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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

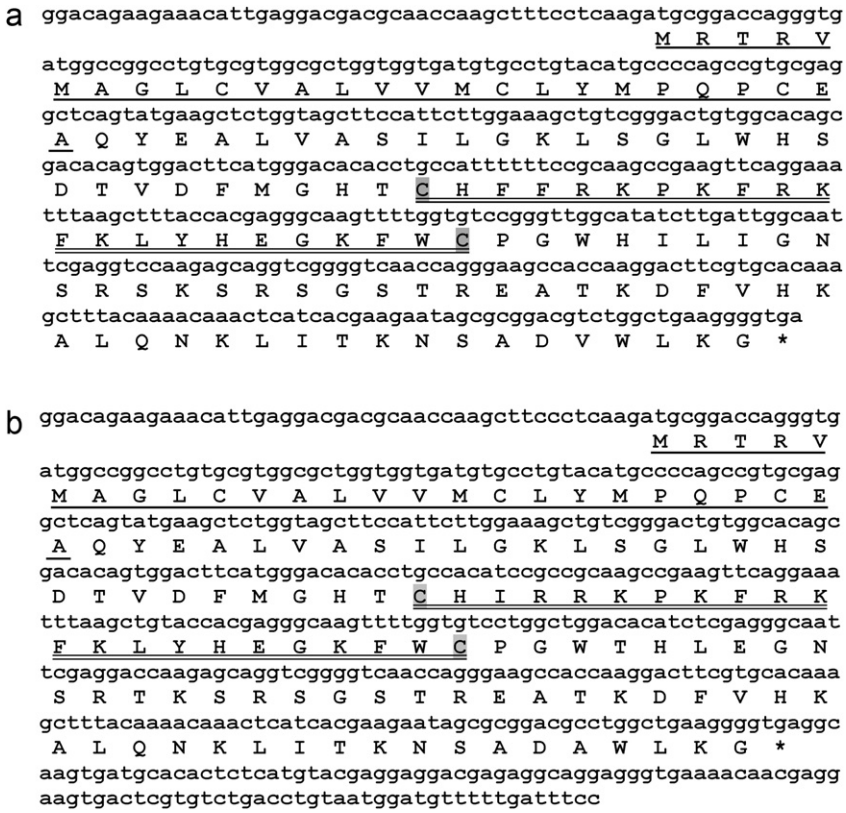

Fig. 1. (a) Nucleotide and amino acid sequences of PpALF2 (JQ899452). The underlined amino acid residues indicate a putative signal sequence. LPS binding domain characteristic of the ALF family is double underlined and the two conserved cysteine residues important for one disulfide bond (loop) formation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

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 GeneDoc. Amino acid sequences of all known ALFs were retrieved from GenBank (NCBI) and phylogenetic and molecular evolutionary analyses was conducted by the neighbor-joining (NJ) method using MEGA version 5 (Tamura et al., 2011). The structural models of the AMPs were created using SWISS-MODEL server. The nucleotide sequences and deduced amino acid sequences of the antimicrobial peptides were submitted to GenBank.

3. Results and discussion

cDNA sequences with homology to the ALFs of crustaceans were identified in the hemocytes of the portunid crabs, *P. pelagicus* and *S. tranquebarica* (Fig. 1a and b). This study reports for the first time an ALF isoform from *S. tranquebarica* designated as StALF (JQ899453). The study also reports the second ALF isoform from *P. pelagicus*, designated as PpALF2 (JQ899452) to distinguish PpALF1 (JQ745295) identified in our previous study (Afsal et al., 2012).

A 417 bp nucleotide sequence representing the complete cDNA sequence of PpALF2 with an open reading frame (ORF) of 372 bp encoding a polypeptide of 123 amino acid residues was obtained by RT-PCR. Similarly, a 522 bp nucleotide sequence representing the complete cDNA sequence of StALF with an open reading frame (ORF) of 372 bp encoding a polypeptide of 123 amino acid residues was also obtained. The obtained ALF sequences encoded for precursor molecules, starting with a signal peptide containing 26 amino acid residues, followed by a highly cationic mature peptide. The conserved characteristics and high similarity with known ALFs showed that PpALF2 and StALF belonged to the ALF family. The predicted molecular mass of both the PpALF2 and StALF was 11.2 kDa with an estimated pI of 10.0. The mature form of PpALF2 and StALF contained 97 amino acid residues; 95–98 being the characteristic range of amino acids reported for ALFs of crustaceans. The sequences of PpALF2 and StALF were submitted in NCBI GenBank under the accession numbers JQ899452 and JQ899453 respectively.

The N-terminus of both PpALF2 and StALF had the consistent features with a signal peptide as defined by SignalP program analysis, with a putative cleavage site located after position 26 (CEA-QY). The mature peptides of PpALF2 and StALF contained the two characteristic conserved cysteine residues forming a disulphide loop, which possess a cluster of positive charges within it. This region of the mature peptide is defined as the LPS-binding domain (Imjongjirak et al., 2007). These conserved cysteine residues were found at the positions C_{29} and C_{50} in PpALF2 and StALF (Fig. 2). The conserved region is believed to be essential for the antimicrobial activity and for the stability of the 3D structure of ALFs (Yang et al., 2009).

The deduced amino acid sequence of PpALF2 was found to be rich in positively charged amino acid residues, lysine (12.4%) and arginine (5.2%). In StALF, the lysine and arginine percentages were 12.4 and 6.2 respectively. In addition, these molecules contained the highly hydrophobic N-terminal region and a consensus amino acid sequence (W/T)CP(G/S)W characteristic of all ALFs. Previous reports have proved that the hydrophobicity is important for the

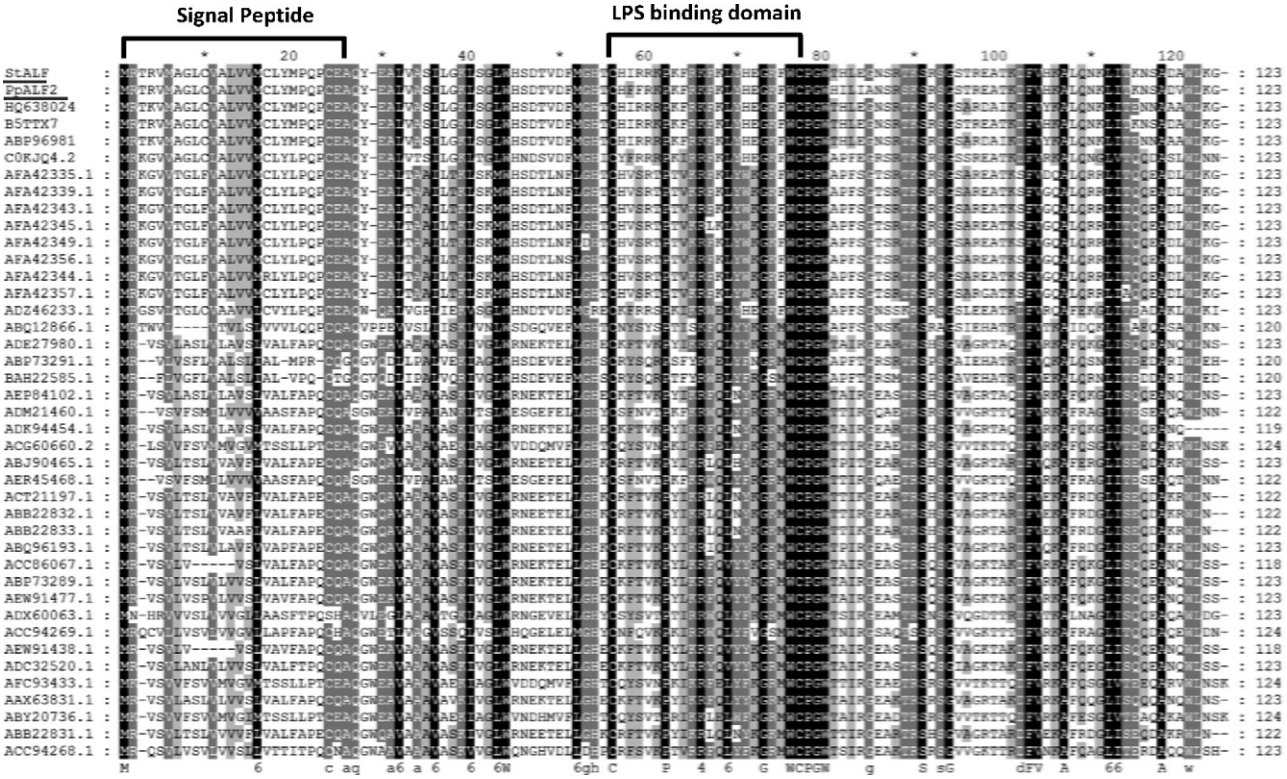

Fig. 2. Multiple alignment of amino acid sequences of PpALF2 (JQ899452) and StALF (JQ899453) with other ALFs (*Scylla serrata* (HQ638024), *S. serrata* precursor (B5TTX7), *Scylla paramamosain* (ABP96981), *Portunus trituberculatus* precursor (C0KJQ4), *Portunus trituberculatus* (AFA42335, AFA42339, AFA42343, AFA42344, AFA42345, AFA42349, AFA42356 and AFA42357), *Eriocheir sinensis* (ADZ46233), *Pacifastacus leniusculus* (ABQ12866), *Fenneropenaeus indicus* (ADE27980 and ADK94454), *Fenneropenaeus chinensis* (AAX63831), *Penaeus monodon* (ABP73289, ABP73291, ACC86067, ADC32520, ADM21460, AER45468, AEW91438 and AEW91477), *Marsupenaeus japonicus* (BAH22585), *Macrobrachium rosenbergii* (ACG60660, AEP84102 and AFC93433), *Litopenaeus schmitti* (ABJ90465), *L. vannamei* (ABB22831, ABB22832, ABB22833 and ACT21197), *Farfantepenaeus paulensis* (ABQ96193), *Procambarus clarkii* (ADX60063), *Homarus americanus* (ACC94268 and ACC94269), *Macrobrachium olfersii* (ABY20736)) obtained using GeneDoc programme Version 2.7.0. The alignment was performed with ClustalW and edited with GeneDoc software. The three levels of shading indicate different degrees of conservation. Black background and white letters correspond to 100% conservation, gray background and white letters correspond to 80% conservation, and gray background and black letters correspond to 60% conservation.

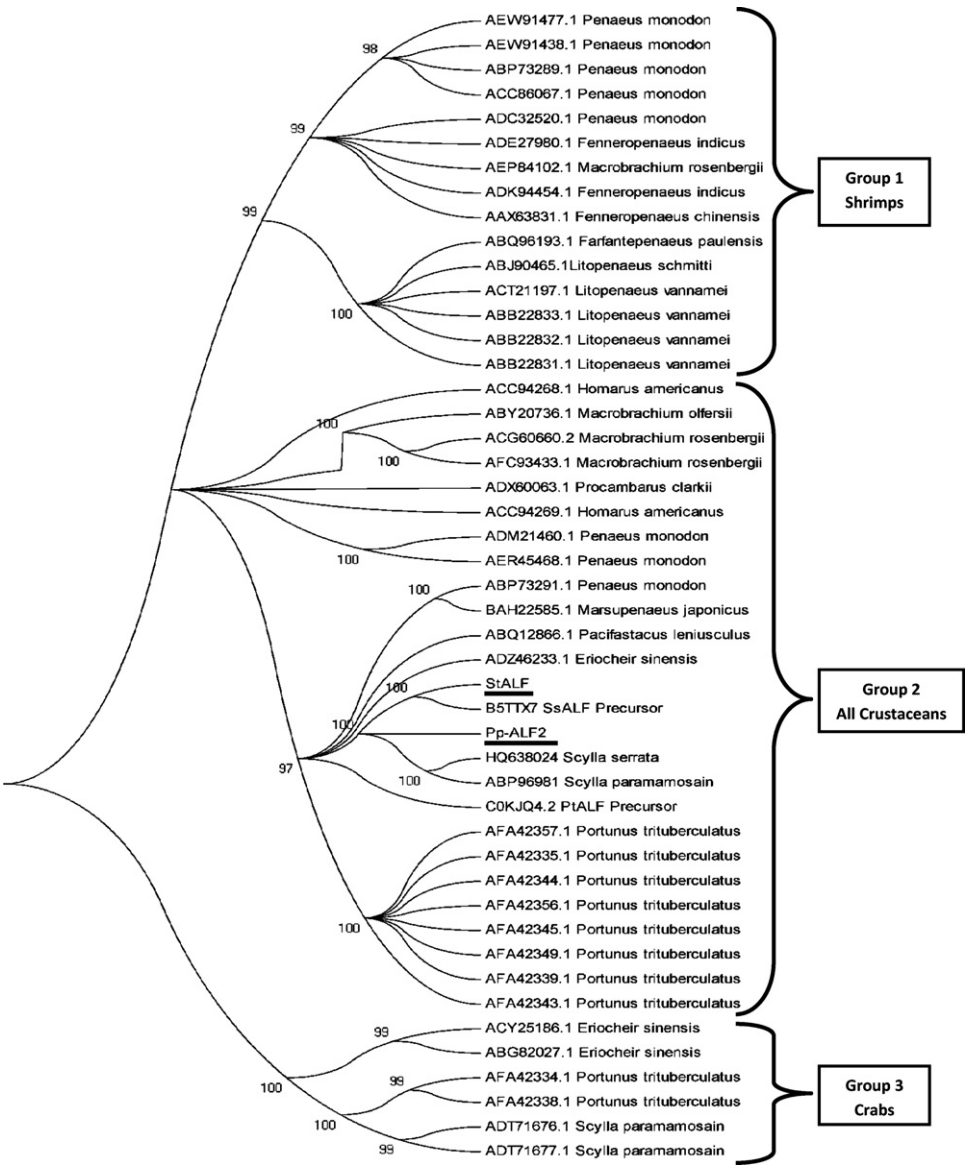

Fig. 3. A bootstrapped neighbor-joining tree obtained using MEGA version 5.0 illustrating relationships between the deduced amino acid sequences of PpALF2 (JQ899452) and StALF (JQ899453) with other ALFs of decapod crustaceans. Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced amino acid sequences.

peptide to bind LPS (Wasiluk et al., 2004). Like other ALF molecules, 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. Synthetic peptides, corresponding to the ALF putative LPS-binding domain of *Limulus* have proved to possess an efficient LPS neutralizing activity (Nagoshi et al., 2006). Likewise, similar synthetic peptides, based on ALF sequences of crustaceans have also showed potent antimicrobial activity (Imjongjirak et al., 2007).

PpALF2 contained a highly hydrophobic amino-terminal region including 10 hydrophobic residues in a total of 38 residues. Moreover, out of the 17 positively charged amino acids, 7 positively charged amino acids and a tryptophan (Trp75) were clustered within the disulfide loop consisting of 22 residues. In contrast, StALF also contained a highly hydrophobic amino-terminal region as found in PpALF2, but contained only 9 hydrophobic residues in a total of 35 residues. Out of the 20 positively charged amino acids, 10 positively charged amino acids and a tryptophan (Trp75) were clustered within the disulfide loop consisting of 22 residues. This characteristic of portunid ALFs was found to be a bit dissimilar to the horseshoe crab and shrimp ALFs, where, out of the 28 residues 12 hydrophobic residues have been reported in the LPS domain and out of the 17 positively charged amino acids, 9 positively charged amino acids and a tryptophan (Trp75) were found to be clustered within the disulfide loop consisting of 22 residues (de la Vega et al., 2008). These features suggest both PpALF2 and StALF to have an amphipathic factor. Also, the similarities in the pI of the two AMPs indicate that antibacterial function or mode of action of both PpALF2 and StALF may also be similar.

BLAST analysis of the deduced amino acid sequences of PpALF2 and StALF revealed significant identities only with the ALFs of crustaceans and not with any other groups including limulids. PpALF2 and StALF shared a significant identity with portunid ALFs (67–93%) and shared low homology with the ALFs of *Eriocheir sinensis*, crayfishes and shrimps (less than 61%) (Table 1).

Multiple alignment of PpALF2 and StALF with members of ALF family revealed that two cysteine residues (C_{29} and C_{50}) were highly conserved in portunid ALFs. Whereas in case of *E. sinensis*, the

Table 1
Result of BLASTp analysis of PpALF2 (JQ899452) and StALF (JQ899453).

GenBank accession no.	Description	Query coverage	Identity to PpALF2	Identity to StALF
ABP96981	Anti-lipopolysaccharide [*Scylla paramamosain*]	100%	87%	93%
ADW11095	Anti-lipopolysaccharide [*Scylla serrata*]	100%	87%	93%
ACM89169	Anti-lipopolysaccharide factor [*Portunus trituberculatus*]	99%	75%	76%
ADZ46233	Anti-lipopolysaccharide factor 3 [*Eriocheir sinensis*]	99%	61%	61%
ABP73291	Anti-lipopolysaccharide factor isoform 2 [*Penaeus monodon*]	96%	44%	45%
BAH22585	Anti-lipopolysaccharide factor 2 [*Marsupenaeus japonicus*]	96%	43%	44%
ABQ12866	Anti-lipopolysaccharide factor [*Pacifastacus leniusculus*]	82%	51%	53%

two conserved cysteine residues were found at C_{27} and C_{48} (Zhang et al., 2010). Twenty residues (H_{30}, R_{33}, K_{34}, P_{35}, K_{36}, F_{37}, R_{38}, K_{39}, F_{40}, K_{41}, L_{42}, Y_{43}, H_{44}, E_{45}, G_{46}, K_{47}, F_{48}, W_{49}) found in between the cysteine residues (C_{29} and C_{50}) in the beta-strand of portunid ALFs were also found to be conserved for the two ALF isoforms, except in case of F_{31}, F_{32} of PpALF2 and I_{31}, R_{32} in case of StALF. The biological functions of residue replacement between C_{29} and C_{50} need to be further investigated. The differences in the sequences of the LPS binding site might indicate the ability of these two ALF isoforms to bind to different microbial cell wall components.

To evaluate the molecular evolutional relationships of PpALF2 and StALF against other ALF family members, a phylogenetic tree was constructed based on the 45 amino acid sequences of ALF members by the neighbor-joining method (Fig. 3). Phylogenetic analysis of ALFs further revealed that ALF sequences were clustered according to species. Phylogenetic trees also 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. PpALF2 and StALF were closely related to crab ALFs rather than to the other groups. There were three distinct groups in the phylogenetic tree. The first and third groups were shrimp and crab ALFs respectively. In the second group, ALFs from all crustaceans viz., crabs, shrimps, lobsters and crayfishes were clustered together. Within this cluster, there were three distinct subgroups of sequences: a subgroup of lobster and shrimp ALFs, a second subgroup of shrimp, crayfish and crab ALFs to which the PpALF2 and StALF belonged to and a third subgroup containing ALFs from the crabs. Phylogenetic tree showed that ALFs from crabs including PpALF2, StALF and ALFs from *P. trituberculatus*, *S. paramamosain*, *S. serrata* and *E. sinensis* were clustered together, and then had a closer relationship with crayfish *Pacifastacus leniusculus* and shrimp ALFs (*P. monodon* and *M. japonicus*). This convergence between crustaceans might imply the complex evolution of ALFs in these groups and potential similarity in biological functions.

Based on the high similarity with other ALFs, the spatial structures of PpALF2 and StALF were established using the SWISS-MODEL prediction algorithm based on the template 2jobA, and they were found to be similar to other known ALFs (Fig. 4a and b). There were four beta-strands and two alpha-helices in the spatial structure of PpALF2 and StALF. The two conserved-cysteine residues (C_{29} and C_{50}) in the beta strands of PpALF2 and StALF formed a disulfide bond that constrained a beta-hairpin loop, in which there were an alternating series of hydrophilic and hydrophobic residues between C_{29} and C_{50} (Hoess et al., 1993). The spatial structural similarity between PpALF2 and StALF implied that they might represent similar biological functions in portunid crabs. The primary structures indicate that PpALF2 and StALF have amphipathic factors which are identical to other ALFs (Zhang et al., 2010). The amphipathic loop structures, believed to be the lipid A (LPS) binding sites (Nagoshi et al., 2006), were identified in the potential tertiary structures of PpALF2 and StALF also. The presence of lipid A (LPS) binding site in PpALF2 and StALF suggest the conservation of LPS binding activity and available antibacterial activity to gram-negative bacteria. Both PpALF2 and StALF are believed to involve in the defense responses of portunids.

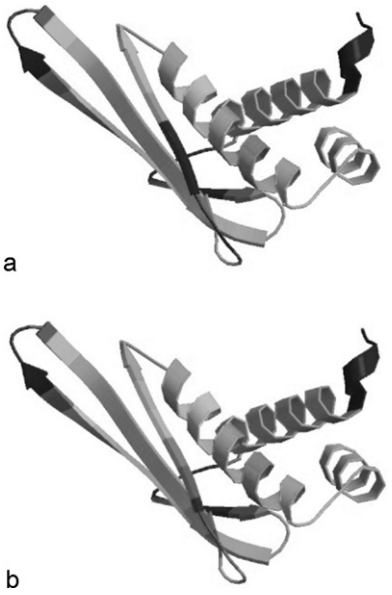

Fig. 4. (a) Structural model of PpALF2 (JQ899452) created using SWISS-MODEL server. (b) Structural model of StALF (JQ899453) created using SWISS-MODEL server.

Thus, from the characteristics of PpALF2 and StALF it is possible to predict that these peptides could function as a broad spectrum antimicrobial peptide against both gram-negative and gram-positive bacteria. Also there is possibly more than one ALF involved in crab immunity against various pathogens. Further investigations on the antimicrobial activities of PpALF2 and StALF against various pathogens will provide a promising drug in crustacean health management.

4. Conclusion

The abundance and diversity of AMPs in invertebrates is considered to be an effective repertoire of defense molecules evolved under the living pressure. This study reports the first ALF from *S. tranquebarica* (StALF) and the second ALF isoform from *P. pelagicus* (PpALF2). Characterization of PpALF2 and StALF revealed that they possess all the vital characteristics needed for an ALF to be a potent antimicrobial agent. The crab ALFs appear to be a good candidate for further investigation for its potential use in larviculture as an alternative to conventional antibiotics. ALFs would surely prove as promising therapeutic or prophylactic agents for health management and disease control in crab aquaculture in the near future.

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