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Molecular cloning and characterization of the lipopolysaccharide and β-1,3-glucan binding protein from red claw crayfish, *Cherax quadricarinatus*

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ABSTRACT

A pattern recognition protein (PRP), lipopolysaccharide and β-1,3-glucan binding protein (LGBP), cDNA was cloned from the hemocytes of red claw crayfish, *Cherax quadricarinatus* by the techniques of homology cloning and RACE. Analysis of nucleotide sequence revealed that the full-length cDNA of 1456 bp has an open reading frame of 1095 bp encoding a protein of 364 amino acids including a 15 amino acid signal peptide. The calculated molecular mass of the mature protein (349 aa) was 39.92 kDa with an estimated pI of 4.50. Sequence comparison of the deduced amino acid sequence of *C. quadricarinatus* LGBP (named as CqLGBP) showed a high identity of 82%, 77%, 76% and 74% with *Procambarus clarkii* LGBP, *Homarus gammanus* BGBP, *Penaeus monodon* BGBP and *Litopenaeus stylirostlis* LGBP, respectively. The CqLGBP sequence contains: (1) two putative integrin-binding motifs, (2) a glucanase motif, (3) two putative N-glycosylation sites, (4) one protein kinase C phosphorylation site, and (5) a putative recognition motif for β-1,3-linkage of polysaccharides. The recombinant CqLGBP were expressed in *Escherichia coli* BL21 with pGEX-6P-2 expression vector. The titer of rabbit anti-CqLGBP serum was above 1:12,800. Microorganism binding assay showed CqLGBP binds two kinds of crustacean pathogens *Spiroplasma eriocheiris* and *Aeromonas hydrophila*. Quantitative RT-PCR method was used to quantify the variation of mRNA transcription level during artificial infection with *S. eriocheiris* and *A. hydrophila*. A significant enhancement of *CqLGBP* transcriptions was apparent post-injection in response to bacterial infection compared to the control group. These data should be helpful to better understand the function of CqLGBP in the crayfish immune system.

1. Introduction

The red claw crayfish, *Cherax quadricarinatus*, is native to Australia and Papua New Guinea, and is a tropical aquaculture species (Karplus et al., 2003). It has become an important species in Jiangsu, Zhejiang, Fujian and Guangdong provinces aquaculture since it was introduced to China. It is an important species in freshwater aquaculture (Fang et al., 2011, 2012). However, the molecular biology study of *C. quadricarinatus* is insufficient. Only a few genes have been reported, such as heat shock protein 70, SOD, etc. (Fang et al., 2011, 2012; Gu et al., 2014; Liu et al., 2011). Therefore, understanding the immune ability of *C. quadricarinatus* and its defense mechanisms has become a primary concern.

Because they lack an adaptive immune system (Loker et al., 2004), invertebrates rely entirely on their innate immune response to protect against invading pathogens (Hoffmann et al., 1999). Recognition of an invading organism as non-self is a critical step in the innate immune response (Medzhitov and Janeway, 1997). In innate immunity, the pattern recognition proteins (PRPs) play an important role by recognizing and binding to common epitopes on the pathogen surface such as β-1,3-glucans (BGs), lipopolysaccharides (LPSs), and peptidoglycans (PGs) (Fearon and Locksley, 1996; Iwanaga and Lee, 2005; Lee et al., 2000). In arthropods, the major proteins that function as PRPs are: (1) β-glucan binding or recognition protein (BGBP or BGRP), (2) LPS and β-1,3-glucan binding protein (LGBP), (3) Gram-negative binding or recognition protein (GNBP or GNRP), and (4) peptidoglycan binding or recognition protein (PGBP or PGRP) (Christophides et al., 2004; Du et al., 2007; Dziarski, 2004; Lee and Söderhäll, 2002). Subsequent to recognition, a series of immune responses are activated by these PRPs,

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including encapsulation, phagocytosis, and nodule formation (Lackie, 1988), clotting cascade, the synthesis of a wide array of antimicrobial peptides, and the prophenoloxidase system (proPO) (Hoffmann et al., 1996; Iwanaga et al., 1994; Lai et al., 2011).

In the present study, we cloned and characterized the LGBP of *C. quadricarinatus*. The main objectives of the present study were to: (1) clone and identify the full-length cDNA of *CqLGBP* from *C. quadricarinatus*, (2) compare its sequence with other known LGBPs and BGBPs from other decapod crustaceans, (3) recombinant express CqLGBP and prepare the antiserum, (4) investigate the *CqLGBP* mRNA transcription in different tissues, and (5) examine the temporal transcription of *CqLGBP* mRNA in *C. quadricarinatus* after stimulation by two aquatic crustacean pathogens, *Spiroplasma eriocheiris* (Wang et al., 2011) and *Aeromonas hydrophila*. The results should help to clarify the roles of CqLGBP in the immune response of *C. quadricarinatus*.

2. Materials and methods

2.1. Animal and RNA isolation

C. quadricarinatus (100 ± 5 g) were purchased from a market in Nanjing, China, and cultivated in 100 l tanks. Intermolt crayfish that appeared to be healthy by visual inspection were used in all experiments. *C. quadricarinatus* was identified to species level by morphology and PCR using 18s sequence (GenBank No. AF235966). The prawns were acclimated for 10 days before the treatment. The hemolymph from crayfish was drawn using a 2-ml syringe, mixed and quickly added into anticoagulant solution (glucose, 2.05 g; citrate, 0.8 g; NaCl, 0.42 g; double distilled water was added to 100 ml, pH 5.0). Samples were immediately centrifuged at 2000 g, 4 °C for 10 min to collect the hemocytes. Total RNA was extracted using TRIzol Reagent (Takara) following the manufacturer's protocol. The total RNA concentration was determined by measuring the absorbance at OD_{260}. Electrophoresis was used to check the RNA integrity.

2.2. cDNA synthesis and gene cloning

A total of 5 μg RNA was reverse-transcribed into cDNA with an M-MLV RTase cDNA Synthesis Kit (Takara, Japan) according to the manufacturer's instruction. The sequences of degenerate primer pairs, *CqLGBP*-F and *CqLGBP*-R (Table 1) were designed based on the highly conserved nucleotide of *LGBP* gene using the CLUSTAL program and used to clone the sequence of the *CqLGBP* gene of *C. quadricarinatus*. PCR amplicons were size separated and visualized on an ethidium bromide stained 1.2% agarose gel. Amplicons of expected sizes were purified with an Agarose Gel DNA Purification Kit (Takara, Japan), and then subcloned into the pMD-19T cloning vector (Takara, Japan). Positive clones containing inserts of an expected size were sequenced using M13 primers, and sequenced at Invitrogen, Shanghai.

Table 1
Primers used for cloning and real-time quantitative analyses of *CqLGBP*.

Name	Sequence (5′–3′)
Primers designed to clone CqLGBP gene	
CqLGBP-F	TCCGGYGGWGGRAACTGGGARTTCCA
CqLGBP-R	GATCAGGTAGAACTTYTGGTCGAANG
CqLGBP-3F1	CGGTCTATCAGCTATACTCGAG
CqLGBP-3F2	GCTCTTCATTAAACCGGATCTG
CqLGBP-3F3	CTGTGGGGGATGAATG
CqLGBP-5R1	CATTCATCCCCCACAG
CqLGBP-5R2	CAGATCCGGTTTAATGAAGAGC
CqLGBP-5R3	CTCGAGTATAGCTGATAGACCG
CqLGBP-ORF-F	GGCCCGGGATGAAGACACTGTGCTTGCTG
CqLGBP-ORF-R	CGGCGGCCGCCTACTGGTCGACACTCTCC
Real-time quantitative primers	
CqLGBP-qPCR-F	CAGCGGTGAGATTGACATT
CqLGBP-qPCR-R	TGGAAACTGTTAGCGAAGG
β-actin-F	ATCACTGCTCTGGCTCCTGCTACC
β-actin-R	CGGACTCGTCGTACTCCTCCTTGG

2.3. Rapid amplification of cDNA ends (RACE) of CqLGBP

The *CqLGBP* gene partial cDNA sequence from *C. quadricarinatus* was extended using 5′ and 3′ RACE (SMART™ cDNA kit). A total of three gene-specific primers (Table 1) were designed based on the gene partial cDNA sequences. The 3′ RACE PCR reaction was carried out in a total volume of 50 μl containing 2.5 μl (800 ng/μl) of the first-strand cDNA reaction as a template, 5 μl of 10× Advantage 2 PCR buffer, 1 μl of 10 mM dNTPs, 5 μl (10 mM) gene-specific primer, 1 ml of Universal Primer A Mix (UPM; Clonetech, USA), 34.5 μl of sterile deionized water, and 1 U 50× Advantage 2 polymerase mix (Clonetech, USA). For the 5′ RACE, UPM was used as forward primers in PCR reactions in conjunction with the reverse gene-specific primers. PCR amplification conditions for both the 3′ and 5′ RACE were as follows: 5 cycles at 94 °C for 30 s, 72 °C for 3 min; 5 cycles at 94 °C for 30 s, 70 °C for 30 s, and 72 °C for 3 min; 20 cycles at 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min. After linking into the vector, the samples were sequenced at Invitrogen.

2.4. Nucleotide and amino acid sequence analysis

The homology search for the nucleotide and protein sequences was performed using the BLAST algorithm at NCBI (http://www.ncbi.nlm.nih.gov/). The deduced amino acid sequences were analyzed with the Expert Protein Analysis System (http://us.expasy.org/tools/). The signal peptide and motif were predicted by the SignalP 3.0 program (http://www.cbs.dtu.dk/services/SignalP/) and Motif scan program (http://hits.isb-sib.ch/cgi-bin/PFSCAN), respectively. The full-length CqLGBP sequence was compared with LGBPs, BGBPs, GNBPs and β-1,3-glucanase (GNs) from other organisms. Amino acid sequences for various species were retrieved from the NCBI and analyzed using the ClustalW Multiple Alignment program (http://www.ebi.ac.uk/clustalw/). An unrooted phylogenetic tree was constructed based on the amino sequence alignment by the neighbor-joining (NJ) algorithm embedded in the MEGA 6 program. The reliability of the branching was tested by bootstrap resampling (1000 pseudo-replicates).

2.5. Protein expression and antibody preparation

CqLGBP-ORF-F and *CqLGBP*-ORF-R were designed to amplify the ORF of the *CqLGBP* gene. The PCR product was digested with restriction enzymes (SmaI and NotI). After digestion with restriction enzymes, the product was ligated into the pGEX-6P-2 expression vector. The resulting recombinant plasmids pGEX-LGBP were transformed to *E. coli* BL21 (DE3) (Trans, China) for protein expression. The recombinant proteins were analyzed by SDS-PAGE. After the denaturation, renaturation and purification by protein PAGE recovery kit (Sangon, China), New Zealand White rabbits were immunized with 100 μg CqLGBP that was homogenized in complete Freund's adjuvant for three times at 2-week intervals. A boost injection in incomplete Freund's adjuvant was given for another week. Rabbit serum was collected 7 days after the last immunization. Antibody titer was determined from the immunized rabbits by ELISA. Optical density was measured at 450 nm with a Model 680 micro-plate reader (Bio-Rad, USA). When the optical density value of a dilution ratio above 2 times that of control and next dilution ratio below 2 times that of control, the antibody titer is defined at this dilution ratio.

2.6. Microorganism binding assay

Two species of microorganisms including *S. eriocheiris* and *A. hydrophila* were used for the assay. Approximately 500 μl of