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Molecular and functional characterization of peptidoglycan-recognition protein SC2 (PGRP-SC2) from Nile tilapia (*Oreochromis niloticus*) involved in the immune response to *Streptococcus agalactiae*

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ABSTRACT

PGRP-SC2, the member of PGRP family, plays an important role in regulation of innate immune response. In this paper, a PGRP-SC2 gene of Nile tilapia, *Oreochromis niloticus* (designated as On-PGRP-SC2) was cloned and its expression pattern under the infection of *Streptococcus agalactiae* was investigated. Sequence analysis showed main structural features required for amidase activity were detected in the deduced amino acid sequence of On-PGRP-SC2. In healthy tilapia, the On-PGRP-SC2 transcripts could be detected in all the examined tissues, with the most abundant expression in the muscle. When infected with *S. agalactiae*, there was a clear time-dependent expression pattern of On-PGRP-SC2 in the spleen, head kidney and brain. The assays for the amidase activity suggested that recombinant On-PGRP-SC2 protein had a Zn^{2+}-dependent PGN-degrading activity. Moreover, our works showed that recombinant On-PGRP-SC2 protein could significantly reduce bacterial load in target organs attacked by *S. agalactiae*. These findings indicated that On-PGRP-SC2 may play important roles in the immune response to *S. agalactiae* in Nile tilapia.

1. Introduction

Innate immunity is the front line of self-defense against infectious non-self in vertebrates and invertebrates. Innate immunity can discriminate between pathogen and self and start a rapid defensive response through pattern recognition receptors (PRRs), which detect pathogens via the recognition of pathogen-associated molecular patterns (PAMPs), including polysaccharides, lipopolysaccharide (LPS), peptidoglycan (PGN), bacterial DNA, double stranded viral RNA and other molecules not normally found on the surface of multicellular organisms [1,2]. Among these PAMPs, PGN, an essential and unique component of all bacterial cell wall, is an ideal target molecule for eukaryotic organisms to detect bacterial invasion. For this purpose, both invertebrate and vertebrate animals possess peptidoglycan recognition proteins (PGRPs), a family of PRRs that recognize and interact with PGN [3,4].

PGRPs are widely distributed in most animal species, from insects to mammals [3]. Based on molecular weight, PGRPs are classified into three types, i.e. short-, intermediate-, and long-PGRPs (PGRP-S, PGRP-I, and PGRP-L, respectively) [5]. In fruit fly, PGRPs are grouped into two classes: PGRP-SA, -SB1, -SB2, -SC1A, -SC1B, -SC2, and -SD, which have short transcripts, and PGRP-LA, -LB, -LC, -LD, and -LE, which have long transcripts [6]. In humans, four types of PGRPs have been identified, which were named PGLYRP-1, PGLYRP-2, PGLYRP-3, and PGLYRP-4 [5].

Structurally, all PGRPs contain a conserved peptidoglycan binding type 2 amidase domain (also called the PGRP domain), and hydrolyzes the amide bond in peptidoglycan [5]. Almost all PGRPs have two closely spaced conserved cysteines in the PGRP domain that form a disulfide bond, which is needed for the activity of PGRPs [7,8]. All amidase-active PGRPs have a conserved Zn^{2+}-binding site

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in the peptidoglycan-binding groove, which consists of two histidines, one tyrosine, and one cysteine [9–11]. Functionally, insect PGRPs activate the Toll or IMD signal transduction pathways and induce proteolytic cascades, thus enhancing production of antimicrobial products and inducing phagocytosis [12]. By contrast, mammalian PGRPs do not act through host signaling pathways but function as bactericides that kill a wide range of bacteria by direct contact [13].

To date, three main types of PGRPs, i.e. PGLYRP-2, PGLYRP-5, and PGLYRP-6, have been identified in teleosts including channel catfish (*Ictalurus punctatus*), grass carp (*Ctenopharyngodon idella*), large yellow croaker (*Pseudosciaena crocea*), rainbow trout (*Oncorhynchus mykiss*), red drum (*Sciaenops ocellatus*), rockfish (*Sebastes schlegeli*) and zebrafish (*Danio rerio*) [14–22]. PGLYRP-2 is homologous to mammal PGLYRP-2, while PGLYRP-5 and PGLYRP-6 are only found in teleost. However, the PGRP homolog of the SC2 type (PGRP-SC2), which was firstly discovered in fruit fly and proved to play an important role in regulation of innate immune response [23,24], has been reported only in tongue sole (*Cynoglossus semilaevis*) [25], and the study of fish PGRP-SC2 expression profiles and functional properties is rather limited.

Nile tilapia (*Oreochromis niloticus*) is one of the most important commercial fishes and widely cultured throughout the world. In recent years, large scale infectious disease caused by *Streptococcus agalactiae* has been severe, resulting in great economic loss in tilapia aquaculture. How to eliminate such severe disease has become an emergency, and this, to most extent, depends on the understanding of the immune responses induced by *S. agalactiae*. In this study, a PGRP-SC2 gene (On-PGRP-SC2) was cloned from Nile tilapia, *O. niloticus*, and its tissue distribution and mRNA expression profile in response to *S. agalactiae* infection and functional properties were investigated. The present results contribute to better understanding of the mechanism of bacterial recognition in tilapia.

2. Materials and methods

2.1. Experimental animals and tissue collection

Samples of Nile tilapia (average weight of 100 ± 10 g) were obtained from a commercial farm in Zhanjiang, Guangdong province, China. Prior to experimentation, fish were acclimated in fiber-reinforced plastic tanks (1000 L each) with a stocking rate of 4 g/L under 28 ± 2 °C for 4 weeks. All tanks were supplied with flow-through aerated sand-filtered water, and a light and dark period of 12 h: 12 h was maintained. *S. agalactiae* ZQ0910, a virulent strain isolated from tilapia was used for infection [26]. The infection experiment was performed by injecting intraperitoneally (i.p.) the tilapia with 0.1 mL of bacteria resuspended in sterilized phosphate buffered saline (PBS) with the concentration of 1×10^7 cells mL^{-1} into the abdominal cavity and the tilapia injected with 0.1 mL of sterilized PBS were used as the control group. Then all processed tilapia were returned to tanks and treated as before. At time points of 0 h, 6 h, 12 h, 24 h, 48 h, 72 h, and 96 h post-infection, eight kinds of tissues including brain, head kidney, spleen, liver, muscle, skin, gill and intestine were collected from the control and infection groups, and immediately frozen by liquid nitrogen, followed by storage at −80 °C until use. Tissues from three individuals were collected and pooled together as a replicate sample, and three replicates were taken for each sampling time point.

2.2. Cloning of cDNA for On-PGRP-SC2

The sequences of all PCR primers used in this study were summarized in Table 1. Total RNA from intestine was extracted using Trizol Reagent (Invitrogen, USA) as described in the manufacturer's instructions. The first-strand cDNA was synthesized from the total RNA using Reverse Transcriptase M-MLV (TaKaRa, Japan) according to the manufacturer's protocol and served as a template to amplify On-PGRP-SC2 partial cDNA sequences by PCR using specific primers designed from our previous study on Nile tilapia transcriptome data (unpublished). To amplify the full-length sequence of On-PGRP-SC2, the first-strand cDNA for 5′/3′-RACE (rapid amplification of cDNA ends) was synthesized with a SMARTer™ RACE cDNA Amplification Kit (Clontech, USA) using intestine RNA as the template and following the manufacturer's protocol. The full-length cDNA of On-PGRP-SC2 was obtained by using 5′/3′-RACE methods with some gene specific primers designed based on the obtained partial sequences of On-PGRP-SC2 cDNA. All the PCR products were ligated into the pMD18-T vector (TaKaRa, Japan) and transformed into competent *Escherichia coli* cells. Then the positive clones were sequenced by sequenced SANGON BIOTECH (Shanghai, China). Finally, the partial sequence, 3'-end and 5'-end were assembled using contigExpress application software.

2.3. Bioinformatics analysis of On-PGRP-SC2

The potential open reading frame (ORF) was analyzed with the ORF Finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.htmL). The protein analysis was conducted with ExPASy tools (http://expasy.org/tools/). Location of domains was predicted using the InterProScan program (http://www.ebi.ac.uk/Tools/pfa/iprscan/). Multiple alignments of On-PGRP-SC2 amino acid sequences were performed with the Clustalw2 program (http://www.ebi.ac.uk/Tools/clustalw2/). The similarity analyses of the determined nucleotide sequences and deduced amino acid sequences were performed by BLAST programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenic trees were constructed by the neighbor-joining method using MEGA 4 software with 1000 bootstrap replications. The three-dimensional (3D) structure prediction was performed by SWISS-MODEL online software at the Expert Protein Analysis System (http://www.expasy.org/). All sequences used in the analysis were listed in Table S1.

2.4. Quantitative analysis of On-PGRP-SC2 mRNA expression

The differential expression levels of On-PGRP-SC2 in pre- and post-infection tissues were measured by fluorescent quantitative real-time PCR using gene-specific primers (Table 1). The first-strand cDNA was synthesized from the DNase treated total RNA using the Reverse Transcriptase M-MLV (TaKaRa, Japan) according to the manufacturer's protocol. The β-actin gene was used as an internal control to normalize the potential variations in RNA loading. The relative expression levels of On-PGRP-SC2 were calculated using Nile tilapia β-actin expression as a reference, and the results were further compared to respective control group expression levels to determine the fold induction [27,28]. The PCR was performed in a 15 μL reaction volume containing 0.5 μL of each primer (10 μM), 1 μL of 10^{-1} diluted cDNA, 3 μL PCR-grade water and 10 μL of 2 × TransStart™ Green qPCR SuperMix (TransGen, China) according to the manufacturer's protocol. Samples were run in triplicate on the Bio-Rad iQ5 Real-time PCR System (Bio-Rad, CA, USA). The relative expression levels of On-PGRP-SC2 were calculated by means of the $2^{-\Delta\Delta Ct}$ method. All quantitative data were presented as the means ± standard deviation (SD).

2.5. Expression and purification of recombinant On-PGRP-SC2 (rOn-PGRP-SC2)

The ORF of On-PGRP-SC2 was amplified by PCR with specific primers, PGRP-SC2-YS and PGRP-SC2-YA (Table 1). The PCR product