A mini review on the short-type peptidoglycan recognition protein in Chinese soft-shelled turtle (Pelodiscus sinensis)

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Abstract. Peptidoglycan recognition proteins (PGRPs) function as the pattern recognition receptor involved in antibacterial innate immunity. Evidence have showed that the molecular structure and function of PGRPs was conserved in vertebrate. However, as the pivotal species in the evolution of vertebrates, reptiles are believed to be the first vertebrates that have escaped from the aquatic environment and are able to adapt to a variety of different terrestrial lives, few studies about the PGRPs in reptiles has been reported. The Chinese soft-shelled turtle, Pelodiscus sinensis, is an ancient, secondary aquatic reptile with high economic value and nutritional value in Asia, which occupies a unique position in the animal kingdom and has important research value. In the latest research, a PGRP gene which was classified into the member of short-type PGRP family was characterized in Pelodiscus sinensis. This paper presented the latest findings on the molecular structure, expression pattern and function feature of PGRP-S from Pelodiscus sinensis, aiming at revealing that PGRP in vertebrates is evolutionarily conserved.

1. Introduction

The innate immune system senses invading pathogens through pattern recognition receptors (PRRs). The PRRs that have been studied in depth include Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-like receptors (RLRs), C-type lectin receptors and peptidoglycan (PGN) recognition proteins (PGRPs)[1, 2]. LPS, lipoprotein, flagellin, PGN, the DNA or RNA of bacterial and viral are collectively referred to pathogen-related molecular patterns, which can be recognized by PRRs. The PRRs recognize PAMPs to initiate the first line of host innate immune defense to withstand infection[1, 2]. PGRPs are an important PRR and a key part of innate immune defense that are conserved from insects to mammals and mainly recognize bacteria and their unique cell wall components, PGN[3]. It is considered that PGRPs play an indispensable role in the host's antibacterial defense mechanism.

PGRP was first discovered from silkworm (Bombyx mori), and then the PGRP homologous genes have been reported in mammals, amphibians, fish and mollusks [3]. PGRPs can be divided into 3 categories according to the transcript length, among them, short type PGRP is ubiquitous in vertebrates [3]. Some of the PGRP have Zn²⁺-dependent amidase activity, and some are antibacterial proteins [3]. Although PGRPs have been identified in many species, as the pivotal species in the evolution of vertebrates, reptiles are believed to be the first vertebrates that have escaped from the aquatic environment and are able to adapt to a variety of different terrestrial lives[4]. However, the molecular and functional characteristics of PGRPs in reptile remain unexplored. The Chinese soft-shelled turtle,
*Pelodiscus sinensis*, is an ancient, secondary aquatic reptile with high economic value and nutritional value in Asia. However, the Chinese soft-shelled turtle farming industry was faced to bacterial diseases such as *E. Tarda* infection which cause serious economic losses. In fact, the research on the innate immunity of the unique reptile species, Chinese soft-shelled turtle, is still limited, and the identification and functional research of its PGRP molecule has not been reported. Hence, the present study was focused on the molecular identification and functional exploration of PGRP-S from Chinese soft-shelled turtle, aiming at revealing that the reptiles PGRP-S is conserved in evolution in terms of function, and filling blank in the study of PGRP in reptiles.

2. Molecular structure characteristics and phylogenetic tree analysis of PGRP-S in *Pelodiscus sinensis*

Total RNA extraction and the first stand cDNA generation was implemented following the TRizol® Reagent (Ambion) protocol and RevertAid™ first strand cDNA synthesis kit (Thermo) instruction, respectively. Using soft-shelled turtle artery (STA) cells cDNA as template to amplify the open reading frame (ORF) of PGRP-S with specific primers using PCR technology. The analysis of signal peptides, putative conserved domains and phylogenetic tree about amplified PGRP molecule was carried out by relational online sites [5]. The whole open reading frame of PGRP-S from *Pelodiscus sinensis* was 807 bp length and encoded a protein with 25 Kd molecular weight. Phylogenetic tree analysis revealed that the PGRP-S belonged to the member of short type PGRPs family. To date, PGRP members have been reported in insects, mollusks, fishes, amphibians, mammals, etc, and the PGRP domain is one of the reported conserved structures of the vertebrate PGRP gene [6-9]. The PGRP-S identified from aquatic reptile, *P. sinensis* suggesting that the structure of PGRP-S was highly conserved from invertebrates to vertebrates.

3. The expression pattern of PGRP-S gene in *Pelodiscus sinensis*

Generally, studying on the constitutive expression and inducible expression of genes in organs/tissues is helpful to find the target tissues or potential functions of genes. In the latest study, the tissues/ organs from Chinese soft-shelled turtles (n=6,100g/individual) were used in investigating the constitutive expression of PGRP-S. The STA cells line was established by predecessors and kept in the laboratory to perform the inducible expression assay[10]. *Edwardsiella tarda* got from Research Professor Xie Hai Xia was used as the pathogen for infection. The expression of PGRP-S gene was detected via qRT-PCR with the primers: 5’-GGTCCACGCGGAGAATAT-3’ and 5’-GGTTCTGCTCGGCTCAAGGAG-3’, following the reaction process: 95 ◦C for 3 min, followed by 45 cycles of 95 ◦C for 10 s, 60 ◦C for 25 s and 72 ◦C for 20 s. It was observed that the PGRP-S transcription of Chinese soft-shelled turtles was most abundant in spleen, which was similar with aquatic animals. In addition, *E. tarda* stimulation resulting in a highly upregulation of this PGRP-S gene. Research have found that PGRP genes were distributed in most organs/tissues with difference among species, and could be induced by PAMP or bacterial. For instance, PGRP6 in intestine, spleen and liver of grass carp (*Ctenopharyngodon idella*) has high expression level, and could response to different microbial ligands stimulation [11]. The PGRP found in amphibians had highest expression in muscle and intestine, they also could be upregulated by microbial ligands stimulation [6, 10]. An interesting finding was that the main organ of green anole lizard (*Anolis carolinensis*) PGLYRP3 distribution was dewlap and skin, which was believed to be involved in terrestrial adaptation [12]. Combining with the constitutive expression and induced expression results of Chinese soft-shelled turtle PSRP-S and other species, it shows that the expression of PGRPs was conserved in vertebrates.

4. Ligand binding activity and antibacterial capability of Chinese soft-shelled turtles PGRP-S

In innate immunity, PRRs are indispensable, because the host can recognize pathogen-related molecular patterns (PAMP) through PRRs. PRRs activate a series of signal pathways after sensing PAMP to initiate the first line of defense. As a PRR, one of the most important features of PGRPs is the recognition and combination of PGN [6, 13]. Study also showed that PGRPs could bind LPS and bacterial [13]. In
present study, the affinity of PGRP-S from Chinese soft-shelled turtles for LPS, PGN and E. Tarda was determined by binding experiments using eukaryotic and prokaryotic recombinant proteins for double verification[6, 14]. Eukaryotic expression plasmid of Chinese soft-shelled turtles PGRP-S was transfected into HEK293T cells for 36h, E.tarda (MOI=0.5) was added to infect the cells, the growth inhibition effect of the PGRP-S protein on E. tarda was verified via plate colony-counting method after E. tarda infection for 3h[6]. For further confirming the antibacterial capability of Chinese soft-shelled turtles PGRP-S against E.tarda, prokaryotic recombinant PGRP-S protein was used to incubate the HEK293T cells while infecting with E.tarda like eukaryotic expression protein, after that using the same plate colony-counting method to calculate the amount of E.tarda in the cells[15]. It was discovered that, in addition to PGN, Chinese soft-shelled turtles PGRP-S showed LPS affinity, as well as Gram-negative bacteria, E.tarda combination activity, and the growth of E.tarda in the cell was inhibited when PGRP-S was present. It is known that PGN is the component of bacterial not only in Gram-positive but also Gram-negative bacteria[3, 16]. Evidence also revealed that PGRPs can bind LPS through the binding sites for LPS outside the peptidoglycan-binding groove of PGRPs [13]. Moreover, PGRPs are also the antibacterial protein, showing a killing effect on Gram-positive and Gram-negative bacteria by activating the systems of B. subtilis CssR–CssS or E. coli CpxA–CpxR [13]. Hence, it is likely that Chinese soft-shelled turtle-PGRP-S possessed the conservative antibacterial mechanism against E. tarda [9, 17-19].

5. Amidase activity of Chinese soft-shelled turtle-PGRP-S
To our knowledge, amidase activity is another classic function of vertebrate PGRP, which promote to split PGN into inactive peptides[17]. The fact that the presence of Zn\(^{2+}\) enhances the activity of PGRP amidase and all amidase-active PGRPs has conserved Zn\(^{2+}\)-binding site [6, 7, 10, 17]. The amidase activity detection of Chinese soft-shelled turtles PGRP-S was conducted in microplate reader to detect the OD\(_{540}\) of prokaryotic recombinant PGRP protein with PGN mixture[10]. The eukaryotic recombinant WT and mutant Chinese soft-shelled turtles PGRP-S protein was used to certificate the Zn\(^{2+}\)-binding ability of PGRP-S. Briefly, the recombinant WT and mutant Chinese soft-shelled turtles eukaryotic expression plasmid expressed in HEK293T cells, respectively. The two proteins incubated with ZnCl\(_2\) buffer in the beads which was coated with flag antibody for overnight. Then the beads were washed with cold PBS and sent to execute microwave digestion. Finally, the concentration of Zn\(^{2+}\) was determined by ICP-MS. In this part of the functional exploration, Chinese soft-shelled turtles PGRP-S was proven to be an amidase-active PGRPs, and conservative Zn\(^{2+}\) binding site mutations weakened the ability of PGRP to bind Zn\(^{2+}\), revealing that the amidase activity of vertebrates is also conserved in evolution.

6. Conclusions
PRRs play a critical and important role in the innate immune system. As one of the PRRs, PGRPs have an antibacterial effect in the host's immune response to bacterial pathogen infection. Reptiles represent key node in vertebrates evolution, the unique evolutionary status makes the research on the immune system of reptiles be exploratory. The identification of PGRP in Chinese soft-shelled turtle provides a new argument for the study of PRRs in the innate immunity of reptiles, and shows that the molecular structure and biological functions of PGRP are conserved in vertebrate evolution. Simultaneously, Chinese soft-shelled turtle-PGRP-S functional research offers a theoretical basis for the defense strategy of Chinese soft-shelled turtle disease.

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