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Molecular cloning and characterization of peptidoglycan recognition proteins from the rockfish, *Sebastes schlegeli*

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ABSTRACT

Peptidoglycan recognition proteins (PGRPs) are innate immune molecules that are structurally conserved through evolution in both invertebrate and vertebrate animals. Here we report the identification and characterization of two long forms of PGRP (SsPGRP-L1 and SsPGRP-L2) from the rockfish, *Sebastes schlegeli*. The deduced amino acid sequences of SsPGRP-L1 and SsPGRP-L2, 466 and 482 residues respectively, contain the conserved PGRP domain and the four Zn^{2+}-binding amino acid residues required for amidase activity. In addition to peptidoglycan-lytic amidase activity, recombinant SsPGRPs have broad-spectrum antimicrobial activity like zebrafish PGRPs. RT-PCR analysis of total RNA shows that the expression patterns of SsPGRP-L1 and SsPGRP-L2 genes are different, though they are widely expressed in the tissues that come in contact with bacteria. Overall, these data suggest that rockfish PGRPs are involved in the innate host defense of *S. schlegeli* against bacterial infections.

1. Introduction

Fish are in intimate contact with their environment, which contain very high concentrations of infectious agents such as bacteria and viruses. However, under normal conditions, fish maintain a healthy state by defending themselves against these potential invaders by complex immune defense mechanisms composed of innate and adaptive immunity [1]. Adaptive immunity of lower vertebrates such as teleost fish are less sophisticated than that of higher animals. In fish, the immune response is also limited in response time by the temperature constraint on fish metabolism [1–3]. Therefore, fish rely heavily on innate immunity for initial protection against infectious agents.

Innate immunity is an evolutionarily ancient form of host defense mechanism, and most multicellular organisms exclusively depend on it [4,5]. The innate immune system uses a variety of germline-encoded receptors (pattern recognition receptors; PRRs) that are expressed on the cell surface, in intracellular compartments, or secreted into the bloodstream and tissue fluids. These receptors have been evolved to recognize conserved products of microbial metabolism produced by microbial pathogens (pathogen-associated molecular patterns; PAMPs) [6]. PAMPs include polysaccharides, lipopolysaccharide, peptidoglycan (PGN), bacterial DNA and double stranded viral RNA and other molecules not normally found on the surface of multicellular organisms [7]. Among them, PGN is an essential component of the cell wall of virtually all bacteria and is an excellent target for recognition by the eukaryotic innate immune response. PGNs are polymers of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) in β (1-4)-linkage, cross-linked by short peptide stems composed of alternating L- and D-amino acids [8,9].

PGN recognition proteins (PGRPs) are innate immune molecules that are structurally conserved through evolution in both invertebrate and vertebrate animals and are a type of PRRs that recognize PGN [10]. The first PGRP was discovered from the hemolymph of the silkworm (*Bombyx mori*) by Ashida and coworkers in 1996 [11]. Subsequently, PGRP homologues have been identified in many species especially in insects and mammals [12–16]. These studies revealed similarities and differences between the vertebrates and invertebrates. Insect PGRPs activate the Toll or IMD signal transduction pathways or induce proteolytic cascades that lead to the protection of insects from infections by generating antimicrobial products and inducing phagocytosis [17]. By contrast, mammalian PGRPs do not act through host signaling pathways but are directly bactericidal [18,19]. In both animal groups, some PGRPs, such as *Drosophila* PGRP-SC1, PGRP-LB, PGRP-SB1 and mammalian PGLYRP-2, can act to attenuate the inflammatory response by degrading

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PGN through *N*-acetylmuramoyl-L-alanine amidase activity, which hydrolyze the lactyl-amide bond between MurNAc and L-Ala in the PGN structure [20–23]. To date several PGRP genes have been identified from teleost fish such as zebrafish (*Danio rerio*) [24] and pufferfish (*Tetraodon nigroviridis*) [25]. However, there is no definitive data on the function of fish PGRPs except that zebrafish PGRPs are bactericidal amidases [24].

Teleost fish occupy a key evolutionary position in the development of the immune responses in that they are the earliest class of vertebrates possessing the elements of both innate and adaptive immunity [26]. Therefore, teleost fish are a good model to study the basic functions of various components of the innate immune response such as PGRPs. Moreover, understanding the innate defense mechanisms of teleost fish may help to develop strategies for the control of fish disease and help the long-term sustainability of fish farming [27]. In this study, we identified two long forms of PGRP (SsPGRP-L1 and SsPGRP-L2) from the rockfish (*Sebastes schlegeli*), which is the most common species of the aquaculture in South Korea, and studied their functions using recombinant proteins.

2. Materials and methods

2.1. cDNA cloning of the genes encoding rockfish PGRPs

Total RNA was extracted from rockfish fingerlings (10 g each, provided from a fish farm near Kunsan, Korea) using a Trizol reagent (Invitrogen, Carlsbad, CA, USA). A first-strand cDNA was synthesized from 1 μg of total RNA using CapFishing Kit (Seegene, Seoul, Korea) according to the manufacturer's procedure. A forward degenerate primer (FDP) was designed based on the conserved sequences of PGRP homologues from *Drosophila*, human, zebrafish and pufferfish, and used as a gene-specific sense primer for 3′-RACE. The 3′-RACE products were cloned into pGEM-T easy vector (Promega, Madison, WI, USA) and the selected clones were sequenced in both directions by using T7 and SP6 primers. Since two different partial cDNA clones were obtained from the 3′-RACE, intragenic primers for each clone were designed (IGP1 and IGP2), and used as a gene-specific primer for 5′-RACE to get the 5′-end of each putative PGRP fragment. The two 5′-RACE products were cloned and sequenced as described above. Then, two pairs of primers were designed based on each tentative full-length cDNA (FL-1F/FL-1R and FL-2F/FL-2R), and the sequences of resulting PCR products (named SsPGRP-L1 and -L2) were verified again. All primers used in the study were listed in Table 1.

Table 1
List of primer sequences used in the study.

Name	Sequence	Length (nt)
FDP	5′-TAY GAR GGH MGH GGH TGG-3′	18
IGP1	5′-CCA GCA TTC CAT GGG GTT GG-3′	20
IGP2	5′-CCA TGC CAT GGA CCT GCT GC-3′	20
FL-1F	5′-ATG GAT AAA GGC TGC TGG AAA GG-3′	23
FL-1R	5′-TCA TTT CTC GAC CTC CCC-3′	18
FL-2F	5′-ATG ACT TTA TTT GGA CTG TCG-3′	21
FL-2R	5′-TCA TTC CCT GAA GTG TTC CC-3′	20
CD-F	5′-AAC GAC ATA GGA TAC AGC-3′	18
CD-R	5′-GTT CAC CAC CTG TCT GTG-3′	18
GSP-1F	5′-TAC ATG GAT TGC CCT CCC-3′	18
GSP-2F	5′-TAC TGG GAC TGC CCT CAA ATC-3′	21
βA-F	5′-GCA TCA CAC CTT CTA CAA TGA GC-3′	23
βA-R	5′-GCT CAT AGC TCT TCT CCA GGG-3′	21
PE-1F	5′-GGA TCC GTA CAT GGA TTG CCC TCC C-3′	25
PE-1R	5′-CTC GAG TTT CTC GAC CTC CCC ATA GT-3′	26
PE-2F	5′-GGA TCC GCA CAA GTA CTG GGA CTG-3′	24
PE-2R	5′-CTC GAG TTC CCT GAA GTG TTC CCA-3′	24

Note: Y = C/T; R = A/G; H = A/C/T; M = A/C.
*Bam*HI and *Xho*I restriction sites are underlined in the primers.

The deduced amino acid sequences of SsPGRP-L1 and -L2 were searched for similarity using BLAST program at the ExPASy Molecular Biology Server (http://www.expasy.org/tools/blast/), and the presence of signal peptide was assumed by the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/). Alignment of multiple sequences was performed using the MEGALIGN program within Lasergene (DNASTAR, Madison, WI, USA) and a phylogenetic tree was constructed using the MEGA4 Neighbor-joining [28]. All sequences used in the analysis were listed in Table 2.

2.2. Northern blot and RT-PCR analysis

The size of SsPGRP mRNA transcripts was determined using Northern blot analysis. 30 μg of whole body total RNA was fractionated by formaldehyde–agarose gel electrophoresis and blotted onto the Immobilon-Ny+ membrane (Millipore, Billerica, MA, USA). A conserved PGRP domain of SsPGRPs was amplified from the cDNA by PCR with CD-F and CD-R (Table 1), labeled with biotin using NEBlot Phototope Kit (NEB, Ipswich, MA, USA), and used as a probe for Northern blot analysis. The biotinylated probe was hybridized to the membrane for 1 h at 68 °C in ExpressHyb solution (CLONTECH, Madison, WI, USA), and detected with Phototope-Star Detection Kit (NEB) according to the manufacturer's protocols.

To determine tissue expression patterns of SsPGRPs, total RNA was extracted from gill, skin, eye, liver, intestine, spleen and head kidney, and 0.5 μg of each RNA was used for semi-quantitative reverse transcription PCR (RT-PCR). Two sets of gene-specific primers (GSP-1F/FL-1R and GSP-2F/FL-2R) were used to specifically amplify SsPGRP-L1 and -L2 fragments, respectively. β-actin gene expression, which was amplified by βA-F/βA-R, was used as an internal control. The RT-PCR was performed using the One Step RNA PCR Kit (AMV) (TaKaRa Bio Inc, Otsu, Japan) with a temperature profile of 50 °C for 30 min, 94 °C for 2 min, followed by 25 cycles of 94 °C for 40 s, 55 °C for 40 s, 72 °C for 60 s, and final extension at 72 °C for 10 min. After amplification, the PCR products were analyzed by 1% agarose gel electrophoresis, and bands were visualized by ethidium bromide staining.

2.3. Expression and purification of recombinant SsPGRP proteins

The PGRP domains of SsPGRP-L1 (amino acids 292–466) and SsPGRP-L2 (amino acids 308–482) were amplified from the SsPGRP-L1 and SsPGRP-L2 full-length cDNA clones by PCR with the gene-specific primer sets (PE1-F/PE1-R for SsPGRP-L1 and PE2-F/PE2-R for SsPGRP-L2). The PCR fragments were digested with *Bam*HI and *Xho*I (restriction sites underlined in the primers) and then subcloned into pET-21b (Novagen, Darmstadt, Germany) digested with the same restriction enzymes. The resultant vectors were named pET-SsPGRP-L1 and pET-SsPGRP-L2, respectively, and transformed into *Escherichia coli* Origami B(DE3) (Novagen). The resulting transformants were each cultured in 1 L of LB medium containing ampicillin (50 μg ml^{-1}) at 37 °C. When the absorbance at 600 nm reached 0.6, the expression of the target protein was induced by adding 1 mM IPTG to the cultures. After 3-h incubation at 30 °C, cells were harvested and disrupted by sonication. The crude lysates were cleared by centrifugation at 15,000 × g for 15 min at 4 °C, and the recombinant SsPGRP proteins were purified from the supernatants by affinity chromatography using His-Bind Agarose Resin (ELPIS, Daejeon, Korea) according to the manufacturer's protocols, except that all buffers included 10 μM $ZnCl_2$. The purified recombinant proteins were dialyzed against 20 mM Tris–HCl (pH 7.9), 150 mM NaCl and 10 μM $ZnCl_2$, and the amount and purity of recombinant proteins were assessed by BCA method and 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), respectively.