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Involvement of a short-type peptidoglycan recognition protein (PGRP) from Chinese giant salamanders *Andrias davidianus* in the immune response against bacterial infection

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ABSTRACT

PGRPs (Peptidoglycan recognition proteins) could recognize peptidoglycan and play vital roles in innate immunity among different animals. Till present, the functions of PGRP have been studied in various animals, but few reports have studied the amphibian PGRPs. In the current research, a short type PGRP was identified from Chinese giant salamander and its involvement in the innate immunity was studied. The ORF of *Ad*PGRP-SC2 cDNA was 573 bp, which encoded 190 amino acids, and contained a PGRP and an amidase_2 domain. The qPCR analysis revealed that *Ad*PGRP-SC2 mRNA transcripts expressed in different tissues, with the highest expression level in muscle, intestine and spleen. Results of immune challenges with peptidoglycan (PGN) demonstrated that expression patterns of *Ad*PGRP-SC2 were significantly up-regulated in erythrocyte and spleen at the early injection stage. The recombinant AdPGRP-SC2 protein was successfully produced and purified, and it could show binding affinity to different bacteria. In the presence of Zn^{2+}, the rAdPGRP-SC2 could exhibit a broad PAMPs binding activities, strongly agglutinate bacteria and exhibit amidase enzyme activity. Collectively, these data indicate AdPGRP-SC2 could act as PRR to recognize the invading microorganisms and as the antimicrobial effectors during the innate immune response of *A. davidianus*.

1. Introduction

The innate immunity is essential for organism to defense against the invasion of microbial pathogens. The immune response is generally proposed to be based on pathogen-associated molecular patterns (PAMPs) which could be recognized byPRRs, and triggered a number of defense responses in a complex manner (Akira et al., 2006). Till present, many PAMPs have been found such as LPS (lipopolysaccharide), PGN (peptidoglycan), bacterial DNA, and so on (Medzhitov, 2007). PGN is the conserved essential component of bacterial cell envelope. It is absence of PGN in eukaryotic cells, so PGN is regarded as a target molecule of PAMPs for recognition by immune system (Davis and Weiser, 2011). Among the higher eukaryotes, several peptidoglycan recognition molecules are present including pattern recognition receptors and effector molecules (Royet and Dziarski, 2007).

PGRPs are kinds of soluble peptidoglycan recognition molecules, and play vital roles in innate immunity (Kang et al., 1998). They are important PRRs that recognize and interact with peptidoglycan, and widely distributed in invertebrates and vertebrates (Liu et al., 2001). Based on the different molecular weights, three different types like S-type (20 kDa), I-type (40–45 kDa), and L-type (90 kDa) are categorized, but the I-type PGRP was only found in mammals (Dziarski and Gupta, 2006). Different types of PGRPs both have more than one PGRP-domain. This motif is reported to be homologous to bacteriophage amidases (Liu et al., 2001), indicating that PGRPs could hydrolyze the amide bond. Two closely conserved Cys residues were found in the center of PGRP domain, which is needed for PGRP structural integrity and activity (Wang et al., 2003; Guan et al., 2005). The amidase-active peptidoglycan-binding grooves have a conserved Zn^{2+}-binding site (Chang et al., 2004). PGRPs have more than three different functions in the innate immune response. They could act as PRRs to recognize different PAMPs, degrade peptidoglycans, and inhibit bacterial growth as

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antibacterial effector (Mellroth and Steiner, 2006). Many studies have demonstrated that PGRPs can recognize the bacterial PGN, LPS, LTA and fatty acids (Yoshida et al., 1996; Sharma et al., 2012). Then they could active the downstream immune responses, including Toll pathway, proPO system, the IMD and JNK pathway (Yoshida et al., 1996; Michel et al., 2001; Steiner, 2004; Maillet et al., 2008). In *Drosophila*, PGRP-SC2 could negatively regulate the Imd/Relish innate immune signaling pathway (Bischoff et al., 2006). In an intracellular bacterial infection *Drosophila* model, PGRP played an important role as the receptor for autophagy and induces the synthesis of antibacterial peptide (Goto et al., 2010). It has also demonstrated that PGRPs could promote phagocytosis and activate the proPO system (Bischoff et al., 2006). Some kinds of PGRPs could also participate in the gut immune response, and play crucial role to keep the microbial homeostasis in intestine (Neyen et al., 2012; Buchon et al., 2013). With the amidases domain, most PGRPs could break down different kinds of PGNs (Zaidman-Rémy et al., 2006). Some PGRPs exhibited direct antimicrobial activity and induced agglutination or phagocytosis (Rämet et al., 2002; Lu et al., 2006; Coteur et al., 2007). The PGRPs play a variety of important functions in the immune response and could effectively prevent the invasive microorganisms.

The Chinese giant salamander (*A. davidianus*) is a kind of *Caudata* amphibian species, widely distributed in China, Japan and America (Zhang et al., 2003). Recent years, it has become a featured economy specie farmed in China. But the characteristic of immune system in *A. davidianus*, especially some PRRs molecular are indistinct. Moreover few studies of PGRPs are focused on amphibians. In the current study, we have investigated the potential functional of PGRP-SC2 in *A. davidianus* immune response. A novel PGRP-SC2 gene was cloned, and its functional properties were investigated. The present study contributes to a better understanding the mechanism of PGRP-SC2 participating in the immune response.

2. Material and methods

2.1. Experimental animals and tissue collections

Samples of *A. davidianus* (average weight of 508.4 ± 10.5 g) were collected from a breeding farms. Before the experiment, all the salamanders were cultured in tanks. The different samples such as skin, heart, liver, intestine, stomach, muscle, pancreas, erythrocyte, spleen, kidney, brain and lung were collected. The small salamanders (163.5 ± 30.5 g) were chosen to inject PGN intraperitoneally with 15 μg/10 g body weight. The spleen and erythrocyte samples were collected at different hours post PGN injection. All the different collected samples were flash-frozen in liquid nitrogen and stored at −80 °C for use.

2.2. Cloning, identification and sequence analysis of AdPGRP-SC2 gene

Total RNA of different tissues were extracted using Trizol under the manufacturer's protocol. cDNA samples were synthesized by PrimeScript Kit. The specific primers were used to amplify the AdPGRP cDNA sequence by *AdPGRP-F* and *AdPGRP-R* (sequences were shown in Table 1). After amplifications, the PCR products were purified and cloned into the pMD19-T simple vector.

The protein sequence of AdPGRP-SC2 was analyzed by NCBI. SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the signal peptide. The multiple alignments was conducted by ClustalW2. The structure of AdPGRP-SC2 was analyzed on the website Swiss model server. The phylogenic tree compared with other PGRPs was constructed by the MEGA software 5.0.

2.3. Expression profiles of AdPGRP-SC2 gene under PGN stimulation

The transcript expression profiles of *AdPGRP-SC2* in tissues were conducted by qPCR. Different primers for qPCR used were shown in Table 1. Before the qPCR, the amplification efficiencies were calculated (Freeman et al., 1999). After qPCR programs, melting curves were conducted to make sure a single PCR product. The PCR products were also sequenced to verify the correct target gene. Two housekeeping genes *EF-1α* and *β-actin* genes were selected as reference gene (Yang et al., 2017a,b). The $2^{-\triangle\triangle Ct}$ method was used to calculate the relative expression level. Data were expressed as mean ± S.D. and analyzed by the one way ANOVA ($p < 0.05$).

Table 1
Details of the primer pairs for gene cloning and real time PCR.

Primers	Sequences (5′-3′)	Efficiency
EF1α-F	GGACAGACCCGTGAACATGC	95%
EF1α-R	CTTCCTTAGTGATCTCCTCGTAGC	
βActin-F	GCCATCAATCGTCCACCG	94.5%
βActin-R	CCGCATCAAGCACCAGAA	
AdPGRP-F	CCCCTCTATTACAGGATGCT	
AdPGRP-R	GGGAATCTGGTGGATAATCA	
qAdPGRP-F	AGGATGCTGCGTATCTTCG	93%
qAdPGRP-R	AGGCGGATTTGGTGGTGC	
ExpPGRP-F	GCCgaattcCCCAACATTATCACCCGCTC	
ExpPGRP-R	CGGctcgagGGCCTGGAGCCAACACTTAG	
M13-F	CGCCAGGGTTTTCCCAGTCACGAC	
M13-R	CACACAGGAAACAGCTATGAC	
T7	TAATACGACTCACTATAGGG	
T7 Ter	TGCTAGTTATTGCTCAGCGG	

After PGN injection, the expression profiles of *AdPGRP-SC2* in spleen and erythrocyte were also analyzed. The qPCR and data analysis were performed as described above.

2.4. Recombinant expression of AdPGRP-SC2 protein

After PCR amplification by *ExpPGRP-F* and *ExpPGRP-R* primers (shown in Table 1), the amplified products were gel-purified and digested completely by *Eco*R I and *Xho I*. The vector pET30a(+) (S-tag and His-tag) was used as expression vector. After digestion by the same restriction enzymes, the PCR products ligated with the pET30a(+) vector. Then *E. coli* BL21 (DE3) plysS were translated into the right sequenced recombinant plasmid. Then *E. coli* BL21 (DE3) with recombinant expression vector pET30a/*Ad*PGRP were cultured in LB medium at 37 °C to mid-logarithmic phase. After IPTG induction, the bacteria fragment were collected and analyzed by SDS-PAGE. Then the induced bacteria were disrupted by sonication and purified by Ni-NTA agarose. The bacterial lysate supernatant was loaded onto Ni^{2+}-chelating column chromatography. After washing by wash buffer (20 mM Tris–HCl, 500 mM NaCl and 20 mM imidazole, pH 7.9), the recombinant fusion protein was eluted with elution buffer (20 mM Tris–HCl, 500 mM NaCl and 400 mM imidazole, pH 7.9). After SDS-PAGE analysis, Coomassie brilliant blue R-250 staining was used to dye the proteins. The recombinant proteins were refolded in dialysate under different concentration of urea (Yang et al., 2017a,b). Bradford method with BSA as standard was performed to calculate the concentration of recombinant protein.

2.5. PAMP binding assay

ELISA was used to measure the different PAMPs binding affinity of rPGRP-SC2 (Bowdish et al., 2004). Briefly, 15 μg LPS, PGN, and mannan (man) were coated to 96-well microtiter plates in 100 μL coating buffer by incubation at 4 °C overnight. The LPS, PGN and man were bought from Sigma Company. With the presence of 1 mmol/L $ZnCl_2$, different concentration of rPGRP-SC2 protein with 100 μL volume were incubated in the plates at 37 °C for 2 h. The BSA protein was used as negative control to be coated onto the ELISA plates. The anti-His Tag mouse monoclonal antibody with HRP-conjugated was diluted