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Antilipopolysaccharide factor (ALF) of mud crab *Scylla paramamosain*: Molecular cloning, genomic organization and the antimicrobial activity of its synthetic LPS binding domain

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Abstract

Antilipopolysaccharide factors (ALFs) are small basic proteins that can bind and neutralize lipopolysaccharide (LPS) and have broad spectrum antimicrobial activities. In this study, we describe the isolation of the full-length cDNA encoding for ALF peptide (ALF*Sp*) of mud crab, *Scylla paramamosain* by sequencing a hemocyte cDNA library and using the rapid amplification cDNA end (RACE) method. A full-length ALF*Sp* cDNA of 614 bp contains an open reading frame (ORF) of 372 bp, encoding 123 amino acid protein with 26 residues signal sequence. The calculated molecular mass of the mature protein is 11.18 kDa. The highly two conserve cysteine residues and putative LPS binding domain were observed in ALF*Sp* peptide. Comparison of amino acid sequences revealed that ALF*Sp* shared high identity with other known ALFs and had an overall similarity of 65, 64, 63, 61 and 59% to those of *Fenneropenaeus chinensis*, *Litopenaeus vannamei*, *Marsupenaeus japonicus*, *Limulus polyphemus*, and *Tachypleus tridentatus*, respectively. A neighbour-joining tree showed a clear differentiation of each species and also indicated that ALF from *S. paramamosain*, *Carcinus maenas* and *Callinectes sapidus* are closely related phylogenetically. The genomic DNA sequence of ALF*Sp* gene consists of 1075 bp containing three exons and two introns. Tissue distribution analysis revealed that ALF*Sp* was abundantly expressed in hemocytes, intestine, and muscle but not in eyestalk. The synthetic ALF*Sp* peptide containing putative LPS binding domain revealed a strong antimicrobial activity against several bacteria especially on the growth of Gram-positive bacteria, *Micrococcus luteus* and Gram-negative bacteria, *Vibrio harveyi* suggested that ALF*Sp* could play an essential role in defense mechanism in *S. paramamosain*.
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Keywords: Mud crab; *Scylla paramamosain*; Antilipopolysaccharide factor; Antimicrobial peptide; LPS binding domain

1. Introduction

The mud crab, *Scylla paramamosain*, is the commercially important crustacean distributed widely throughout the Indo-Pacific region. Mud crabs are commonly found in estuaries and mangrove areas. Southern Thailand has long been a center for the capture and culture of the mud crab. Mud crabs are quite hardy organisms. Little is known of disease problems in the juvenile or grow-out phase of their culture. However, susceptibility to infection has been identified during the larval stages. Several bacterial and viral pathogens have been reported to infect crabs (Johnson, 1983).

Antimicrobial peptides (AMPs) are important components of the natural defenses of most living organisms against microbial invasion. In crab species, several AMPs have been found and characterized. The 6.5 kDa proline-rich cationic protein, with homology to bovine bactenecin 7 at the *N*-terminus has been isolated from the shore crab, *Carcinus maenas* (Schnapp et al., 1996). Callinectin is a cationic antimicrobial peptide of 3.7 kDa isolated from the blue crab *Callinectes sapidus* (Khoo et al., 1999). A cysteine-rich 11.5 kDa antibacterial peptide, carcinin, has been isolated from the granular hemocytes of the shore crab

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C. maenas (Relf et al., 1999). Recently, scygonadin, an anionic antimicrobial peptide has been isolated from seminal plasma of the mud crab, *Scylla serrata* (Wang et al., 2007).

Antilipopolysaccharide factors (ALFs) are small proteins that can bind and neutralize lipopolysaccharide (LPS). The first ALF, originally called LALF, were identified from the amebocyte of the horseshoe crab *Limulus polyphemus* (Tanaka et al., 1982). LALF is a 11.8 kDa protein which binds and neutralizes LPS and has a strong antibacterial effect on Gram-negative *R*-type bacteria (Morita et al., 1985). From the crystal structure analysis, the LPS binding domain of LALF is presented in a loop conformation (Hoess et al., 1993). Synthetic peptide analogues of LALF have been made and the site of the activity and specific sequence needed to bind and neutralize LPS have been determined (Kloczewiak et al., 1994).

Recently, several ALFs have been isolated and characterized from hemocytes in five species of shrimps; *Fenneropenaeus chinensis*; ALF*Fc* (Liu et al., 2005, *Marsupenaeus japonicus*; M-ALF (Nagoshi et al., 2006), *Penaeus monodon*; ALF*Pm*3 (Somboonwiwat et al., 2005), *Litopenaeus setiferus* (Gross et al., 2001) and *Pacifastacus leniusculus* (Liu et al., 2006). Nevertheless, a number of ALFs from horseshoe crabs and shrimps are well characterized, little is known about the ALF in hemocytes of portunid species. To date, no ALF sequences have been identified in mud crab *S. paramamosain*. Although, some of ALFs were discovered in EST library of shore crab, *C. maenas* and blue crab, *C. sapidus* (Towle and Smith, 2006), these ALF molecules were not precisely characterized. Moreover, the antimicrobial activity of these proteins was not clarified.

Therefore, in the present study, the EST library was constructed from hemocytes of *S. paramamosain*. We reported for the first time the molecular cloning and characterization of the full-length cDNA of the ALF*Sp* gene by RACE-PCR. The genomic structure of the ALF*Sp* gene was determined. The synthetic LPS binding domain of ALF*Sp* peptide was evaluated for ability to inhibit the growth of various pathogenic bacteria. The expression profile of ALF*Sp* in different tissues of *S. paramamosain* was also investigated.

2. Material and methods

2.1. Sample preparation

Mud crabs (*S. paramamosain*) were purchased from local fish markets in Bangkok, Thailand. The hemolymph was collected in an anticoagulant solution of 10% (w/v) of trisodium citrate dihydrate pH 4.6. Hemocytes were isolated by centrifugation at 800 g for 10 min at 4 °C and immediately preserved in liquid nitrogen.

2.2. RNA isolation and genomic DNA extraction

Total RNA was extracted from hemocytes using TRIzol Reagent (Gibco-BRL, USA). Contaminant DNA was eliminated by incubation of the RNA with 1 unit of DNase I for 15 min at 37 °C. The mRNA was isolated by QuickPrep mRNA purification kit (Amersham Biosciences, USA). Genomic DNA was extracted from muscle tissue by homogenized in lysis buffer (100 mM Tris–HCl, 50 mM EDTA, and 100 mM NaCl; pH 8.0, 1% SDS, 200 μg/ml Proteinase K). The mixture was extracted with standard phenol/chloroform method.

Table 1
Primer sequences used for amplification of *S. paramamosain* ALF*Sp* gene

Primer	Sequence (5′–3′)
ALF1	5′-GGACAGAAGAAACATTGAGGACGACGCA-3′
ALF2	5′-ACCAAGCTTCCCTCAAGATGCG-3′
ALF3	5′-GGAAATCAAAAACATCCATTACAGGTCA-3′
ALF1n	5′-ACCAAGCTTCCCTCAAGATGCG-3′
ALF3n	5′-CTTCCTCGTTGTTTTCACCCTCCTG-3′
ALF4	5′-CAGTATGAAGCTCTGGTAGCTTCCATTC-3′
ALF5	5′-CTTCCTCGTTGTTTTCACCCTCCTG-3′
EF-1α-F	5′-GGTGCTGGACAAGATGAAGGA-3′
EF-1α-R	5′-CGTTCCGGTGATCATGTTCTTGATG-3′
pUC1	5′-CCGGCTCGTATGTTGTGTGGA-3′
pUC2	5′-GTGGTGCAAGGCGATTAAGTTGG-3′

2.3. Construction of an EST library

Three micrograms of mRNA were reverse-transcribed to cDNA using a SuperScript™ Plasmid System for cDNA Synthesis and Plasmid Cloning Kit (Gibco BRL, USA). Size-fractionated cDNA was ligated to a *Not* I/*Sal* I digested pSPORT1. The ligated product was transformed into *Escherichia coli* JM109. The insert sizes were verified by colony PCR using pUC1 and pUC2 primers (Table 1). Plasmid DNA was extracted and sequenced with M13 forward or reverse primers on an automated DNA sequencer (Macrogen Inc., Korea).

2.4. Rapid amplification of cDNA end (RACE)-PCR

The full-length cDNA of ALF*Sp* gene was isolated through RACE-PCR. The 3′-RACE cDNA were performed with SMART RACE cDNA Amplification kit (Clontech, USA) according to the manufacturer's protocol. Briefly, aliquots of mRNA (3.0 μg) were pre-heated for 5 min at 70 °C and immediately cooled on iced water for 10 min. The first strand cDNA was synthesized using PowerScript reverse transcriptase with 3′-RACE CDS primer for 2 h at 42 °C. Gene specific primers (ALF1 and ALF2) were designed from EST sequence of an ALF*Sp* homologue from the *S. paramamosain* hemocyte cDNA library. RACE-PCR was performed with primer ALF1 and universal primer A mix, followed by nested PCR with a second set of primer ALF2 and nested universal primer A. The amplification reaction is described in Amparyup et al. (2007). PCR conditions were as follows: 5 cycles consisting of 94 °C for 45 s, 65 °C for 45 s and 72 °C for 1.30 min; and 25 cycles consisting of 94 °C for 45 s, 60 °C for 45 s and 72 °C for 1.30 min. The final extension was carried out at 72 °C for 10 min. The expected DNA fragment was eluted from agarose gel and ligated to pGEM®-T Easy vector (Promega, USA). The ligation product was transformed to *E. coli*. The recombinant clone was identified by colony PCR. Plasmid DNA was extracted and sequenced.

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