Anti-Lipopolysaccharide Factor from Banana Shrimp (*Fenneropenaeus merguiensis*) Exhibits Antibacterial Activity

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ABSTRACT

Anti-lipopolysaccharide factor (ALF) is a type of antimicrobial peptide that plays an important role in the innate immune system of crustaceans such as shrimps and crabs. In the present study, anti-lipopolysaccharide factor isoform 3 (ALF3) was identified and characterized from banana shrimp (*Fenneropenaeus merguiensis*) named as FmALF3. The full-length cDNA of FmALF3 was 643 bp. The open reading frame of FmALF3 was 372 bp and encoded a polypeptide of 123 amino acids with a signal peptide of 25 amino acid residues. Its calculated molecular mass is 13.67 kDa with an isoelectric point of 9.88. FmALF3 is a cationic ALF, that consists of lipopolysaccharide-binding domain and two highly conserved cysteine residues at position 55 and 76. It shared 96.7% similarity with FiALF-B from *F. indicus*. The mRNA transcript of FmALF3 was detected in all examined tissues with the highest expression in hemocytes. The temporal expression of FmALF3 mRNA in hemocytes after *Vibrio parahaemolyticus* injection was analyzed by quantitative real-time PCR (qRT-PCR). FmALF3 was up regulated at 3 h and reached the highest level at 12 h. After that, the expression level of FmALF3 was decreased at 24, 48 and 72 h. The recombinant of FmALF3 (rFmALF3) was expressed in *Escherichia coli* BL21*(DE3) and purified. The purified rFmALF3 could inhibit the growth of Gram-positive and Gram-negative bacteria. Therefore, all these results indicated that FmALF3 is an inducible protein and could play a key biological role in the innate immune defense of *F. mergueinsis*.

Keywords: Anti-lipopolysaccharide factor, Antimicrobial peptide, *Fenneropenaeus merguiensis*, Innate immunity, *Vibrio parahaemolyticus*

INTRODUCTION

Shrimp aquaculture is a vital industry in Thailand, with the banana shrimp (*Fenneropenaeus merguiensis*) being a valuable species in the Penaeidae family (Chimnuala *et al*., 2022). However, the industry has faced a significant challenge in recent years due to the emergence of a destructive shrimp disease known as early mortality syndrome (EMS) or acute hepatopancreatic necrosis disease (AHPND). This disease is caused by *Vibrio parahaemolyticus* strains carrying a plasmid encoding the *pirAB* binary toxin gene (*Vp*AHPND). The rise of AHPND has severely impacted Thailand's shrimp industry, necessitating urgent attention and strategic interventions (Lightner *et al*., 2012; Zorriehzahra and Banaederakhshan, 2015).

Crustaceans, including banana shrimp, possess an innate immune system encompassing both humoral and cellular immune responses (Vazquez *et al*., 2009). This immune system is their primary defense mechanism against invading pathogens (Chungjit *et al*., 2016). A crucial element

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of their innate immune system is the presence of antimicrobial peptides (AMPs). Most AMPs are typically possess cationic properties and are characterized as small peptides (Guaní-Guerra *et al*., 2010). These peptides encompass a diverse group of essential proteins that can eliminate or neutralize various pathogens (Hoffmann *et al*., 1999). AMPs are commonly referred to as natural antibiotics due to their rapid and effective antimicrobial activity against a wide range of microorganisms, including Gram-positive and Gram-negative bacteria, fungi, protozoa and certain enveloped viruses (Rosa and Barracco, 2010). The majority of AMPs have an amphipathic molecular structure both hydrophilic and hydrophobic surfaces. These peptides disrupt membrane integrity and form microbial membrane pores (Antony *et al*., 2011). AMPs play a crucial role in the innate immune system of crustaceans, serving as a significant component of their defense mechanisms.

Shrimps have been shown to possess several types of AMPs, including anti-lipopolysaccharide factor (ALF), crustin, penaeidin and stylicin, each with distinct properties and functions in combating microbial pathogens (Tassanakajon *et al*., 2013). Among these molecules, ALFs exhibit significant antimicrobial properties, demonstrating a wide-ranging effectiveness against Gram-negative and Gram-positive bacteria, fungi, parasites and viruses (Li *et al*., 2015). ALF is a type of AMPs that is widely distributed among crustaceans (Tassanakajon *et al*., 2013; Destoumieux-Garzón *et al*., 2016). They are characterized by their small size and basic nature and contain a conserved disulfide loop formed by cysteine residues, known as the lipopolysaccharide binding domain (LPS-BD) (Zhou *et al*., 2019b). The LPS-BD serves as the functional domain responsible for the antimicrobial properties of ALFs. It possesses the ability to recognize and bind to specific components present in the cell walls of microorganisms, such as lipopolysaccharide (LPS) in Gram-negative bacteria and lipoteichoic acid (LTA) in Gram-positive bacteria (Matos *et al*., 2018). The first discovery of ALF occurred in the hemocytes of the horseshoe crab, *Limulus polyphemus*, and was initially referred to as LALF (Tanaka *et al*., 1982; Morita *et al*., 1985). After that, numerous ALFs have been identified and characterized in various crustacean species, including *Marsupenaeus japonicus* (Zhang *et al*., 2022), *Litopenaeus vannamei* (Tinwongger *et al*., 2019), *F. chinensis* (Yang *et al*., 2015), *Penaeus monodon* (Methatham *et al*., 2017; Zhou *et al*., 2019a), *Macrobrachium rosenbergii* (Srisapoome *et al*., 2018), *Procambarus clarkii* (Yin *et al*., 2021), *Portunus trituberculatus* (Wu *et al*., 2019) and *Scylla paramamosain* (Long *et al*., 2021). In penaeid shrimp, ALFs can be categorized into seven distinct groups (group A-G) based on the similarity of their LPS-BD sequences and net charge properties (Matos *et al*., 2018). Among these groups, ALFs from A, D, E and G possess anionic properties. In contrast, B, C and F are exclusively composed of cationic peptides due to their highly positive charge (pI>9). Notably, ALFs belonging to group B have demonstrated potent antimicrobial activities against various pathogens, including viruses (Tassanakajon *et al*., 2018). For instance, in black tiger shrimp, the recombinant ALF*Pm*3 (rALF*Pm*3) belonging group B exhibits broad-spectrum antimicrobial activity against filamentous fungi, Gram-positive and Gram-negative bacteria, including the pathogenic bacteria *V. harveyi*, which causes vibriosis and Vp_{AHPND}, responsible for early mortality syndrome (Somboonwiwat *et al*., 2005; Supungul *et al*., 2017). rALF*Pm*3 is capable of binding to LPS and LTA, essential components of bacterial cell membranes, thereby inducing membrane permeabilization and cell lysis in *V. harveyi* and Vp_{AHPND} (Somboonwiwat *et al*., 2008; Jaree *et al*., 2012; Supungul *et al*., 2017). Moreover, rALF*Pm*3 has been demonstrated for its anti-white spot syndrome virus (WSSV) activity. It effectively inhibits WSSV propagation in crayfish hematopoietic cell culture and in shrimp (Tharntada *et al*., 2009). Recent studies have experimentally confirmed the interaction between ALF*Pm*3 and five WSSV structural proteins (Suraprasit *et al*., 2014; Methatham *et al*., 2017). Further, transmission electron micrographs have revealed that incubation with rALF*Pm*3 disrupts the stability of the WSSV envelope and nucleocapsid structures, leading to the disintegration of WSSV virions (Methatham *et al*., 2017). In Chinese shrimp, the recombinant protein ALFFc (group B) strongly inhibits the growth of Gram-positive and Gram-negative bacteria and the propagation of WSSV through the activity of a synthetic peptide of ALFFc LPS-BD (Guo *et al*., 2014). Additionally, the rFcALF5 has shown antibacterial activity and has been observed to inhibit WSSV infectivity in shrimp. Similarly, similar findings have been reported for rALF*Pm*3, where rFcALF5 and its synthetic LPS-BD peptide have been found to directly interact with the envelope protein VP24 of WSSV (Yang *et al*., 2015).

So far, numerous studies have focused on the identification, molecular characterization, expression analysis and functional analysis of ALFs in various penaeid shrimp species. However, there is currently no report regarding the presence of ALFs, especially group B in *F. merguiensis*. In the present study, we successfully isolated a cDNA sequence encoding ALF isoform 3 (FmALF3) from *F. merguiensis*. Subsequently, we investigated the mRNA distribution of FmALF3 in various tissues, examined the expression profile following bacterial challenge and conducted recombinant protein expression and purification of rFmALF3. The objective of this study was to gain insights into the immune defense mechanisms of banana shrimp and elucidate the specific roles of FmALF3.

MATERIALS AND METHODS

Experimental shrimp, tissue collection and infection experiment

Healthy *Fenneropenaeus merguiensis* shrimp weighing between 15 and 20 g were obtained from Songkhla province, Thailand. They were used for gene cloning, tissue sample collection and *Vibrio parahaemolyticus* challenge. The shrimp were cultured in tanks filled with aerated seawater at ambient temperature and a salinity level of 30 parts per thousand (ppt). The shrimp were fed a commercial shrimp pellet feed from Gold Coin Specialities Co., Ltd. (Songkhla, Thailand) with 38% crude protein and 5% crude lipid to satiation every 6 h. Hemolymph of the banana shrimp was withdrawn from the ventral sinus with a 1 mL syringe containing 300 µL of anticoagulant solution (450 mM NaCl, 10 mM KCl, 10 mM EDTA, 10 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM HEPES, pH 7.3). Hemocytes were separated from the plasma by centrifugation at 800×g for 10 min at 4 °C and stored at -80 °C. To investigate the tissue distribution of FmALF3, various tissues, including heart, hepatopancreas, lymphoid, intestine, stomach, gill, and muscle, were collected from three healthy *F. merguiensis* shrimp. For the *V. parahaemolyticus* infection experiments, shrimp were intramuscularly injected with 100 µL of 0.85% NaCl containing *V. parahaemolyticus* (2×10^7 CFU·mL^{-1}). Five shrimp were randomly sampled from each group at 0, 3, 6, 12, 24, 48 and 72 h post-injection (hpi) and the hemocytes were collected and preserved for subsequent analysis. All samples were stored at -80 °C until RNA extraction. The protocol of this study was approved by the Institutional Animal Care and Use Committee, Prince of Songkla University (PSU) (Ethic no. AQ055/2023).

Cloning of full-length cDNA sequence of FmALF3

Total RNA was extracted from hemocytes using a Ribozol RNA extraction reagent (Ameresco). RNA concentration was quantified based on its absorbance at 260 nm (A_{260}), while its purity was assessed through the A_{260}/A_{280} ratio. They were treated with RNase-Free DNase I (Promega) to eliminate residual genomic DNA. First-strand cDNA was synthesized from 2 µg of total RNA by using an Improm-II™ Reverse Transcription System (Promega). The internal fragment of FmALF3 was amplified using the primers FmALF3-F1 and FmALF3-R1 (Table 1), utilizing the first-strand cDNA derived from hemocytes. The purified PCR product was then cloned into the pGEM®-T Easy vector (Promega) and transformed into competent *Escherichia coli* cells DH5α strain, following the manufacturer's protocol. Positive clones were identified through blue/white colony screening and confirmed using colony PCR. Single white colonies were grown overnight for plasmid preparation. Plasmid DNA was purified using Plasmid Miniprep Kit (Omega) and sequenced with T7 and SP6 primers. To obtain a full-length FmALF3 cDNA sequence, rapid amplification of cDNA ends (RACE) was carried out to get the 5′ and 3′ termini from hemocyte cDNA using GeneRacer™ kit (Invitrogen). The 5′ RACE PCR reaction was performed using a GeneRacer 5′ primer and FmALF3-R2 primer (Table 1), while the 3′ RACE reaction was monitored using a gene specific

Table 1. Primers used in this study.

Primers	Sequence (5´-3´)	Sequence information
FmALF3-F1	ATGCGAGTGTCCGTGCTG	internal fragment
FmALF3-R1	AGCCTCCTGTTGAGAGATG	internal fragment
FmALF3-F2	ATGCGTGTTTCCGTGTTGGCAAGCCTG	3′ RACE
FmALF3-R2	CGGAGTGACTGCGCGTGCTGGCTTCT	5′ RACE
FmALF3-*Bam*H I-F	GGATCCATGCGCGTCTCCGTGTTG	recombinant protein
FmALF3-*Eco*R I-R	GAATTCCTATGAGTTAAGCCACTG	recombinant protein
FmALF3-QF	TCTCATCTCTCAACAGGAGG	qRT-PCR
FmALF3-QR	TAGAGCTTCCATTGCCAACT	qRT-PCR
18S rRNA-QF	GCAGGCGCGCAAATTAC	qRT-PCR
18S rRNA-QR	TGCGAGGCCCCGTTC	qRT-PCR

FmALF3-F2 primer and GeneRacer 3′ primer. The PCR products were analyzed and sequenced similarly to the preceding mention. A full-length FmALF3 cDNA was obtained by overlapping the sequences of three fragments.

Sequence analysis of FmALF3

The cDNA sequence and deduced amino acid sequence of FmALF3 were subjected to analysis using the BLAST algorithm online (http://www.ncbi.nlm.nih.gov/BLAST/) for sequence similarity comparisons. ExPASy online software was utilized to calculate the molecular weight and predict the pI values. The SMART tool (http://smart.embl-heidelberg.de/) was employed to identify the protein domain. The presence of a signal peptide in FmALF3 was predicted using the SignalP 4.0 program (Petersen *et al*., 2011). Multiple sequence alignment was conducted using ClustalW2 (https://www.ebi.ac.uk/Tools/msa/clustalw2/) to assess sequence conservation across related proteins. Furthermore, the phylogenetic tree was constructed and analyzed using MEGA7 software (Kumar *et al*., 2016) to elucidate the evolutionary relationships among various ALF proteins.

Tissue distribution of FmALF3

In unchallenged shrimps, the mRNA levels of FmALF3 in different tissues, such as the heart, hepatopancreas, lymphoid, intestine, stomach, gill, and muscle, were evaluated using SYBR Green quantitative real-time PCR (qRT-PCR). Total RNA was extracted from various tissues using the same procedure as hemocytes. To examine the expression of FmALF3, qRT-PCR was conducted using an MX3005P QPCR system (Stratagene, USA). To amplify FmALF3 fragment, gene-specific primers FmALF3-QF and FmALF3-QR (Table 1) were employed. Furthermore, 18S rRNA primers 18S rRNA-QF and 18S rRNA-QR (Table 1) were used to amplify 18S rRNA gene fragment as the internal control. Negative controls without a cDNA template were included in this experiment. Cloning FmALF3 and 18S rRNA for the standard material was carried out using the same primers as previously described. A standard curve was generated by preparing a series of five dilutions of plasmid DNA containing the FmALF3 and 18S rRNA fragments. By establishing a linear relationship between the known DNA concentrations and the corresponding cycle threshold (Ct) values, the standard curve enabled the estimation of the absolute quantities of FmALF3 and 18S rRNA transcripts in the samples. The PCR amplifications were performed in triplicate, with a total volume of 20 µL. Each reaction mixture contained 1 µL of cDNA from each tissue, 4 µL of 5X Evagreen Master mix and 0.5 µL of each primer. The PCR amplification was performed under the following conditions: one cycle at 95 °C for 12 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 20 s and 72 °C for 20 s. Finally, a concluding cycle was performed at 95 °C for 1 min, 55 °C for 30 s and 95 °C for 30 s. Dissociation curve analysis was conducted at the end of each PCR reaction to ensure that only a single PCR product was amplified and detected.

Expression pattern analysis of FmALF3 upon Vibrio parahaemolyticus challenge

V. parahaemolyticus strains mentioned in this study were obtained from diseased shrimps and their identification was performed by Dr. Nion Chirapongsatonkul, who is a co-author of this research paper. The bacteria were overnight cultured in tryptic soy broth (TSB) medium containing 1.5% NaCl at 37 °C, harvested by centrifugation at 1,000×g and 4 °C for 30 min and resuspended in sterile 0.85% NaCl. For the bacterial challenge experiment, the abdomen segment of each shrimp was intramuscularly injected with 100 µL of *V. parahaemolyticus* (2×10^{7} CFU·mL^{-1}). The hemocytes were collected and extracted for total RNA at 0, 3, 6, 12, 24, 48 and 72 h, then cDNAs were synthesized. qRT-PCR analysis was performed with the same condition as the tissue distribution assay. The experiment was performed in triplicate. The standard curve was created using five serial dilutions of plasmid containing the FmALF3 and 18S rRNA fragments. The quantities of 18S rRNA expression at different times were used to normalize FmALF3 mRNA expression after bacterial exposure. The mean standard error (SE) of relative mRNA level was used to represent all data using SPSS® statistical software, the differences between each group were examined using one-way analysis of variance (one-way ANOVA) and Duncan's multiple range test. P values of less than 0.05 were considered significant ($p<0.05$).

Recombinant expression and protein purification

Primers were designed to obtain the mature FmALF3 peptide based on the predicted sequence encoding a FmALF3 protein (Table 1). These primers were used to amplify the FmALF3 gene from cDNA prepared from unchallenged shrimp hemocytes. The PCR reactions were carried out under the following conditions: initial denaturation at 95 °C for 2 min for one cycle, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 45 s and extension at 72 °C for 2 min. Finally, a final elongation step was performed at 72 °C for 5 min for one cycle. The PCR product was then analyzed using 1.5% agarose gel electrophoresis. After purification, the PCR product was digested at the specified restriction sites, cloned into the expression vector pET32a (+), and then transformed into *E. coli* BL21*(DE3). The cells were grown in LB broth at 37 °C until the logarithmic phase (OD_{600} of 0.4–0.6). To induce protein expression, isopropyl thiogalactoside (IPTG) was added to the cultures at a final concentration of 0.5 mM and the cultures were then incubated for 3 h. Subsequently, the cells were harvested by centrifugation at 8,000×g, 4 °C for 10 min and the pellets were collected. Recombinant FmALF3 (rFmALF3) was expressed as an inclusion body. Denaturation of the protein was performed under denaturing conditions. The rFmALF3 protein was then renatured into a soluble form and dialyzed using a dialysis buffer (0.1 M Tris-HCl, pH 7.5, 5 mM EDTA, 5 mM L-Cysteine). The empty pET32a (+) vector was employed for the expression of recombinant thioredoxin (rTRX), serving as a negative control. The rFmALF3 and rTRX proteins were purified using a Ni-NTA column (Qiagen®, Germany) following the manufacturer's protocol under native conditions. The purified protein solution was subsequently dialyzed to ensure the complete removal of imidazole. The concentration of rFmALF3 and rTRX protein was quantified using the Bradford Protein assay. The purities of purified rFmALF3 and rTRX were verified by 12% SDS-PAGE according to the method of Laemmli (1970).

Antimicrobial activities of FmALF3

The bacterial strains used in this study were derived from the Division of Biological Science, PSU. It consisted of Gram-positive bacteria (*Staphylococcus aureus* ATCC29213 and *Micrococcus luteus* PSU414) and Gram-negative bacteria (*E. coli* ATCC25922, *V. parahaemolyticus* PSU414 and *V. harveyi* PSU414). The agar well diffusion method was utilized to preliminarily evaluate the antimicrobial activity of the rFm ALF3 against the Gram-positive bacteria *S. aureus* ATCC29213, the Gram-negative bacteria *E. coli*

ATCC25922 and the pathogenic bacterium *V. parahaemolyticus* PSU414. The bacteria were grown overnight and inoculated at a 1:100 ratio in TSB medium at 37 °C to obtain turbidity of 0.2 at 600 nm. After that, 100 µL of each bacterium culture was added to warm melting TSB in phosphate buffer, pH 6.2, containing 1.3% agar and mixed, then poured into a 100 mL plate. Wells (5 mm in diameter) were punched after the gel had solidified. In each well of various bacteria agar plates, the rFmALF3, ampicillin (positive control) and rTRX (negative control) were dropped and incubated at 30 °C for 18 h. The clear zone diameters were measured. The experiment was performed in triplicate. The minimal inhibitory concentration (MIC) was determined by the resazurin-based 96-well plate microdilution method as previously described (Elshikh *et al.*, 2016) with slight modification. Briefly, the examined bacteria, including *S. aureus* ATCC29213, *M. luteus* PSU414, *E. coli* ATCC25922, *V. parahaemolyticus* PSU414, and *V. harveyi* PSU414, were overnight cultured in TSB, then inoculated in Mueller-Hinton broth (MHB) at 37 °C for 3 h to achieve turbidity of 0.5 McFarland (1×10^8 CFU·mL^{-1}), which was subsequently corrected to 1×10^6 CFU·mL^{-1}. Serial two-fold dilutions of the rFmALF3 or rTRX in MHB were added with 50 µL of the bacteria mentioned above and incubated at 37 °C for 18 h. Streptomycin and rTRX were used as positive and negative controls, respectively. After overnight incubation, each well was added 25 µL of 0.015% resazurin (w/v) and incubated for another 2 h. The minimal bactericidal concentration (MBC) was assessed by dropping 10 µL of rFmALF3/MHB into MHA medium from wells with no bacterial growth observed by turbidity. After 18 h of incubation at 37 °C, the number of colonies on the plates was counted. If no bacterial colonies were formed from the wells, the recombinant protein concentration of the wells would be the MBC value.

RESULTS

Sequence analysis and multiple sequence alignment of FmALF3

In this study, a full-length cDNA encoding ALF (FmALF3) was identified and characterized from the hemocytes of banana shrimp *Fenneropenaeus merguiensis*. The FmALF3 cDNA sequence has been deposited in Genbank #OR246893. The full-length cDNA sequence of FmALF3 was 643 bp. It consisted of 5′ untranslated region (UTR) of 40 bp, 3′ UTR of 231 bp and the putative polyadenylation consensus signal (AATAAA). The ORF of FmALF3 contained 372 bp, encoding 123 amino acids. The deduced FmALF3 protein sequence was composed of a signal peptide, an LPS-BD and two conserved cysteine residues which form one disulfide loop in peptide leading to formation of β-sheet structures. The LPS-BD is flanked by two conserved cysteine residues (Cys^{55} and Cys^{76}) that were predicted to form a disulfide bond (Figure 1). The molecular mass of the deduced mature peptide was 13.67 kDa. It is highly cationic, with a theoretical pI of 9.88. The multiple alignments of nine shrimp ALF proteins showed that FmALF3 possesses both a signal peptide and a characteristic LPS-BD, as depicted in Figure 2. Additionally, FmALF3 exhibits complete conservation of the two conserved cysteine residues responsible for forming a disulfide bridge. Notably, the disulfide loop region contains a prominent clustering of positively charged amino acid residues. A neighbor-joining phylogenetic tree was constructed using MEGA7 software to elucidate the evolutionary relationships between FmALF3 and 30 other shrimp ALF sequences. Based on this analysis, all the ALFs were categorized into seven distinct groups. Notably, FmALF3 was classified within the cationic ALFs group B, demonstrating a close evolutionary relationship with FiALF-B from *F. indicus* (Figure 3).

Tissue distribution of FmALF3

The mRNA transcript of FmALF3 exhibited detectable expression in multiple tissues, including hemocytes, heart, hepatopancreas, intestine, lymphoid, stomach, gill and muscle. Among these tissues, the highest level of FmALF3 expression was observed in the hemocytes and gill, as illustrated in Figure 4. This distribution pattern suggested that FmALF3 plays a significant role in the immune defense mechanisms of banana shrimp, particularly in the hemocytes and gill tissues, which are likely exposed to bacterial encounters and actively involved in immune responses.

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                                        M  R  V  S  V  L  A  S  L  V  L  A  V  S  L  V  A  L  F  A
  1  TTCTGTTAGTTACTTGAAAAGGGCTTTCCTAGTTTAGAAGATGCGCGTCTCCGTGTTGGCAAGCCTGGTGCTGGCGGTGTCCCTGGTGGCACTCTTCGCC
      P  Q  C  Q  A  Q  G  W  E  A  V  A  A  A  V  A  S  K  I  V  G  L  W  R  D  E  K  T  E  L  L  G  H
101  CCGCAGTGCCAGGCCCAAGGGTGGGAGGCGGTGGCAGCGGCCGTCGCCAGCAAGATTGTTGGGCTGTGGAGGGACGAGAAAACTGAACTCCTGGGCCACG
     E  C  K  F  T  V  K  P  Y  L  K  R  F  Q  L  H  Y  K  G  R  M  W  C  P  G  W  T  A  I  R  G  E  A  S
201  AGTGCAAGTTCACCGTCAAGCCTTACCTTAAGAGGTTCCAGTTGCACTACAAGGGCAGGATGTGGTGCCCAGGCTGGACGGCTATCAGAGGAGAAGCCAG
       T  R  S  H  S  G  V  A  G  R  T  A  Q  D  F  V  R  K  A  F  Q  K  G  L  I  S  Q  Q  E  A  N  Q  W
301  CACACGCAGTCACTCCGGGGTGGCTGGACGGACAGCCCAAGACTTCGTTCGGAAGGCTTTCCAGAAAGGTCTCATCTCTCAACAGGAGGCCAACCAGTGG
      L  N  S
401  CTTAACTCATAGGCCCTTTGCTCAGCCGCAATCGGCAATGGAGGCTCTACCATTATGATCTCATGTGTGAAGGCATTCATTATGTGAAAATTTTGCTGTG
501  CACAAAATAATATATCATAAACTTGTAATAATCTGTCTTAAAAGGATTCTTATACGCTGCTGTCAGTAATGCACAGTACATTCTTTGAAACGTGACTGAT
601  GTTAGTAAACGACAACTTGTCGTAAATAAAAAAAAAAAAAAAA
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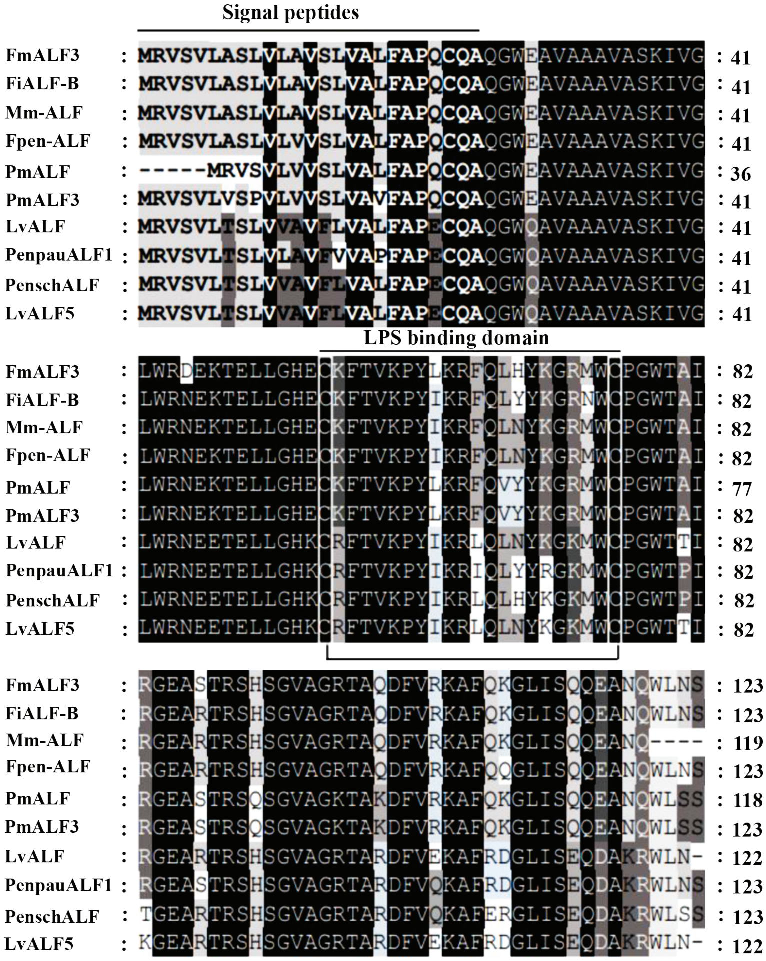
Figure 1. The full-length nucleotide and deduced amino acid sequences of FmALF3 cDNA from *Fenneropenaeus merguiensis*. The start codon, stop codon and polyadenylation signal are bolded in black and red; the signal peptide is italicized; the LPS-BD is boxed, and the two cysteine residues (positions 55 and 76) involved in the formation of the disulfide loop are highlighted in yellow.



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FmALF3      : MRVSVLASLVLAVSLVALFAPQCQAQGWEAVAAAVASKIVG : 41
FiALF-B     : MRVSVLASLVLAVSLVALFAPQCQAQGWEAVAAAVASKIVG : 41
Mm-ALF      : MRVSVLASLVLAVSLVALFAPQCQAQGWEAVAAAVASKIVG : 41
Fpen-ALF    : MRVSVLASLVLVVSLVALFAPQCQAQGWEAVAAAVASKIVG : 41
PmALF       : -----MRVSVLVVSLVALFAPQCQAQGWEAVAAAVASKIVG : 36
PmALF3      : MRVSVLVSPVLVVSLVAVFAPQCQAQGWEAVAAAVASKIVG : 41
LvALF       : MRVSVLTSLVVAVFLVALFAPECQAQGWQAVAAAVASKIVG : 41
PenpauALF1  : MRVSVLTSLVLAVFVVAPFAPECQAQGWQAVAAAVASKIVG : 41
PenschALF   : MRVSVLTSLVVAVFLVALFAPECQAQGWQAVAAAVASKIVG : 41
LvALF5      : MRVSVLTSLVVAVFLVALFAPECQAQGWQAVAAAVASKIVG : 41

FmALF3      : LWRDEKTELLGHECKFTVKPYLKRFQLHYKGRMWCPGWTAI : 82
FiALF-B     : LWRNEKTELLGHECKFTVKPYIKRFQLYYKGRNWCPGWTAI : 82
Mm-ALF      : LWRNEKTELLGHECKFTVKPYIKRFQLNYKGRMWCPGWTAI : 82
Fpen-ALF    : LWRNEKTELLGHECKFTVKPYIKRFQLNYKGRMWCPGWTAI : 82
PmALF       : LWRNEKTELLGHECKFTVKPYLKRFQVYYKGRMWCPGWTAI : 77
PmALF3      : LWRNEKTELLGHECKFTVKPYLKRFQVYYKGRMWCPGWTAI : 82
LvALF       : LWRNEETELLGHKCRFTVKPYIKRLQLNYKGKMWCPGWTTI : 82
PenpauALF1  : LWRNEETELLGHKCRFTVKPYIKRIQLYYRGKMWCPGWTPI : 82
PenschALF   : LWRNEETELLGHKCRFTVKPYIKRLQLHYKGKMWCPGWTPI : 82
LvALF5      : LWRNEETELLGHKCRFTVKPYIKRLQLNYKGKMWCPGWTTI : 82

FmALF3      : RGEASTRSHSGVAGRTAQDFVRKAFQKGLISQQEANQWLNS : 123
FiALF-B     : RGEARTRSHSGVAGRTAQDFVRKAFQKGLISQQEANQWLNS : 123
Mm-ALF      : RGEARTRSHSGVAGRTAQDFVRKAFQKGLISQQEANQ---- : 119
Fpen-ALF    : RGEARTRSHSGVAGRTAQDFVRKAFQQGLISQQEANQWLNS : 123
PmALF       : RGEASTRSQSGVAGKTAKDFVRKAFQKGLISQQEANQWLSS : 118
PmALF3      : RGEASTRSQSGVAGKTAKDFVRKAFQKGLISQQEANQWLSS : 123
LvALF       : RGEARTRSHSGVAGRTARDFVEKAFRDGLISEQDAKRWLN- : 122
PenpauALF1  : RGEASTRSHSGVAGRTARDFVQKAFRDGLISEQDAKRWLNS : 123
PenschALF   : TGEARTRSHSGVAGRTARDFVQKAFERGLISEQDAKRWLSS : 123
LvALF5      : KGEARTRSHSGVAGRTARDFVEKAFRDGLISEQDAKRWLN- : 122
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Figure 2. Multiple alignment of deduced amino acid sequence of FmALF3 (WLW39507.1) with other reported shrimp ALFs: FiALF-B (ADE27980.1); MmALF (QJW82622.1); PmALF (ACC86067.1); PmALF3 (AEW91477.1); Fpen-ALF (QCQ82547.1); LvALF (XP_027206930.1); LvALF5 (ACT21197); Penpau ALF1 (ABQ96193.1); PenschALF (ABJ90465.1). The likely signal peptide is in bold. Black indicates complete conservation. Grey indicates conservation in four or more species. The putative disulfide linkages are shown by solid lines.

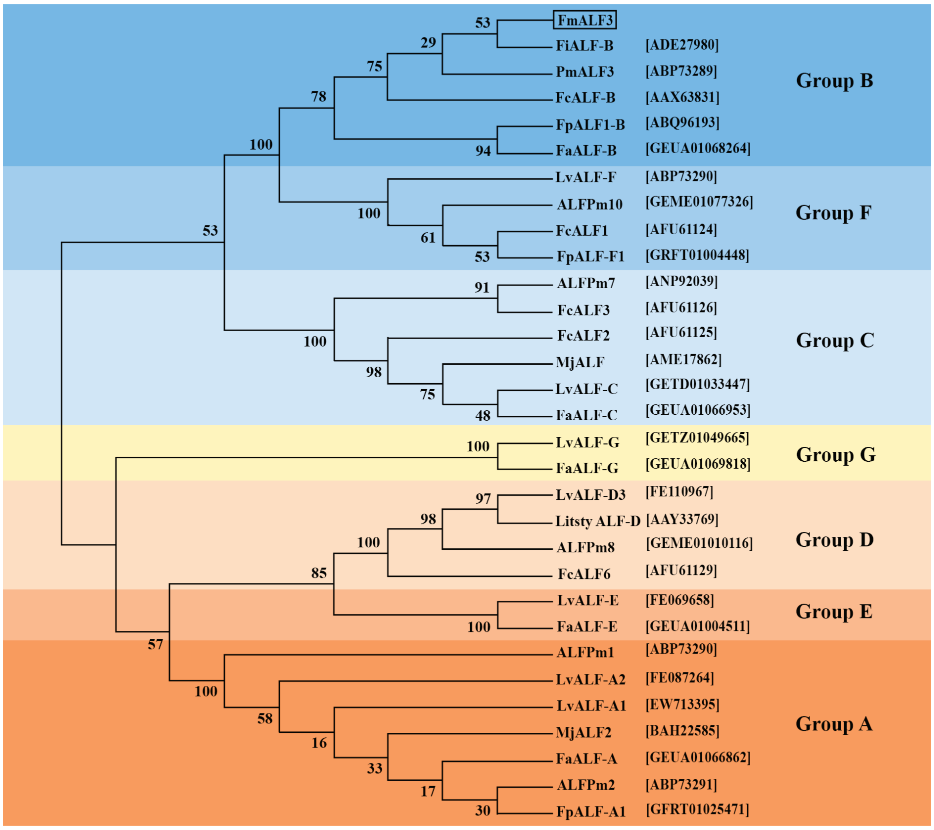

Figure 3. Phylogenetic analysis of various shrimp ALFs based on their protein sequences (FmALF3 was marked in box). The reproducibility of the results was performed with one thousand bootstraps.

Note: Fc = *Fenneropenaeus chinensis*; Fi = *F. indicus*; Fp = *F. penicillatus*; Fa = *Farfantepenaeus aztecus*; Lv = *Litopenaeus vannamei;* Litsty = *L. stylirostris*; Mj = *Marsupenaeus japonicus*; Pm = *Penaeus monodon*

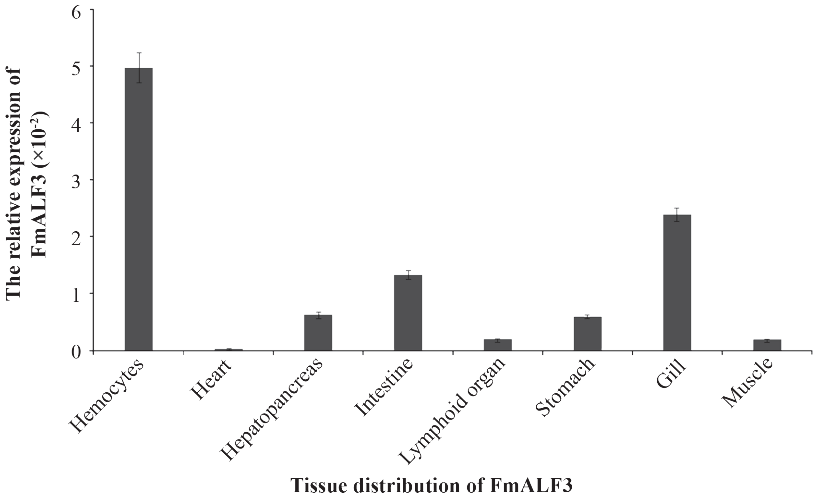

Figure 4. Tissue distribution of FmALF3 in various tissues of healthy *Fenneropenaeus merguiensis* by qRT-PCR. Bars represent mean±SD (n = 3).

FmALF3 was upregulated by Vibrio parahaemolyticus challenge

In the present study, we investigated the expression profile of the FmALF3 gene in the hemocytes of banana shrimp following injection with *Vibrio parahaemolyticus*. Our findings revealed a significant and dynamic modulation of FmALF3 gene expression in response to *V. parahaemolyticus* stimulation. Specifically, we observed a noticeable upregulation of FmALF3 gene expression, which commenced at 3 and 6 h and peaked at 12 h. However, as the experimental time progressed, the expression level of the FmALF3 gene gradually declined at 24, 48 and 72 h, respectively. These results highlighted the intricate regulatory mechanism governing the FmALF3 gene during *V. parahaemolyticus* infection, suggesting its pivotal role in the shrimp's immune response against this bacterial pathogen (Figure 5).

Recombinant protein expression and purification

To study the biological activities of FmALF3, the rFmALF3 and rTRX proteins were expressed in *E. coli* BL21*(DE3). The whole cells, collected at 3 h after 0.5 mM IPTG induction were analyzed by 12% SDS-PAGE. rFmALF3 was expressed as inclusion bodies. And then, rFmALF3 and rTRX proteins were purified. The results from SDS-PAGE of purified rFmALF3 and rTRX protein revealed only one band (Figure 6).

Antimicrobial activity of FmALF3

To assess the inhibitory effects of rFmALF3 using the agar well diffusion method, the diameter of the inhibition zone was measured and compared to the control group. The experimental results demonstrated that rFmALF3 exhibited inhibitory activity against microorganisms, including *S. aureus* ATCC29213, *E. coli* ATCC25922 and *V. parahaemolyticus* PSU414, forming distinct inhibition zones (Figure 7). The size of the inhibition zone caused by rFmALF3 in *E. coli* ATCC25922 and *V. parahaemolyticus* PSU414 was comparable to that of ampicillin. However, in the case of *S. aureus* ATCC29213, the inhibition zone produced by ampicillin was larger than that of rFmALF3, indicating varying degrees of effectiveness against different bacterial strains. In addition, no inhibition zones appeared for negative control rTRX (Figure 7). In the biological activity testing of rFmALF3 against bacterial pathogens, the MIC and MBC values were determined for five representative bacterial strains. The results demonstrated that rFmALF3 exhibited inhibitory properties against *S. aureus* ATCC29213, *M. luteus* PSU414, *E. coli* ATCC25922, *V. parahaemolyticus* PSU414, and *V. harveyi* PSU414, with corresponding MIC values of 1.32, 0.66, 2.63, 1.32, and 2.63 µM, respectively. Furthermore, rFmALF3 exhibited bactericidal activity against *S. aureus* ATCC29213, *M. luteus* PSU414 and *E. coli* ATCC25922, as indicated by an MBC value of 2.63 µM. However, it could not kill *V. parahaemolyticus* PSU414 and *V. harveyi* PSU414 at a concentration of 2.63 µM (Table 2).

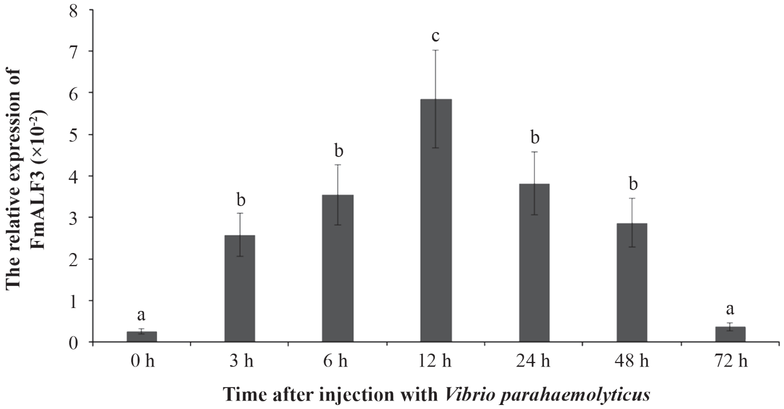

Figure 5. Temporal expression profile of FmALF3 in hemocytes after *Vibrio parahaemolyticus* challenge analyzed by qRT-PCR. All data are presented as the mean±SE of relative mRNA expression. Different letters above bars represent significant ($p<0.05$) difference.

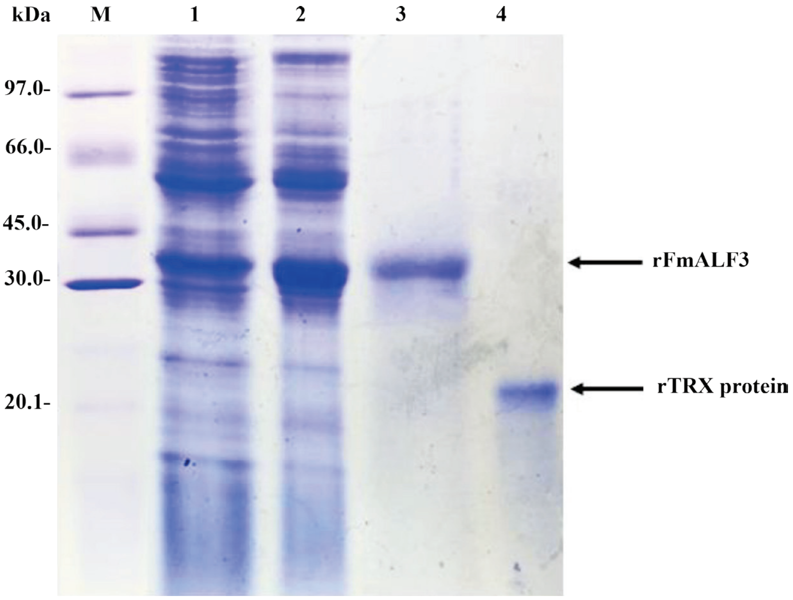

Figure 6. Patterns of recombinant FmALF3 protein expression analyzed by 12% SDS-PAGE. Lane M: molecular weight markers; lane 1: total proteins of *Escherichia coli* without IPTG induction; lane 2: total proteins of *E. coli* after 3 h with IPTG induction; lane 3: purified rFmALF3; lane 4: purified rTRX protein

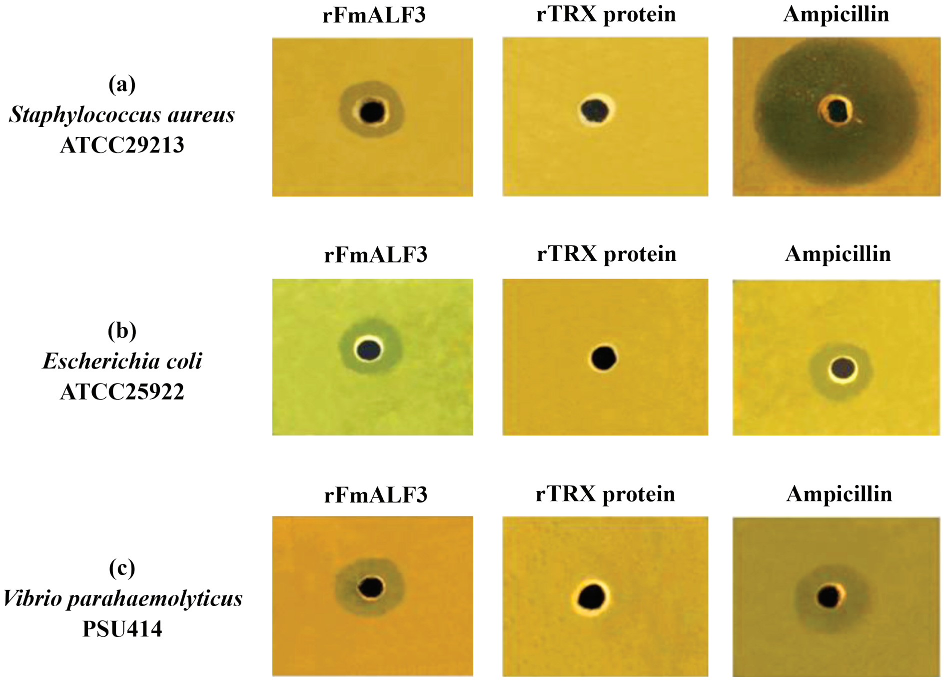

Figure 7. The inhibition zone of rFmALF3 for (a) *Staphylococcus aureus* ATCC29213; (b) *Escherichia coli* ATCC 25922; (c) *Vibrio parahaemolyticus* PSU414. The rTRX protein and Ampicillin were used as a negative and positive control, respectively.

Table 2. Antimicrobial activities of rFmALF3.

Microorganisms	**MIC (µM)**	**MBC (µM)**
Gram-positive bacteria		
Staphylococcus aureus ATCC29213	1.32	2.63
Micrococcus luteus PSU414	0.66	2.63
Gram-negative bacteria		
Escherichia coli ATCC25922	2.63	2.63
Vibrio parahaemolyticus PSU414	1.32	>2.63
Vibrio harveyi PSU414	2.63	>2.63

Note: MIC = minimal inhibitory concentration; MBC = minimal bactericidal concentration

DISCUSSION

In the present study, we successfully identified and characterized a full-length cDNA encoding anti-lipopolysaccharide factor (ALF), designated as FmALF3, from the hemocytes of the banana shrimp *Fenneropenaeus merguiensis*. The deduced protein sequence of this ALF exhibited a conserved structural arrangement comprising a signal peptide, an LPS-BD, the consensus sequence WCPGW and two conserved cysteine residues positioned at each end of the LPS-BD (Figure 1). Notably, these cysteine residues played a crucial role in forming a disulfide loop within the peptide, contributing to the formation of β-sheet structures in solution. A comparative analysis was performed to assess the similarity of the amino acid sequences of FmALF3 with other ALF proteins from diverse shrimp species. The results revealed significant sequence similarities between FmALF3 and ALFs from *F. indicus* (ADE27980.1), *Metapenaeus monoceros* (QJW82622.1), *P. monodon* (ACC86067.1), *F. penicillatus* (QCQ82547.1), *L. vannamei* (XP_027206930.1) and *Pleoticus schmitti* (ABJ90465.1), which were found to be 96.7%, 95.8%, 93.3%, 95.2%, 83% and 80%, respectively.

In this study, a phylogenetic analysis was conducted to investigate the evolutionary relationships of FmALF3 by comparing its amino acid sequences with those of other ALF proteins from various shrimps. The results revealed that the phylogenetic tree could be divided into two distinct clusters based on the charge properties of the ALFs. The first cluster included cationic ALFs from groups B, C and F, while the second cluster comprised anionic ALFs from groups A, D, E and G. FmALF3 was classified within the cationic cluster, explicitly belonging to group B, which showed a high positive charge. The calculated pI value for FmALF3 was 9.88, indicating that it is a highly positively charged peptide (pI>9). This charge distribution can be attributed to the abundance of positively charged amino acids, such as Lysine (Lys) and Arginine (Arg), in the FmALF3 (Figure 2). Notably, the phylogenetic analysis demonstrated that FmALF3 exhibited the closest evolutionary relationship with FiALF-B from *F. indicus*. Furthermore, it also exhibited a close evolutionary relationship with PmALF3 and FcALF-B from other shrimp species (Supungul *et al*., 2002; Liu *et al*., 2005). This finding suggested that the evolution of FmALF3 in banana shrimp is in line with other ALF proteins found in the Penaeus shrimp.

Crustaceans exhibit diverse expression profiles for different isoforms of ALF. In this study, the tissue distribution analysis of FmALF3 demonstrated its expression in multiple tissues, including hemocytes, heart, hepatopancreas, intestine, lymphoid, stomach, gill and muscle. Notably, the highest expression of FmALF3 was observed in hemocytes, suggesting a potential association with the immune function of these cells. Hemocytes play crucial roles in pathogen recognition and phagocytosis (Johansson *et al*., 2000). Similar patterns of tissue distribution have been observed in other shrimp species. For instance, in the giant freshwater prawn (*M. rosenbergii*), ALF is expressed in hemocytes, gill, heart, intestine, hepatopancreas, muscle, eye stalk and brain (Arockiaraj *et al*., 2014). In *L. vannamei*, ALF is expressed in hemocytes, gill, heart, muscle, intestine and hepatopancreas (Zhan *et al*., 2015). Likewise, in the kuruma prawn (*M. japonicus*), ALF is expressed in the stomach, muscle, intestine, gill, hepatopancreas, heart, hemocytes and eyestalks (Zhang *et al*., 2020). These findings collectively suggest that ALFs in shrimps are expressed in a wide range of tissues that encounter bacteria in the surrounding water, thus contributing to the defense of shrimp against bacterial infections.

In response to *V. parahaemolyticus* injection, the expression level of FmALF3 in hemocytes significantly increased after 3 h, reaching its peak at 12 h and subsequently declined at 24, 48 and 72 h. These temporal expression patterns exhibit similarity to the observed profile of PtALF6 in the swimming crab *P. trituberculatus*, where PtALF6 expression increased at 6 and 12 h after *V. alginolyticus* injection, followed by a decrease at 24 h (Liu *et al*., 2013). Similarly, ALF*Pm*3 displayed increased expression at 6 h after *V. harveyi* injection, while *Mj*ALF-E2 from *M. japonicus* exhibited elevated expression levels following *V. anguillarum* injection (Ponprateep *et al*., 2009; Jiang *et al*., 2015). The expression patterns of ALFs after *Vibrio* spp. challenge indicated a significant increase immediately after stimulation (0 h), with the highest levels reached at 12 and 24 h. This dynamic regulation of ALF's expression during this period strongly suggests their involvement in the stimulation of shrimp immune processes against bacterial infection. These findings strongly support the notion that FmALF3 is synthesized in response to bacterial infection, affirming its crucial role in the defense mechanisms of *F. merguiensis* against bacterial pathogens.

To investigate the potential biological function of rFmALF3, its antimicrobial activity was evaluated through both agar well diffusion and liquid growth inhibition assays. The results demonstrated that rFmALF3 effectively inhibited the growth of five different bacterial strains, *S. aureus* ATCC29213, *M. luteus* PSU414, *E. coli* ATCC25922, *V. parahaemolyticus* PSU414 and *V. harveyi* PSU414. These findings are consistent with prior research on ALFs in other shrimp species. For instance, the peptide ALF*Pm*3 exhibited broad-spectrum antimicrobial activity, suppressing the growth of both Gram-positive and Gram-negative bacteria, including *V. harveyi*, a pathogenic bacterium affecting shrimp (Somboonwiwat *et al*., 2005). Additionally, MnALF4 in Mantis shrimp displayed robust inhibitory activity against both Gram-positive and Gram-negative bacteria (Tang *et al*., 2020). The observed inhibition zones for *S. aureus* ATCC29213, *E. coli* ATCC25922 and *V. parahaemolyticus* PSU414 in the agar well diffusion method further confirmed the antimicrobial efficacy of rFmALF3. Other relevant studies have also explored the functional properties of ALFs. They are known to interact with bacterial membranes through various mechanisms, including translocation into the bacterial cytoplasm and disruption of membrane integrity (Kamysz *et al*., 2003; Rosa and Barracco, 2010). Thus, the results of this study suggested that rFmALF3 exhibits potent antimicrobial activity, potentially altering surface structures and inducing membrane destabilization (Jaree *et al*., 2012). These findings highlight the antimicrobial potential of rFmALF3, positioning it as a promising candidate for further exploration and potential application in the development of novel antimicrobial agents for combating bacterial infections in shrimp and aquaculture industries.

CONCLUSION

In this research, we identified and characterized FmALF3 from *Fenneropenaeus merguiensis*. Sequence analysis of FmALF3 suggested that the amino acid property composed basic amino acid with positive charge and functional domain called LPS-BD. FmALF3 was mainly expressed in hemocytes and upregulated after *V. parahaemolyticus* stimulation. The antimicrobial activities of rFmALF3 showed strong exhibition activities to Gram-positive bacteria *S. aureus* ATCC 29213 and *M. luteus* PSU414 and Gram-negative bacteria *E. coli* ATCC25922, *V. parahaemolyticus* PSU414 and *V. harveyi* PSU414. So, FmALF3 was an effective AMP of shrimp that could be developed and applied in aquaculture in the future.

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