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An anti-lipopolysaccharide factor from red swamp crayfish, *Procambarus clarkii*, exhibited antimicrobial activities *in vitro* and *in vivo*

Chen Sun [a,b], Wen-Teng Xu [a,b], Hong-Wei Zhang [a,b], Li-Ping Dong [a,b], Ting Zhang [a,b], Xiao-Fan Zhao [a,b], Jin-Xing Wang [a,b,*]

[a] The Key Laboratory of Plant Cell Engineering and Germplasm Innovation of Ministry of Education, School of Life Sciences, Shandong University, Jinan, Shandong 250100, China
[b] Shandong Provincial Key Laboratory of Animal Cells and Developmental Biology, School of Life Sciences, Shandong University, Jinan, Shandong 250100, China

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ABSTRACT

The anti-lipopolysaccharide factors (ALFs) are a group of effector molecules of innate immunity in arthropods, exhibiting binding and neutralizing activities to lipopolysaccharides. In this study, an ALF cDNA sequence (*PcALF1*) was identified from red swamp crayfish, *Procambarus clarkii*. The deduced peptide of *PcALF1* was conserved; it manifested the signal peptide and lipopolysaccharide (LPS)-binding domain, especially the two conserved cysteine residues at both ends of the domain. Transcripts of *PcALF1* were detected in multiple tissues. Results of quantitative real-time PCR exhibited that the expression level of *PcALF1* was induced by virus and Gram-positive and Gram-negative bacteria. Purified recombinant protein of PcALF1 revealed multiple biological activities: it gave all the tested bacteria and fungi a tight binding; it could bind microbial polysaccharides (LPS, LTA, and β-glucan) as well. *In vitro*, the antimicrobial activity assay was demonstrated as a broad spectrum against Gram-positive and Gram-negative bacteria and a fungus. The rPcALF1 also exhibited a clearance activity on *Vibrio anguillarum* in a dose-dependent manner *in vivo*

1. Introduction

In the process of evolution, both vertebrates and invertebrates have developed a system, referred to as innate immunity, as their first line of defense against diverse pathogens [1–3]. As the counterpart of adaptive immunity, innate immunity refers to an ancient defense mechanism in terms of phylogeny [4,5]. Once invaded by pathogens, a series of humoral and cellular immune responses of this defense mechanism is activated, including the production of antimicrobial peptides (AMPs) [6,7]. AMPs are groups of proteins or peptides that function as innate immune effector molecules, and are widespread in multicellular organisms, including microorganisms, protozoa, invertebrate, vertebrate, and plants [8]. As an essential component of innate immune response, AMPs exhibit a broad spectrum of antimicrobial activity against bacteria, fungi, parasites, and viruses [9]. Thus, AMPs have become a focus of innate immunity field. More than 1500 AMPs have been identified, as mentioned in the Antimicrobial Sequences Database (http://aps.unmc.edu/AP/main.php). Among many AMPs, the anti-lipopolysaccharide factors (ALFs), a family of proteins, have attracted attention of researchers.

ALF is a basic peptide exhibiting binding and neutralizing activities to lipopolysaccharides (LPS) [10]. The first ALF was found in amebocytes from *Limulus polyphemus* and *Tachypleus tridentatus*, a potent anticoagulant inhibiting the endotoxin-mediated activation of the coagulation cascade [11]. Thereafter, an increasing number of studies have been reported on the identification and characterization of ALFs in arthropods, consequently providing additional pertinent information. All these ALFs usually have a signal peptide and a conserved LPS-binding domain; within this domain formed between two preserved cysteine residues, there is a cluster of positively charged amino acids [12–14]. These typical characteristics are closely associated with the biological activities of ALFs, especially in relation to pathogens.

The expression of *Ss*ALF in the Indian mud crab, *Scylla serrata*, was demonstrated as having increased upon the LPS challenge [14]. Similar induction by LPS was also observed in the Kuruma prawn, *Marsupenaeus japonicus* [15,16]. Bacterial or fungal challenges could also induce the expression of ALF genes [12,13,15,17–19]. However, in *Litopenaeus vannamei*, the expression of *Lv*ALF1 did not change significantly when challenged with *Vibrio penaeicida* and *Fusarium oxysporum*. A slight but significant increase of *Lv*ALF1 mRNA level was detected in the white spot syndrome virus (WSSV)-infected

* Corresponding author. The Key Laboratory of Plant Cell Engineering and Germplasm Innovation of Ministry of Education, School of Life Sciences, Shandong University, Jinan, Shandong 250100, China. Tel/fax: 86 531 88364620.
E-mail address: jxwang@sdu.edu.cn (J.-X. Wang).

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L. vannamei [20]. Another similar up-regulated expression was reported for the freshwater crayfish, *Pacifastacus leniusculus*, after it was challenged by both active and UV-inactivated virus [21].

Many investigations have revealed that the ALF proteins manifest as a broad spectrum of anti-bacterial activity. LALF, the ALF from *L. polyphemus*, exhibited anti-bacterial activity against Gram-negative bacteria, but not against Gram-positive bacteria [10]. The experiment on ALP from *Penaeus monodon* demonstrated a spectrum of anti-fungal activity and anti-bacterial activities against both Gram-positive and Gram-negative bacteria [22]. The purified rEsALF presented bactericidal activity against both Gram-positive and Gram-negative bacteria [13]. Similarly, the recombinant *Ss*ALF expressed activity against both Gram-positive and Gram-negative bacteria [14]. A recent study on ALF from the Chinese mitten crab, *Eriocheir sinensis*, indicated that the purified EsALF2 displayed anti-bacterial activity against a Gram-negative bacterium and anti-fungal activity, but not against Gram-positive bacteria [12].

Corresponding with the expression patterns of ALFs, the anti-viral activities were also investigated by previous studies. In crayfish, *P. leniusculus*, the reduction of ALF transcripts by RNAi was accompanied by the increase in VP28 transcripts, suggesting that ALF affects WSSV replication [21]. A recent study showed that pre-incubation of WSSV with rALF*Pm*3 could decrease the VP28 expression in haematopoietic cell cultures of *P. leniusculus* and reduce the propagation of WSSV in *P. monodon* [23]. ALFs presented multiple biological activities against Gram-positive and Gram-negative bacteria, fungi, and even virus.

The ALFs displayed different functions in different animals. We have obtained several ESTs by random sequencing of the cDNA library from the red swamp crayfish, *Procambarus clarkii*. In order to understand their functions, an ALF cDNA was cloned and its expression patterns and functions were analyzed. Its transcription level was induced by virus, and Gram-positive and Gram-negative bacteria in hemocytes. Binding assay revealed that rPcALF1 could bind with all of the tested bacteria, fungi, and microbial polysaccharides (LPS, LTA, and β-glucan). *In vitro* experiments demonstrated the antimicrobial activity of rPcALF1 against Gram-positive and Gram-negative bacteria and a fungus. *In vivo*, rPcALF1 exhibited an activity of clearance of *Vibrio anguillarum* in a dose-dependent manner.

2. Materials and methods

2.1. Immune challenge of crayfish and collection of tissues and hemocytes

Red swamp crayfish *P. clarkii*; 10–15 g per crayfish) were purchased from an aquaculture market in Jinan, Shandong Province, China. They were cultured in tanks in the laboratory with air-pumped fresh water and placed at room temperature. For the bacterial challenge, *V. anguillarum* (a gift from Institute of Oceanology, Chinese Academy of Sciences) and *Staphylococcus aureus* (from Shandong Agricultural University) were used to introduce a challenge of Gram-positive or Gram-negative bacteria, respectively. Two kinds of bacteria (20 μL, 2×10^7 cells per crayfish) were injected into the third abdominal segment of the cultured crayfish. For the viral challenge, WSSV (50 μL, 3.2×10^7 copies per crayfish) was injected into the third abdominal segment of the crayfish. The WSSV inoculum was prepared and quantified according to previously described methods [24,25]. The control was injected with 20 μL crayfish saline buffer (CFS) (0.2 M NaCl, 5.4 mM KCl, 10 mM $CaCl_2$, 2.6 mM $MgCl_2$, and 2 mM $NaHCO_3$; pH 6.8) [26].

The hemolymph was extracted from the ventral sinus using 1/10 volume of the ice-cold anticoagulant buffer (0.14 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, and 10 mM EDTA, pH 4.6) [27,28] at 0, 12, 24, 48, 72, and 96 h after challenge. The hemocytes were then obtained by centrifugation (800 × *g*, 5 min, 4 °C) from the collected hemolymphs. The hearts, hepatopancreas, gills, stomachs, and intestines were also collected for further study.

2.2. Total RNA isolation and cDNA synthesis

Total RNA was extracted from hemocytes, hearts, hepatopancreas, gills, stomachs, and intestines with Unizol reagent (Biostar, Shanghai, China) according to manufacturer protocol. First-strand cDNAs were reverse-transcribed using the RevertAid First Strand cDNA Synthesis kit (Fermentas, Burlington, Canada).

2.3. Sequence analysis

Nucleotide sequence homology and comparisons of deduced amino acid sequence were accomplished by using the online program, BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The cDNA translation and deduced protein prediction were achieved with ExPASy (http://www.au.expasy.org/), while signal sequence and domain prediction utilized SMART (http://www.smart.embl-heidelberg.de/). The software programs, ClustalW and GENDOC, were used to perform alignments. The phylogenetic tree was constructed with MEGA 4 [29]. Sequence comparisons of cDNAs were carried out using the DNAMAN 5.2.2 software.

2.4. Semi-quantitative RT-PCR and qRT-PCR

The expression of *PcALF1* in different tissues was analyzed with semi-quantitative RT-PCR using primers, PcALF1 RT-F and PcALF1 RT-R (Table 1). PCR reaction of *PcALF1* was performed as follows: 2 min at 94 °C, 26 cycles of 94 °C for 30 s, 57 °C for 45 s, and 72 °C for 45 s. A final extension was carried out at 72 °C for 10 min. The 18S RNA was introduced as an inner reference with the primers 18S RNA RT-F and 18S RNA RT-R (Table 1).

Quantitative real-time PCR (qRT-PCR) was employed to study the expression profile of *PcALF1* in hemocytes following the methods described previously [30]. The cDNAs from hemocytes of unchallenged crayfish and from those of crayfish challenged with *S. aureus*, *V. anguillarum*, WSSV, and CFS at 12, 24, 48, 72, and 96 h after the challenge were used as templates. The same primers with the semi-quantitative RT-PCR of *PcALF1* were used in the qRT-PCR. The qRT-PCR was programmed at 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, 59 °C for 30 s, and 72 °C for 20 s. Next, they were melted from 60° to 95 °C. All samples were repeated in triplicate for the qRT-PCR analysis. The data obtained were calculated using $2^{-\Delta\Delta Ct}$, as described previously [31], and subjected to statistical analysis. Unpaired sample *t*-test was conducted, and a significant difference was accepted if $P < 0.05$.

2.5. Expression and purification of PcALF1 and preparation of antiserum

A pair of primers, PcALF1 ExF and PcALF1 ExR (*Eco*R I and *Xho* I sites are underlined; Table 1), were designed to amplify the cDNA

Table 1
Sequences of the primers used in this study.

Primer	Sequence (5′–3′)
PcALF1 RT-F	GAAGCGATGACGAGGAGCAAT
PcALF1 RT-R	GACGGGTTGGCACAAGAGC
PcALF1 ExF	TACTCAGAATTCCAGGTCCTGGAGGGTCTG
PcALF1 ExR	TACTCACTCGAGCTACCCATCAAGCCATGCCT
18S RNA RT-F	TCTTCTTAGAGGGATTAGCGG
18S RNA RT-R	AAGGGGATTGAACGGGTTA

The *Eco*RI and *Xho*I sites are underlined.