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Identification of an anti-lipopolysacchride factor possessing both antiviral and antibacterial activity from the red claw crayfish *Cherax quadricarinatus*

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ABSTRACT

It is well-known that anti-lipopolysacchride factors (ALFs) are involved in the recognition and elimination of invading pathogens. In this study, the full-length ALF cDNA sequence of the red claw crayfish *Cherax quadricarinatus* (termed *CqALF*) was cloned from a suppression subtractive hybridization library constructed using red claw crayfish hematopoietic tissue cell (Hpt cell) cultures following challenge with white spot syndrome virus (WSSV). The full-length cDNA sequence of *CqALF* was 863 bp, and the open reading frame encoded 123 amino acids with a signal peptide in the N-terminus and a conserved LPS-binding domain. Unlike most ALFs, which are highly expressed in haemocytes, high expression levels of *CqALF* were detected in epithelium, the stomach and eyestalks, while lower expression was detected in Hpt, nerves, the heart, muscle tissue, gonads, haemocytes, intestines, gills and the hepatopancreas. To further explore the biological activities of *CqALF*, mature recombinant *CqALF* protein (rCqALF) was expressed and purified using a eukaryotic expression system, and an antimicrobial activity test was carried out. rCqALF clearly exerted antiviral activity, as evidenced by the severe disruption of the envelope of intact WSSV virions following co-incubation of virions with rCqALF. Additionally, pre-incubation of WSSV with rCqALF resulted in both a significant reduction in WSSV replication in red claw crayfish Hpt cell cultures and an increased survival rate among animals. Furthermore, rCqALF was effective against both Gram-negative bacteria and Gram-positive bacteria, particularly *Shigella flexneri* and *Staphylococcus aureus*. A membrane integrity assay suggested that rCqALF was unlikely to disrupt bacterial membrane integrity compared to cecropin P1. Taken together, these data suggest that *CqALF* may play an important role in immune defence in the crustacean *C. quadricarinatus*.

1. Introduction

Antimicrobial peptides (AMPs) are small cationic molecules that are ubiquitously found in all kingdoms of living organisms and are among the first lines of immune defence against microbial invasion [1]. Unlike traditional antibiotics, AMPs rarely result in bacterial drug-resistance [2]. Therefore, as potentially bioactive substances, AMPs are expected to replace traditional antibiotics. To date, many AMPs, including astacidin [3], crustin [3–5] and anti-lipopolysacchride factor (ALF) [6], have been characterized from crayfish species. ALF is a cationic AMP capable of binding and neutralizing lipopolysaccharide (LPS) [7] and is a crucial bioactive substance in crustacean innate immunity. Since ALF was first identified from the American horseshoe crab *Limulus polyphemus* [8], numerous ALFs have been found in other crustaceans such as *Pacifastacus leniusculus*, *Litopenaeus vannamei*, *Fenneropenaeus chinensis*, *Homarus americanus* and *Scylla paramamosain* [6,9–12]. Although different ALF isoforms possess low similarity, they exhibit a common feature: the LPS-binding domain, a disulphide loop formed by two conserved cysteine residues, which is considered to be the key functional domain for ALF antibacterial activity [7]. Both recombinant ALF proteins and synthetic peptides of the LPS-binding domain exhibit different antimicrobial activities against Gram-positive and Gram-negative bacteria [13,14]. In addition to

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antibacterial activity, ALF antiviral activities have also been reported. For instance, gene silencing of ALF in *P. leniusculus* via RNA interference specifically resulted in higher rates of white spot syndrome virus (WSSV) replication [6]. A synthesized LPS-binding domain of *L. polyphemus* was able to inhibit the entry of HIV-1, HCV and HSV1 into host cells [15]. The recombinant protein ALFPm3 exerts antiviral activity by interacting with WSSV structural proteins [16]. All of these data suggest that ALFs play important roles in immune defence against microbial infection. Thus, further functional characterization of *CqALF* in terms of antimicrobial activity, particularly antiviral properties, will bring new insight for disease control in aquaculture.

The red claw crayfish *C. quadricarinatus*, introduced to China from Australia in the 1990s, is an important aquaculture crustacean species with high economic value. It has been reported that red claw crayfish can be infected by WSSV, a mortal pathogen of both shrimp and crayfish aquaculture, and crayfish Hpt cell cultures are a good cell model for the study of WSSV infection [17,18]. Previously, a partial sequence of *CqALF* was screened from a suppression subtractive hybridization (SSH) library constructed from hematopoietic tissue (Hpt) cell cultures of *C. quadricarinatus* post- WSSV challenge [17]. Investigation of the role of *CqALF* in immunity, particularly its anti-WSSV activity, will be useful for WSSV disease control in aquaculture. In the present study, the full-length cDNA sequence of *CqALF* and its gene expression profiles in different tissues were investigated. Furthermore, recombinant *CqALF* protein (rCqALF) was expressed and purified with a eukaryotic expression system and assessed against both WSSV and bacteria in an antimicrobial activity test. The results indicated that *CqALF* may play an important role in the innate immune defence against microbial infection in the crustacean *C. quadricarinatus*.

2. Materials and methods

2.1. Animals and tissue collection

Healthy *C. quadricarinatus* crayfish, averaging 48 ± 5 g in body weight, were obtained from the Tenglong Company, Xianyou, Fujian Province, China, and acclimatized in aerated freshwater at 26 °C for one week before sample collection. Haemocytes were obtained with a sterile syringe and centrifuged for 10 min at 1000*g* (4 °C). Other tissues (stomach, gonad, muscle, nerve, intestine, heart, Hpt, hepatopancreas, gill, epithelium and eyestalk) were sampled from three random individuals for total RNA isolation.

2.2. RNA extraction and cDNA synthesis

Total RNA from tissues (described above) was isolated using Trizol reagent (Roche, Mannheim, Germany) according to the manufacturer's instructions. RNase-Free DNase I (Ambion, Austin, Texas, USA) was used to eliminate genomic DNA contamination in the extracted RNA following the manufacturer's protocol. The extracted RNA was evaluated with a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) and analysed by 1.0% agarose gel electrophoresis. Then, cDNA was synthesized using a PrimeScript™ RT Reagent Kit (TaKaRa) according to the manufacturer's instructions.

2.3. Gene cloning of the full-length CqALF cDNA

A partial *CqALF* cDNA sequence of *C. quadricarinatus* was isolated from an SSH library upon WSSV infection in our previous study [17]. To obtain the full-length cDNA sequence of *CqALF*, 5′- and 3′-RACE were carried out using a SMART RACE cDNA Amplification Kit (Clontech, Madison, Wisconsin, USA) following the manufacturer's protocol. The gene-specific primers for RACE, RACE3F and RACE5R, are shown in Table 1. The PCR conditions were as follows: 5 min at 94 °C; 30 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 60 s; and 72 °C for 7 min. All amplified PCR products were gel-purified using a Gel Extraction Kit (Dongsheng Biotech, Co., Ltd., Beijing, China), and the expected DNA fragments were ligated into a PMD18-T vector (TaKaRa). The vectors were transformed into competent *E. coli* DH5α cells, and the recombinants were identified via resistance selection on ampicillin-containing LB plates incubated overnight at 37 °C. Bacterial colony PCR was performed to screen positive colonies as previously described [19]. The positive clones were picked for sequencing at Shanghai Genewindows Biotech Co. Ltd (China). Then, the full-length cDNA sequence of *CqALF* was assembled using SeqMan software.

2.4. Bioinformatics analysis

The *CqALF* open reading frame (ORF) and amino acid sequence were analysed using the ExPASy translate tool (http://web.expasy.org/translate/). Protein sequences homologous to *CqALF* were identified by BLAST searching (http://blast.ncbi.nlm.nih.gov/Blast). The signal peptide was predicted using the SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). The conserved domain was identified using the Conserved Domain database of NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi/). Putative glycosylation site analysis was carried out using the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). An ALF phylogenetic tree was constructed with Mega 6.06 using the Neighbour-Joining method based on sequence alignments of *CqALF* with other homologous amino acid sequences.

2.5. CqALF transcript distribution in various tissues

As mentioned earlier, twelve tissues were dissected from three random healthy crayfish and prepared for total RNA isolation and cDNA synthesis as described above. To determine the *CqALF* mRNA transcript levels in the different tissues, qRT-PCR was performed using an ABI PCR machine (Applied Biosystems 7500, UK). A pair of specific primers (Q-F/Q-R in Table 1) was designed using Primer Premier 5.0 to amplify a target product from the cDNA, and the crayfish 16S ribosomal gene (GenBank: AF135975.1) was employed as an internal standard. *CqALF* expression levels relative to 16S gene expression were quantified using the $2^{-\Delta\Delta Ct}$ method [20]. The primers designed for 16S gene amplification (16S-F and 16S-R) are shown in Table 1. qRT-PCR reactions were performed in a 96-well PCR plate. The reaction volume consisted of 20 μl of mixture containing 10 μl of SYBR Green Master (2×) (Roche, USA), 0.5 μl of each primer (10 μM), 5.0 μl of cDNA, and 4 μl of sterile water. The qRT-PCR program was as follows: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The amplification specificities of *CqALF* and 16S were confirmed by

Table 1
Primers used in the experiments.

Primers	Sequences (5′–3′)
RACE3F	ATCTGTCGCTTGTTGCTGAGTG
RACE5R	GAGAAGCATCAACGCGGAGC
16S-F	AATGGTTGGACGAGAAGGAA
16S-R	CCAACTAAACACCCTGCTGATA
Q-F	GCTGGACTGTGGGATAATGGC
Q-R	GATGCTTCTCCTCTGATGGTGGT
E-F	CCGGAATTCCAGATTACAGAGGCTCTGG
E-R	GCTCTAGATTAATGATGATGATGATGGTGAGTTTTCAAAAAATCTGTTGC
IE1-F	CTGGCACAACAACAGACCCTACC
IE1-R	GGCTAGCGAAGTAAAATATCCCCC