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Two isoforms of anti-lipopolysaccharide factors identified and characterized from the hemocytes of portunid crabs, *Portunus pelagicus* and *Scylla tranquebarica*

V.V. Afsal[a], Swapna P. Antony[a], E.R. Chaithanya[a], I.S. Bright Singh[b], Rosamma Philip[a,*]

[a] Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, CUSAT, Fine Arts Avenue, Kochi 682016, Kerala, India
[b] National Center for Aquatic Animal Health (NCAAH), CUSAT, Fine Arts Avenue, Kochi 682016, Kerala, India

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ABSTRACT

Anti-lipopolysaccharide factors (ALFs), a type of cationic antimicrobial peptides (AMPs), and their derivatives are becoming predominant candidates for potential drugs in viral and bacterial diseases. This study reports the first ALF from the mud crab *Scylla tranquebarica* (StALF, JQ899453) and the second ALF isoform from the blue swimmer crab *Portunus pelagicus* (PpALF2, JQ899452). Both sequences encoded for precursor molecules, starting with a signal peptide containing 26 amino acid residues, followed by a highly cationic mature peptide, containing two conserved cysteine residues flanking a putative lipopolysaccharide (LPS)-binding domain. BLAST analysis revealed that both PpALF2 and StALF exhibited significant similarity with crustacean ALF sequences. The predicted molecular mass of the mature ALFs was 11.2 kDa with an estimated pI of 10.0. PpALF2 and StALF also showed the typical pattern of alternating hydrophobic and hydrophilic residues in their putative disulphide loop, suggesting that they comprise the same functional domain. Phylogenetic analysis showed that PpALF2 and StALF have similar evolutionary status and they were phylogenetically ancient immune effector molecules which may play an essential role in the host defense mechanism. The spatial structures of PpALF2 and StALF possessed four beta-strands and two alpha-helices. The results indicated that there were more than one ALF involved in crab immunity against various pathogens. ALFs would provide candidate promising therapeutic or prophylactic agents in health management and diseases control in crustacean aquaculture.

1. Introduction

Crustaceans live in the aquatic environment where they are exposed to a large number of micro-organisms causing health hazards. Since there is no adaptive immunity in crustaceans, the whole burden of anti-pathogen defense falls on the innate immune system, and the antimicrobial peptides (AMPs) play an important role in invertebrate innate immune defense. Anti-lipopolysaccharide factors (ALFs) are a type of cationic AMPs, that are evolutionarily conserved across a wide range of marine invertebrates, including the ancient horseshoe crabs and crustaceans and are found to possess broad spectrum activities against gram-positive and gram-negative bacteria, fungi, and even virus (Antony et al., 2011; Liu et al., 2006; Ponprateep et al., 2012). Crustacean ALFs have also been proved to possess cell-penetrating ability and anti-cancer activity. ALFs belong to the group of single domain AMPs with a signal peptide at the N-terminal region followed by a conserved LPS-binding domain. The LPS-binding domain, which is the characteristic feature of ALFs, are formed between two conserved cysteine residues which form a disulphide loop, and contain a cluster of positively charged residues within it (Hoess et al., 1993). This typical structure makes ALFs capable of binding and neutralizing lipopolysaccharides (LPS).

The first ALF was isolated from the amoebocytes of the horseshoe crab *Limulus polyphemus* (Tanaka et al., 1982) and found to have a strong antibacterial effect on gram-negative R-type bacteria. Reports on crustacean ALFs have been increasing in recent years viz., in shrimps (Tharntada et al., 2008); crabs (Afsal et al., 2011, 2012); lobsters (Beale et al., 2008) and crayfishes (Sun et al., 2011). Some decapods have also been reported to express multiple ALF isoforms which vary in length or sequence and display different biological activities.

Crab culture is facing constraints in production due to severe health problems resulting in large scale mortality. Understanding the defense mechanisms of crab may be effective in the development of better disease control strategies in farming. The identification and characterization of immune effectors are believed to be helpful for elucidation of immune defense mechanisms and disease control in crab aquaculture because of their potential use as therapeutic agents and genetic improvement as biomarkers on disease-resistant strain selection. Many AMPs have been identified and characterized in crabs till date

* Corresponding author. Tel.: +91 484 2368120; fax: +91 484 2381120.
E-mail addresses: rosammap@gmail.com, rose@cusat.ac.in (R. Philip).

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viz., callinectin, carcinin, scygonadin, crustin and ALF. There into, crustin and ALF are the most important AMPs in crabs due to their peculiar antimicrobial function. The growing number of relatively conserved ALF genes identified with apparently conserved functions being characterized across taxa seems to indicate the likely importance of ALF in crabs besides the AMPs of the crustin family. Though there are several studies, regarding the molecular characterization, gene organization, expression analysis and functional studies of ALFs in crustaceans, there is hardly any record on ALFs from the blue swimmer crab *Portunus pelagicus* and the mud crab *Scylla tranquebarica*, the decapod crustaceans belonging to the brachyuran family Portunidae. *P. pelagicus* and *S. tranquebarica* are among the widely cultured crab species having high commercial value. However, molecular structure, feature and phylogentic studies on AMP genes are still lesser in *P. pelagicus* and *S. tranquebarica*. The present study is an attempt to identify and characterize sequences coding for ALFs in the hemocytes of these two commercially important crabs. The identification of ALFs in these crabs will bring interesting insight into the crab defense mechanisms as well as disease control in crab culture systems.

2. Materials and methods

Live specimens of the blue swimmer crab, *P. pelagicus* was obtained directly from the Cochin Barmouth region and the mud crab, *S. tranquebarica* from a culture site along the stream of Cochin Backwaters in Vypeen, India. Hemolymph was collected from the base of abdominal appendages using specially designed capillary tubes (RNase-free) rinsed with pre-cooled anticoagulant solution (RNase free Sodium citrate (10%), pH 7.0).

Total RNA was extracted from the hemocytes using TRI Reagent (Sigma) following manufacturer's protocol. RNA was quantified by spectrophotometry at 260 and 280 nm. Only RNAs with absorbance ratios (A_{260}:A_{280}) greater than 1.8 were used for the present work. First strand cDNA was generated in a 20 μl reaction volume containing 5 μg total RNA, 1× RT buffer, 2 mM dNTP, 2 μM oligo d(T_{20}), 20 U of RNase inhibitor and 100 U of M-MLV Reverse transcriptase (Fermentas, Inc.). The reaction was conducted at 42 °C for 1 h followed by an inactivation step at 85 °C for 15 min. PCR amplification of 1 μl of cDNA was performed in a 25 μl reaction volume containing 1× standard Taq buffer (10 mM Tris–HCl, 50 mM KCl, pH 8.3), 3.5 mM $MgCl_2$, 200 μM dNTPs, 0.4 μM each primer and 1 U Taq DNA polymerase (Fermentas Inc.). PCR amplifications were performed using the forward primer (5′-ggacagaagaaacattgaggacgacgca-3′) and reverse primer (5′-ggaaatcaaaaacatccattacaggtca-3′), designed using GeneTool software based on consensus sequences of ALFs in GenBank. The thermal profile used was 94 °C for 2 min followed by 35 cycles of 94 °C for 15 s, 60 °C for 30 s and 68 °C for 30 s and a final extension at 68 °C for 10 min. PCR products were analyzed by electrophoresis in 1.5% agarose gels in TBE buffer, stained with SYBR Safe and visualized under UV light. The PCR products were purified and sequenced with ABI Big Dye Terminator Cycle Sequencing Kit and analyzed in the ABI prism 377 Automated DNA sequencer at SciGenom, India.

The sequence homology and the deduced amino acid sequence comparisons were carried out using BLAST algorithm (BLASTn and BLASTp) at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast). Gene translation and prediction of deduced proteins were performed with ExPASy (http://www.au.expasy.org/). The signal peptide was predicted

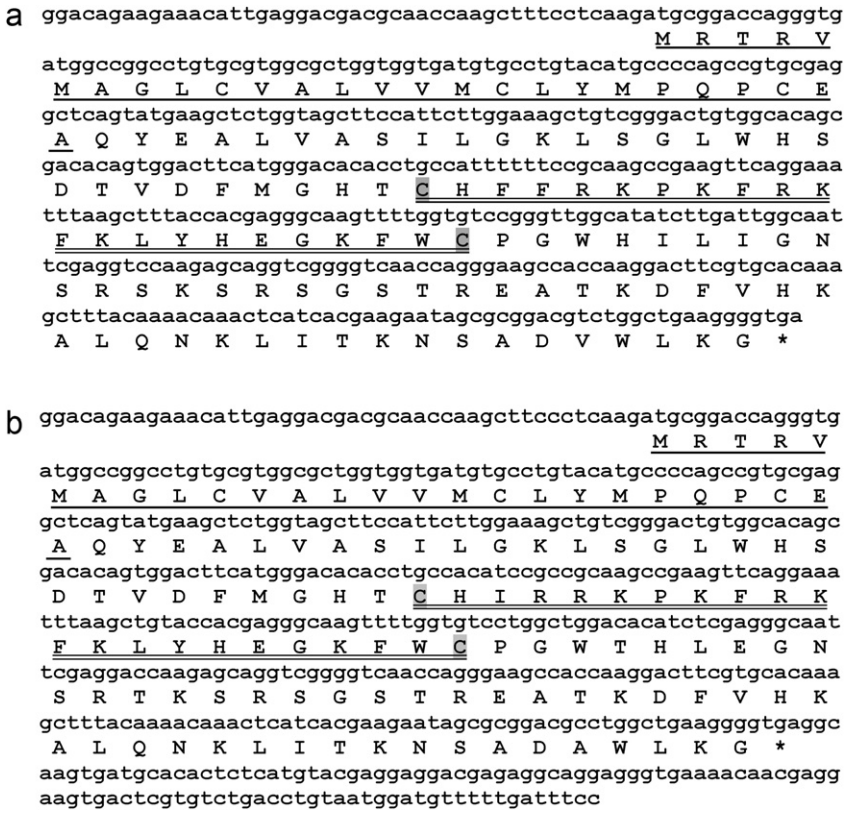

Fig. 1. (a) Nucleotide and amino acid sequences of PpALF2 (JQ899452). The underlined amino acid residues indicate a putative signal sequence. LPS binding domain characteristic of the ALF family is double underlined and the two conserved cysteine residues important for one disulfide bond (loop) formation is highlighted in gray. An asterisk is the stop codon. (b) Nucleotide and amino acid sequences of StALF (JQ899453). The underlined amino acid residues indicate a putative signal sequence. LPS binding domain characteristic of the ALF family is double underlined and the two conserved cysteine residues important for one disulfide bond (loop) formation is highlighted in gray. An asterisk is the stop codon.