue from a healthy rock bream. RbPGRP2 mRNA was found to be distributed in all examined tissues (Fig. 3). In particular, the highest expression was observed in the liver (1470.8 fold) compared to the control (PBLs).

To evaluate the response of gene mRNA expression in rock bream when exposed to pathogens, we observed RbPGRP2 mRNA expression profiles at 0, 1, 3, 6, 12, 24, 36 and 48 hpi after exposure to *E. piscicida*, *S. iniae* and RSIV (Fig. 4). In the kidney, it showed the highest expression shortly after exposure (at 1 and 3 hpi, by 12.3 and 49.9 fold, respectively) compared to the control (0 h) during *E. piscicida* and *S. iniae* infection, while it was significantly downregulated during RSIV infection (Fig. 4A). In the spleen, the results showed that *E. piscicida* and RSIV infections significantly increased mRNA expression of RbPGRP2 at early time points (3 hpi, by 5.8 and 24.7 fold, respectively), and *S. iniae* infection was the highest at 48 hpi (42.3 fold) (Fig. 4B). In the gills, its expression peaked at 6, 1 and 48 hpi (38.7, 29.7 and 41.2 fold, respectively) after *E. piscicida*, *S. iniae* and RSIV infection, respectively (Fig. 4C). Finally, RbPGRP2 expression in liver tissue was significantly upregulated at 1, 12 and 48 hpi (87.9, 203.6 and 20.8 fold, respectively) after infection with *E. piscicida*, *S. iniae* and RSIV (Fig. 4D).

3.3. Purification and identification of rRbPGRP2

RbPGRP2 with the His-tag expressed in *E. coli* was purified by chromatography on a Ni-NTA resin. An analysis of the purified protein

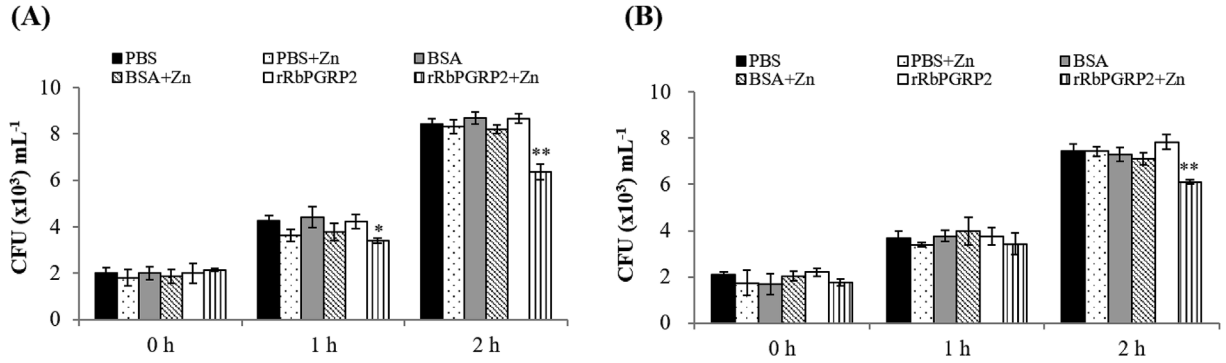

Fig. 6. Antibacterial activity of rRbPGRP2 against (A) *Streptococcus iniae* and (B) *Edwardsiella piscicida* in the presence and absence of Zn^{2+}. Bacteria were incubated with different concentrations of rRbPGRP2, BSA or PBS at 27 °C. Data are the mean of three independent assays and are shown as the mean ± SD. Asterisks indicate significant differences (*$P < 0.05$ and **$P < 0.01$) compared to the control (PBS).

by SDS-PAGE yielded a major band corresponding to a protein of approximately 52.9 kDa (Supplementary fig. 1).

3.4. Bacterial binding assay

The specificity of the His-tag antibody was demonstrated by western blotting and verified that the His-tag antibody recognized rRbPGRP2 (Supplementary fig. 2).

The binding index of rRbPGRP2 for bacteria was increased in a concentration-dependent way in the presence of Zn^{2+}, and significantly increased binding index with all of the bacteria used in the experiment was confirmed (Fig. 5). In particular, binding index of rRbPGRP2 to *S. iniae* was relatively high in the absence of Zn^{2+} (Fig. 5B).

3.5. rRbPGRP2 inhibits the growth of bacteria

The growth of both *E. piscicida* and *S. iniae* was significantly inhibited by rRbPGRP2 in a time and Zn^{2+} dependant manner (Fig. 6). In addition, growth inhibition was more effective against *S. iniae* (Fig. 6A) than *E. piscicida* (Fig. 6B). Meanwhile, no significant inhibition was observed in other groups including the control group (PBS).

4. Discussion

PGRP genes have highly conserved residues that form disulfide bonds and Zn^{2+}-binding sites that are located in/near the C-terminal PGRP domain [17,20–22]. In this study, we have identified four conserved cysteine residues (Cys306, Cys339, Cys345 and Cys426) and four Zn^{2+}-binding sites (His331, Tyr367, His442 and Cys450) in the amino acid sequence analysis of RbPGRP2. Phylogenetic analysis confirmed that RbPGRP2 belongs to the group of PGRP2 in bony fish. These results have demonstrated that RbPGRP2 can function as an *N*-acetylmuramoyl-L-alanine amidase.

PGRP2 is a serum amidase that is primarily expressed in the liver and is secreted into the bloodstream [23]. However, it is rarely expressed in the skin and eyes of healthy humans [22–24]. In teleost fish, PGRP2 was most highly expressed in the liver [15,17]. Similarly, in our study we found that RbPGRP2 mRNA was expressed ubiquitously in all tissues analysed under healthy conditions, especially in the liver. Moreover, in teleost fish, PGRP2 was strongly expressed in the eggs and developing embryo [15,25]. These results suggest that PGRP2 plays an important role as an innate immune gene during the development of adaptive immunity from egg to adult fish.

Previous studies have shown that PGRP2 mRNA was induced by bacterial infection in cultured human corneal epithelial cells and stimulated to the highest level by poly I:C in human epithelial cells [26,27]. In *Drosophila*, both sigma virus and Gram-negative bacteria have an outer lipid bilayer and glucose residues that might induce PGRP in the immune system [28]. In teleost fish, PGRP2 mRNA expression is known to be induced after bacterial infection but its expression after virus infection is not clear [15–17]. In this study, the expression of RbPGRP2 mRNA was significant upregulated by virus infection as well as by bacterial infection. PGRPs are involved in the activation of the Toll and IMD pathways, which are also involved in antiviral immunity [29–31]. Moreover, PGRPs are upregulated by fungi and parasites as well as bacteria and viruses [32,33], which proves that PGRPs have a broad pathogen recognition spectrum. These results indicate that RbPGRP2 may be involved in the initial response to the invasion of various pathogens as well as bacteria.

PGRP2 recognizes bacterial PGN in the host and activates the toll or IMD signalling pathway to perform a variety of functions that include induction of antimicrobial peptides, activation of the prophenoloxidase cascade, induction of phagocytosis, and hydrolysis of bacterial PGN [22,34–36]. The ability of PGRP2 to bind to microbial components of the cell membranes has been previously reported [9,16]. Zn^{2+} ions are required for its amidase activity, and PGRP2 is also known to be Zn^{2+} dependent [16,37]. In this study, we have found that rRbPGRP2 binds to both Gram-positive and -negative bacteria in a concentration-dependent manner in the presence of Zn^{2+} ions, especially to the Gram-positive bacteria *S. iniae*. These results suggest that RbPGRP2 plays an important role in the recognition of and binding to invading bacterial cells in the rock bream. Inflammatory cytokines induced by the recognition of PGN act on the liver to produce acute-phase proteins, which are secreted into the serum [38,39]. Our results showed a rapid increase in its expression in the liver after bacterial infection, which is expected to play an important role via secretory proteins similar to acute phase proteins.

Finally, we confirmed that rRbPGRP2 significantly inhibits the proliferation of Gram-positive and -negative bacteria in the presence of Zn^{2+} ions. Zebrafish PGLYRP-2 more efficiently killed Gram-positive bacteria than Gram-negative bacteria [25], which is similar to our results. The recombinant protein of human PGRP2 showed Zn^{2+}-dependent PGN-lytic amidase activity that hydrolysed the amide bond between the *N*-acetylmuramic acid and L-alanine of pro-inflammatory PGN [22,40]. Meanwhile, bactericidal PGRPs did not hydrolyse insoluble peptidoglycan, uncross-linked soluble polymeric peptidoglycan, synthetic peptidoglycan fragments or heat-killed bacteria [41]. In the case of RbPGRP2, the bacterial growth inhibition or sterilization mechanism is unclear. Therefore, further research is needed to confirm the mechanism of action of RbPGRP2 during bacterial infections.

In conclusion, we identified and characterized RbPGRP2 from rock bream in this study. Purified rRbPGRP2 interacted with both Gram-positive and -negative bacteria and showed antimicrobial activity, especially against the Gram-positive bacteria *S. iniae*. These observations suggest a role for RbPGRP2 against bacterial infection in the rock bream immune system.

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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.2018.11.025.

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