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# A new isoform of anti-lipopolysaccharide factor identified from the blue swimmer crab, *Portunus pelagicus*: Molecular characteristics and phylogeny

V.V. Afsal [a], Swapna P. Antony [a], V.N. Sanjeevan [b], P.R. Anil Kumar [a], I.S. Bright Singh [c], Rosamma Philip [a,*]

[a] Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, CUSAT, Fine Arts Avenue, Kochi-16, Kerala, India
[b] Centre for Marine Living Resources and Ecology, 6th Floor, C-Block, Kendriya Bhavan, P.B. No. 5415, CSEZ P.O., Kochi-682 037, India
[c] National Center for Aquatic Animal Health (NCAAH), CUSAT, Fine Arts Avenue, Kochi-16, Kerala, India

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ABSTRACT

Anti-lipopolysaccharide factors are small proteins that bind and neutralize lipopolysaccharide and exhibit potent antimicrobial activities. This study presents the molecular characterization and phylogenetic analysis of the first ALF isoform (Pp-ALF1; JQ745295) identified from the hemocytes of *Portunus pelagicus*. The full length cDNA of Pp-ALF1 consisted of 880 base pairs encoding 293 amino acids with an ORF of 123 amino acids and contains a putative signal peptide of 24 amino acids. Pp-ALF1 possessed a predicted molecular weight (MW) of 13.86 kDa and theoretical isoelectric point (*pI*) of 8.49. Two highly conserved cysteine residues and putative LPS binding domain were observed in Pp-ALF1. Peptide model of Pp-ALF1 consisted of two α-helices crowded against a four-strand β-sheet. Comparison of amino acid sequences and neighbor joining tree showed that Pp-ALF1 has a maximum similarity (46%) to ALF present in *Portunus trituberculatus* followed by 39% similarity to ALF of *Eriocheir sinensis* and 38% similarity to ALFs of *Scylla paramamosain* and *Scylla serrata*. Pp-ALF1 is found to be a new isoform of ALF family and its characteristic similarity with other known ALFs signifies its role in protection against invading pathogens.



## 1. Introduction

Fifteen divergent families of AMPs or single peptides sharing common molecular features with the known AMP families displaying antimicrobial activities against a number of specific microorganisms have been identified and characterized in a number of decapod crustaceans (Rosa and Barracco, 2010). Anti-lipopolysaccharide factors (anti-LPS factors or ALFs) are the potent antimicrobial peptides that can bind and neutralize lipopolysaccharides (LPS) forming a key effector molecule of the innate immune system in crustaceans. ALFs contain a signal peptide and a conserved LPS-binding domain consisting of a conserved cluster of positively charged residues within the disulfide loop (Hoess et al., 1993; Muta et al., 1987; Somboonwiwat et al., 2008; Yang et al., 2009). These peptides are part of a very well-characterized family of crustacean AMPs with different subgroups and variants that are either encoded by distinct genes or generated by alternative mRNA splicing (Tharntada et al., 2008). The high diversity of the variants (subgroups and isoforms) found in most crustacean AMP classes can presumably confer a broad spectrum of activity to a single AMP family. Limulus ALF has proved to possess strong antibacterial activity, especially on the growth of G-negative R type bacteria (Wang et al., 2002). Crustacean ALFs are also multifunctional proteins exhibiting a potent (MIC<6.25 μM) and broad spectrum antimicrobial activity against a large number of both Gram-positive and Gram-negative bacteria, including several opportunistic/pathogenic *Vibrio* species, fungi and human enveloped virus (Carriel-Gomes et al., 2007; Somboonwiwat et al., 2005; Yedery and Reddy, 2009). ALF has been reported to have a specific role in reducing white spot syndrome viral replication in crayfishes and shrimps (Antony et al., 2011; Liu et al., 2006). Although more than 200 penaeidin and crustin relative sequences have been registered in the GenBank to date, information on ALFs of crustaceans is comparatively less. ALFs were first purified from the hemolymph cells (amoebocytes) of two marine chelicerate arthropods, the horseshoe crabs, *Limulus polyphemus* and *Tachypleus tridentatus* (Tanaka et al., 1982). In crustaceans, homologues to horseshoe crab ALFs were first identified from the hemocytes of the penaeid shrimp species *Litopenaeus setiferus* (Gross et al., 2001) and *Penaeus monodon* (Supungul et al., 2002) using an EST approach. To date, the genes encoding ALFs have been identified only in decapod crustaceans, penaeid shrimps (Ponprateep et al., 2012; Tassanakajon et al., 2010), freshwater prawns (Lu et al., 2009; Ren et al., 2012; Rosa et al., 2008), crayfish (Liu et al., 2012), lobster (Beale et al., 2008) and crabs (Afsal et al., 2011; Imjongjirak et al., 2007; Li et al., 2008; Liu et al., 2012; Yedery and Reddy, 2009; Yue et al., 2010). However, there is hardly any published work on major AMP families present in the blue swimmer crab, *Portunus pelagicus*. This species is a large Portunid crab found in shallow waters between 10 and 50 m depth, including areas near reefs, mangroves, sea grass and algal beds. *P. pelagicus* is

* Corresponding author. Tel.: +91 4842368120; fax: +91 4842381120.
*E-mail addresses:* rosammap@gmail.com, rose@cusat.ac.in (R. Philip).

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similar to *Portunus trituberculatus* in its general appearance but, easily distinguished because it has four frontal teeth (three in case of *P. trituberculatus*) and possesses three spines in the merus of chelipeds (four in case of *P. trituberculatus*). *Portunus sanguinolentus* is easily distinguished by their three prominent maroon to red spots on posterior 1/3 of carapace. *P. pelagicus* is a highly prized commercially important crab and their characteristic features such as fast growth, ease of larviculture, high fecundity and relatively high tolerance to both nitrate and ammonia make this species ideal for aquaculture.

Studies on characterization and structure elucidation of ALFs in *P. pelagicus* may be useful in understanding the immune defense mechanisms in crabs giving new insights into disease control in crab aquaculture. This paper presents the molecular characterization and phylogenetic analysis of a new isoform of ALF identified from *P. pelagicus* and this could be the first report of an antimicrobial peptide from the species.

## 2. Materials and methods

Healthy adults of *P. pelagicus* (~250 g body weight) were collected from the backwater stream along Vypeen, Kochi, India. Hemolymph was collected from the base of abdominal appendages using specially designed capillary tubes (RNase-free) rinsed using pre-cooled anticoagulant solution (RNase free 10% sodium citrate, pH 7.0). Total RNA was extracted from the hemocytes using TRI Reagent (Sigma) following the manufacturer's protocol. RNA was quantified by spectrophotometry at 260 and 280 nm. 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 primers were designed using GeneTool software based on consensus sequences of already reported ALFs of decapods. Amplifications were performed using the primers Pp-ALF1-F (5′-agggagtgggtgatgagcta-3′) and Pp-ALF1-R (5′-tacggctattacgatccaaca-3′). 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. Purified PCR products were sequenced at SciGenom, India.

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gttgttatcatctgttgcccttgctgcttccctccaccttcttccctcatgatgaaaaaa
                                                      M  K  K
ggagtggccgccctgctgcggctgctgctggtgatgaggctgctgctgccgccccttct
 G  V  A  A  L  L  R  L  L  L  V  M  R  L  L  L  P  P  P  S
tgcgatcctcatgacgatcctctggcttcttctcttcttgaaatcctcgctgggcggtgc
 C  D  P  H  D  D  P  L  A  S  S  L  L  E  I  L  A  G  R  C
cacaactcctctgatgacttggtgggcccctgctgcttcctccgccgcccccctatcata
 H  N  S  S  D  D  L  V  G  P  C  C  F  L  R  R  P  P  I  I
agaatatttctgctgcaccacgagggctttttttggtgtgctgggtggccgcctttcgat
 R  I  F  L  L  H  H  E  G  F  F  W  C  A  G  W  P  P  F  D
gggtcgacgaggacaaacaggacggcggcgtcctcggaggacgccaccgacgacttgcgg
 G  S  T  R  T  N  R  T  A  A  S  S  E  D  A  T  D  D  L  R
cgcgctttttacacaaactactcatcccacaggatgatgctccgcggcggatgaacaac
 R  A  F  L  H  K  L  L  I  P  Q  D  D  A  P  R  R  M  N  N
taaagaagaagagatgagctgcggaggacgacgaggagaataatgataacaacatagaaa
 *
ttgccggacgacttgttattatatatttttttcctcagactagttggtggatgctgcgt
tgttgatgcaatgatggaagtagagaacgtgaaggaacgactatgatgtgaagataccac
ctcaaaaaatttttttttattatttttttatgaggtagcctcgtcatttttttcttctct
tttcctccaccatcgccgttgatgctagactggtggacgacaatgaagagataaagaagt
agatgatattattatttctaccactacatctttttatatgaggaacaaggagggccgcc
tcctctttttgttgttgtggtagtgggacgactatctttttttttgtgtgtgggagga
agaaagaaaagaaaaatatctttatttgtacttgagctgt
```

**Fig. 1.** Nucleotide and amino acid sequences of Pp-ALF1 from the hemocyte of the blue swimmer crab, *Portunus pelagicus* (JQ745295). 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.

**Table 1**
Result of BLASTp analysis of Pp-ALF1 (JQ745295).

| Accession no. | Description of the AMP | Query coverage | E value | Max identity |
|---|---|---|---|---|
| ADU25043 | Anti-lipopolysaccharide factor isoform 2 [*Portunus trituberculatus*] | 88% | 3e −23 | 46% |
| ADU25072 | Anti-lipopolysaccharide factor [*Portunus trituberculatus*] | 77% | 1e −20 | 46% |
| ADZ46233 | Anti-lipopolysaccharide factor 3 [*Eriocheir sinensis*] | 85% | 7e −16 | 39% |
| ABP96981 | Anti-lipopolysaccharide factor [*Scylla paramamosain*] | 94% | 5e −18 | 38% |
| ADW11095 | Anti-lipopolysaccharide factor [*Scylla serrata*] | 92% | 8e −18 | 38% |

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 protein were performed with ExPASy (http://www.au.expasy.org/). The signal peptide was predicted by SignalP program (http://www.au.expasy.org/). The multiple sequence alignments were performed with amino acid sequences of known ALFs from decapod crustaceans using CLUSTALW and GENDOC. Amino acid sequences of all known ALFs were retrieved from the NCBI GenBank and phylogenetic tree was constructed by the Neighbor-Joining (NJ) method using MEGA version 4.0 (Tamura et al., 2007). The structural models of the AMPs were created using SWISS-MODEL server. The nucleotide and deduced amino acid sequences of the antimicrobial peptide were submitted to NCBI GenBank.

## 3. Results and discussion

In the present study, an AMP belonging to ALF family was identified and characterized from the hemocytes of *P. pelagicus*, herein after referred to as Pp-ALF1. An 880 bp fragment cDNA encoding an ORF of 123 amino acids was obtained from the mRNA of *P. pelagicus* hemocyte by RT-PCR (Fig. 1). BLAST analysis of the nucleotide sequences revealed the relation of Pp-ALF1 to other ALFs present in decapod crustaceans. Pp-ALF1 showed only 46% similarity to ALF isoforms present in *P. trituberculatus* followed by 39% similarity to ALF of *Eriocheir sinensis* and 38% similarity to ALFs of *Scylla paramamosain* and *Scylla serrata* (Table 1). Analysis with the SignalP software revealed the presence of a signal peptide with 24 amino acid residues at the N-terminal region of the Pp-ALF1 (Fig. 1). The mature peptide consisted of 123 amino acid residues with a predicted

**Fig. 2.** Structural model of Pp-ALF1 (JQ745295) of *Portunus pelagicus* created using SWISS-MODEL server.

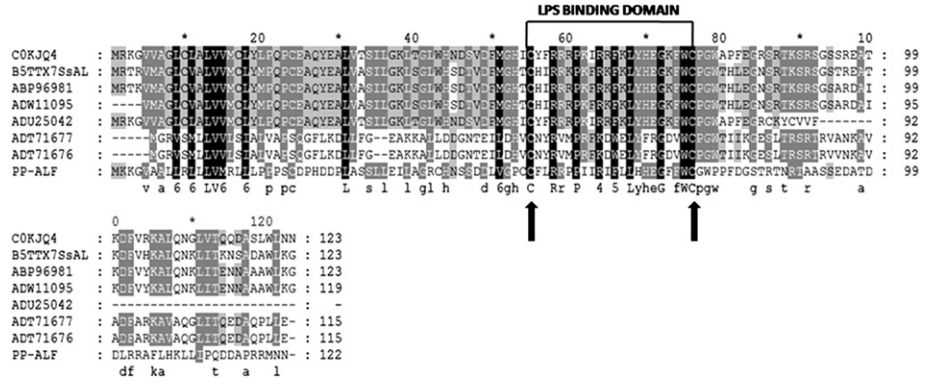


**Fig. 3.** Multiple alignment of nucleotide sequence of the Pp-ALF1 (JQ745295) with other ALFs obtained using GeneDoc program Version 2.7.0. The LPS-binding domains are enclosed with a bracket. The conserved cysteine residues are marked with arrowheads below the alignment. Black and gray indicate conserved sequences.

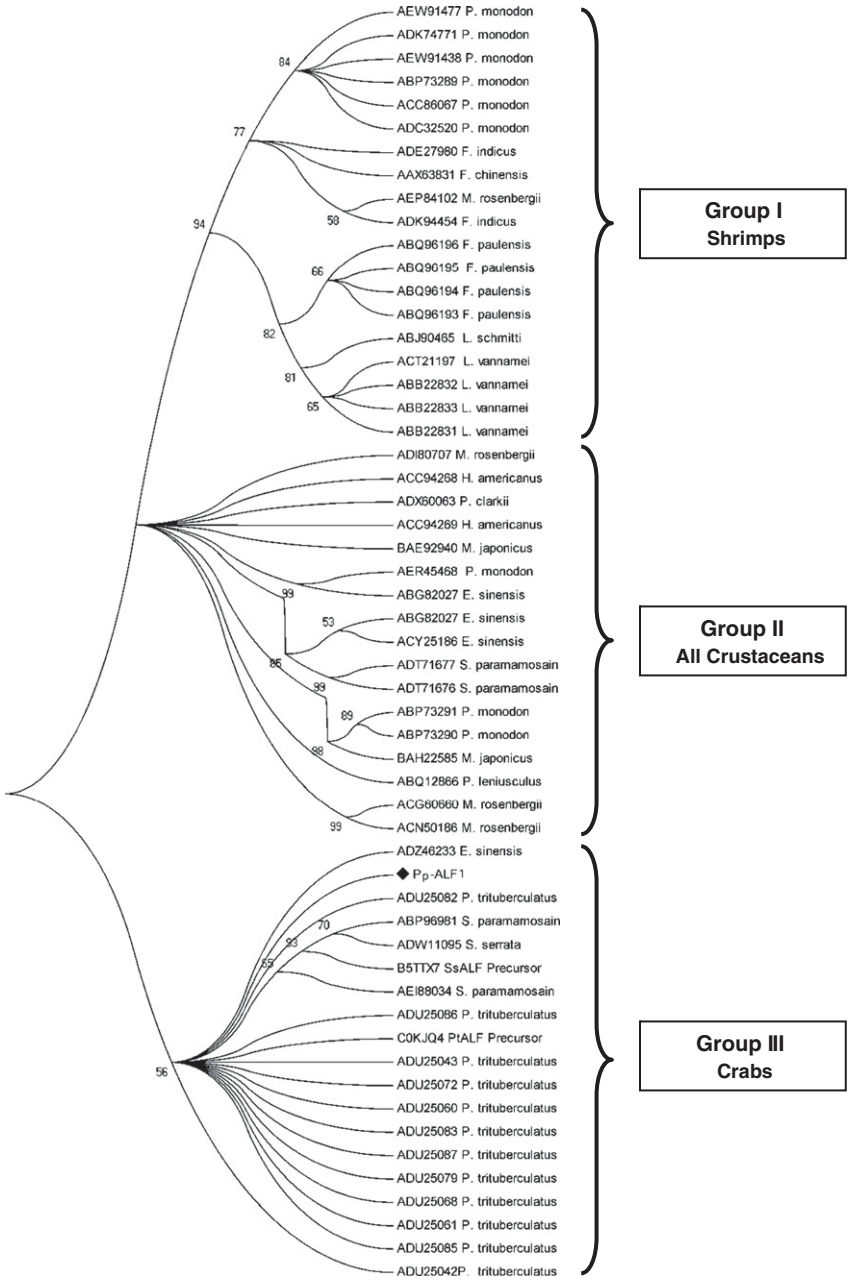


**Fig. 4.** A bootstrapped neighbor-joining tree obtained using MEGA version 4.0 illustrating relationships between the deduced amino acid sequences of the Pp-ALF1 (JQ745295) with other ALFs of decapod crustaceans. Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.

molecular weight (MW) of 13.86 kDa. The Pp-ALF1 was highly cationic and the isoelectric point (*pI*) was estimated to be 8.49 as predicted by the PROTPARAM software. The sequence was deposited in the NCBI GenBank under accession number JQ745295.

Sequence comparison of Pp-ALF1 peptide revealed that it possessed a signal peptide and conserved amino acid residues in the region of LPS binding domain (Fig. 1). The deduced amino acid sequence of Pp-ALF1 showed a 24 amino acid domain from residue 54 through 77 that was necessary for LPS binding and neutralization (Imjongjirak et al., 2007). The Pp-ALF1 molecule also showed the conservation of two cysteine residues at positions $Cys^{55}$ and $Cys^{76}$, important for one disulfide bond (loop) formation in the peptide (Fig. 1). The deduced amino acid sequence of Pp-ALF1 was found to be rich in positively charged amino acid residues, arginine (10.6%) forming a cluster within the disulfide loop regarded as the functional domain of ALF as described by Imjongjirak et al. (2007). Peptide model of Pp-ALF1 created using SWISS-MODEL server consisted of two α-helices crowded against a four-strand β-sheet. Two of the β-strands are in turn linked by a disulfide bond to form an amphipathic loop rich in cationic amino acid side chains (Fig. 2). Multiple alignment performed for Pp-ALF1 with other ALFs revealed the presence of conserved regions within the sequence (Fig. 3).

The phylogenetic relationship between Pp-ALF1 and other ALFs of decapod crustaceans was analyzed using neighbor-joining (NJ) method (Fig. 4). The phylogenetic tree clearly showed that the ALF sequences clustered according to species. The crab and shrimp ALFs were found to cluster separately. The tree could be broadly divided into three major groups. Group I consisted of ALFs from shrimps; whereas Group II consisted of ALFs from all crustaceans including shrimps, crabs, crayfishes and lobsters. Group III was found to consist of ALFs found in crabs alone and Pp-ALF1 belonged to this group (Group III). The analysis showed that Pp-ALF1 is more closely related to crab ALFs rather than to the shrimp, crayfish and lobster ALFs. Also, within the crab cluster, ALFs of *Scylla* sp. grouped together and were found to be different from that of the *Portunus* and *Eriocher* sp. Also, Pp-ALF1 was found to be different from the other ALF isoforms of *P. trituberculatus*. The bootstrap distance tree calculated clearly indicated Pp-ALF1 to be a new isoform of the ALF family. The detection of ALF in the blue swimmer crab indicated Pp-ALF1 to be an immune effector molecule that may play an essential role in defense mechanism of these crabs. Also, molecular phylogenetic tree based on amino acid sequence suggests that all the ALF members are of the same ancestral origin, which has subsequently diverged at different phases of evolution (Fig. 4).

To conclude, this report presents the characterization and phylogenetic analysis of the first ALF isoform (Pp-ALF1) from the commercially important *P. pelagicus*. Discovery of novel AMPs and its antimicrobial spectrum might pave way to unravel the obscurity of crustacean immunity. Further research on the expression profile of these molecules in response to various environmental conditions and microbial infection would reveal their role in the protection of the animals from the onslaught of diseases.

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