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Structural Characteristics, Prokaryotic Expression and Activity Analysis of Antimicrobial Peptide ALF*Pm10* from *Penaeus monodon*

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Abstract
Antimicrobial peptides are crucial component of the invertebrates' innate immune system. Anti-lipopolysaccharide factors is a major type of AMPs in crustaceans, which can generally participate in the prevention of diseases. In this study, a new ALF named ALF*Pm10* was obtained by analyzing the transcriptome of hepatopancreas of *Penaeus monodon*. It consists of a signal peptide of 25 amino acids and a mature peptide of 98 amino acids. It contains an LBD domain and share 89% homology with ALF1 from *Penaeus chinensis*. The expression sequence of ALF*Pm10* was designed according to the amino acid sequence of its mature peptide and the preference of *E. coli* codon. Firstly, SUMO fusion tag was combined with ALF*Pm10* and connected to prokaryotic expression vector pColdIV. Then the recombinant vector pColdIV-SUMO-ALF*Pm*10 was constructed and transformed into prokaryotic expression host strain *E.coli* BL21 (DE3). Induced by 0.4 mM IPTG for 9 h, the recombinant protein of SUMO-ALF*Pm10* was expressed in highest level and with high solubility. Moreover, ALF*Pm10* could be purified by affinity chromatography with the concentration of 335 μg/mL. The sequence of ALF*Pm10* was conformed by LC–MS after removing SUMO tag by SUMO enzyme. Moreover, ALF*Pm*10 at 24 μM showed significantly antibacterial activity against six testing bacteria within 12 h. In a word, the above research would provide a great foundation to further study the functions of ALFs.

Keywords Antimicrobial peptides · Anti-lipopolysaccharide factors, ALFs · Prokaryotic expression · Affinity chromatography · Antibacterial activity

Introduction

Antimicrobial peptides (AMPs), as a crucial immune molecule, play a very important role in innate immune defense (Hancock et al. 2006; Li et al. 2021a, b). Considered as a natural antibiotic, AMPs widely exist in natural organisms and have broad-spectrum antimicrobial activity against various bacteria, fungus, viruses and parasites (Bulet et al. 2004). Compared with traditional antibiotics, AMPs is not easy to cause bacterial resistance (Li et al. 2012), so that it is the most promising new antibiotic. So far, more than 2960 AMPs have been found, of which 2184 are from animals (Moravej et al. 2018; Lin et al. 2021).

Invertebrates rely on innate immunity to defend invasive microbial pathogens because of its lack of adaptive immunity. Innate immunity is composed of cellular immunity and humoral immunity, and AMPs is one of the main component of humoral immunity. Pattern recognition proteins (PRPs) on the cell surface will trigger the signal transduction pathway once they detect the invasion of pathogens, afterwards, AMPs will be produced to kill pathogens (Tassanakajon et al. 2018).

At present, various AMPs have been found in crustaceans, including anti-lipopolysaccharide factors (ALFs), penaeidins, crustins, lysozymes and hemocyanin derived peptides (Tassanakajon et al. 2013; Destoumieux-Garzón et al. 2016; Zhou et al. 2019). There are three main classes of AMPs found in shrimp, namely penaeidins, crustins and ALFs (Gross et al. 2001; Supungul et al. 2002).

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ALFs were first identified from the blood lymphocytes of *Limulus polyphemus* and *Tachypleus tridentatus* (Supungul et al. 2002). It could bind and neutralize bacterial lipopolysaccharide (LPS). Also, it could inhibit the coagulation cascade caused by LPs and thus it is named for anti-lipopolysaccharide factor (Tanaka et al. 1982; Morita et al. 1985). ALF is generally composed of 120–150 amino acids, including a signal peptide sequence and a mature peptide sequence. Its secondary structure consists of multiple amino acids α-helix and β-sheet (Supungul et al. 2002). All ALFs contain a two-cysteines conserved domain, which is composed of 22 amino acids, called lipopolysaccharide binding domain (LBD) with the activities of binding LPS and antibacterial (Hoess et al. 1993). For example, ALF2 and LBD of ALF7 from *Penaeus chinensis* inhibit the growth of bacteria, while ALF1, ALF2 ALF5 and ALF7 inhibit the growth of White Spot Syndrome Virus (WSSV) (Li et al. 2015). It is reported that ALF*Pm3* found in *P. monodon* has antimicrobial activity against Gram-positive bacteria *Bacillus megaterium*, Gram-negative bacteria *Vibrio harveyi* and fungus *Fusarium oxysporum* (Somboonwiwat et al. 2005). Also, ALF*Pm6* found in *P. monodon* has extraordinary inhibitory effect on Gram-positive bacteria and Gram-negative bacteria (Kamsaeng et al. 2017).

In conclusion, AMPs is a crucial component of invertebrate immune system, while it is expressed in low level in vivo. Therefore, in order to study its function, we should obtain it by genetic engineering techniques. Because of AMPs' inhibitory effects on bacteria and fungus, and facilitation to purify, fusion tags are often added for expression. In this study, the gene sequence of AMPs ALF*Pm10* from *P. monodon* was obtained by alignment of transcriptome sequencing data with the AMPs' database. Recombinant expression in *Escherichia coli* after binding ALF*Pm10* with SUMO fusion protein and analysis of its antimicrobial activity were carried out to preliminarily explore its function, so as to provide a basis for subsequent application.

Materials and Methods

Vectors and Strains

Receptive *E.coli* BL21 (DE3) was purchased from Shanghai Weidi Biotechnology Co., Ltd. pColdIV carrier was purchased from General Biology (Anhui) System Co., Ltd.

Bacillus sp. T2, *Aeromonas hydrophyla*, *Vibrio parahaemolyticus*, *Bacillus*, *Streptococcus agalactiae*, *Vibrio harveyi* are owned by A5-428 laboratory, College of life and Marine Sciences, Shenzhen University;

Main Reagents

Ampicillin (AMP), trimethylolaminomethane (Tris), Isopropyl-1-thio-β-D-galactopyranosid (IPTG), sodium chloride (NaCl) and imidazole; Plasmid Extraction Kit was purchased from Takara company; 5 × Protein loading buffer and Broadford kit were purchased from Sangong Bioengineering (Shanghai) Co., Ltd; 96 well plate was purchased from kejing (Ningbo) Biotechnology Co., Ltd; dialysis bag (3.5kd) was purchased from Shanghai Beibo Biotechnology Co., Ltd; SUMO enzyme was purchased from General Biology (Anhui) System Co., Ltd.

Identification and Bioinformatics Analysis of ALF*Pm10* from *Penaeus monodon*

After stressing *P. monodon* by *Vibrio parahaemolyticus* for 3 h, the total RNA of hepatopancreas was extracted and sequenced (SRA: PRJNA473435). And a new AMPs' gene would be obtained after analysis of the transcriptome sequences with NT, NR, KOG, KEGG, Swissprot. ORF Finder was used to analyze the structure of the gene and to obtain the open reading frame and amino acid sequence of the gene. The amino acid signal peptide was predicted using Singal 4.0. DNAman 6.0 was used to display the gene information.The isoelectric point and relative molecular weight of mature peptides were predicted by Protparam. The secondary structure was predicted by Phyre2 web portal. The NCBI database Blastp was used to carry out homology analysis on AMP ALF*Pm*10, and the phylogenetic tree was constructed by MEGA X.

ALF*Pm10* Gene Synthesis and Vector Construction

The gene sequence of ALF*Pm*10 mature peptide was fused with SUMO fusion protein with 6 × His label at the C-terminal. *Nde*I and *Sac*I cut sites were added at both ends of the sequence respectively. The codon was optimized according to the codon preference of *E. coli*, and the gene sequence were obtained through chemical synthesis method. The synthesized gene was then linked to pColdIV vector by *Nde*I and *Sac*I cut sites to obtain the recombinant plasmid named pColdIV-SUMO-ALF*Pm*10.

PCR Verification of Recombinant Plasmid

The recombinant plasmid was constructed through PCR with forward primer pColdIV-F and reverse primer pColdIV-R. pColdIV-F was as 5′- ACGCCATATCGCCGAAAGG-3′ and pColdIV-R was as 5′- GGCAGGGATCTTAGATTCTG-3′.

The DNA of ALF*Pm10* as template, the PCR was performed with 94 ℃, 3 min; 30 cycles of 94 ℃, 20 s, 58 ℃, 20 s and 72 ℃, 40 s; 72 ℃, 5 min. The PCR product was checked by electrophoresis on a 1% agarose gel.

Prokaryotic Expression and Soluble Analysis of SUMO-ALF*Pm10*

Extracted by plasmid extraction kit, the recombinant construct was transformed into *E.coli* BL21 (DE3) by thermal activation method to obtain monoclonal colony. Select positive monoclonal colonies, inoculate them in LB medium containing Amp, cultivate them at 37℃ with shaking at 200 rpm overnight (12–16 h); On the next day, the overnight cultivated bacterial solution was added into the fresh LB broth at the ratio of 1:100, and then was cultivated at 37℃ with shaking at 200 rpm until the absorbance reached 0.4–0.6 at 600 nm. Add IPTG to the cultured at a final concentration of 1.0 mM. The induction was performed at 16 ℃ for 6 h, and take 1 mL bacterial solution as the control before induction. After induction, take 1 mL induced bacterial solution. Cells were harvested by centrifuging the remaining bacterial solution at 8000×g for 10 min, resuspended in Balance Buffer (50 mM Tris, 200 mM NaCl, pH = 8.0), and then disrupted by ultrasonication for 40 min (Power: 195 w, open for 2 s, stop for 4 s). After that, the supernatant and sediment were separated by centrifugation at 10,000× g, 4 ℃ for 30 min. The bacterial solution before induction, the bacterial solution after induction for 6 h, and the supernatant and sediment of the disrupted bacterial solution were added respectively into 5 × Protein Loading Buffer, boiled at 100℃ for 10 min, and then analyzed the expression and solubility of SUMO-ALF*Pm10* by 4–20% SDS-PAGE.

Optimization the Prokaryotic Expression Conditions of SUMO-ALF*Pm10*

In prokaryotic expression, induction time and IPTG concentration were the main conditions affecting protein expression. In order to obtain the best expression condition of SUMO-ALF*Pm10*, the induction time and IPTG concentration should be optimized. The sampling points were set to 0 h, 1.5 h, 3 h, 4.5 h, 6 h, 7.5 h and 9 h; the gradient values of IPTG final concentration were set to 0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM and 1.0 mM. The induced expression was performed according to Method 1.5, and then analyzed by 4–20% SDS-PAGE to determine the optimal expression conditions of SUMO-ALF*Pm10*.

Separation and Purification of SUMO-ALF*Pm10*

After determining the protein was soluble, the cells were harvested, and then resuspended in 4 mL Balance Buffer. The supernatant was collected by ultrasonication, filtered into a new 50 ml centrifuge tube using 0.45 μm filter membrane, and then separated and purified by Nickel Column affinity chromatography (BIO-RAD NGC SCOUT, USA). Balance the column with Balance Buffer before loading. The sample was loaded after the absorbance reached stable at 280 nm. After the absorbance decreased and reached stable at 280 nm, the miscellaneous proteins were removed with Washing Buffer (50 mM Tris, 200 mM NaCl, 100 mM imidazole), then the sample was collected with Elution Buffer 1 (50 mM Tris, 200 mM NaCl, 200 mM imidazole) and Elution Buffer 2 (50 mM Tris, 200 mM NaCl, 500 mM imidazole). Elutes were further analyzed by 4–20% SDS-PAGE.

The eluted protein was put into the dialysis bag, soaked in Balance Buffer, and dialyzed at 4℃ for 16 h to remove imidazole. After that the BSA standard curve was made according to the instructions of Broadford kit, the protein concentration of ALF*Pm10* was calculated according to the standard curve. The purified SUMO-ALF*Pm10* was finally stored at −80 ℃.

SUMO Fusion Label Excision and LC–MS Identification

Due to the large molecular weight of SUMO fusion label, it might affect the function of AMP ALF*Pm10*. Therefore, SUMO Enzyme was used to separate SUMO fusion label from ALF*Pm10*. According to the instructions of SUMO Enzyme, mix a certain quality of SUMO-ALF*Pm10* with a certain quality of SUMO Enzyme at 4℃ for 16 h for enzyme digestion. The enzyme digestion was analyzed by 4–20% SDS-PAGE.

After SDS-PAGE, the gel strip of ALF*Pm10* without SUMO fusion label was cut, extracted and placed in 1.5 mL EP tube. 50% acetonitrile was added into the EP tube and shaken to decolorize overnight at 37℃, 200 rpm/min. Discard the supernatant, add 100% acetonitrile to whiten and harden the gel, discard the supernatant. After trypsin (25 μg/mL) hydrolysis, add Covering Solution (25 mM $NaHCO_3$ dissolved in 1000 mL ddH_2O) and water bath at 37 ℃ for 16 h. Afterward the solution was transferred to a new 1.5 mL EP tube and mixed with the Extract (acetonitrile and ddH_2O were mixed in the ratio of 1:4 and 0.5% of the total volume of formic acid). Protein powder was obtained by ultrasonication for 10 min, centrifugation and vacuum drying for 4 h. Dissolve the protein powder with LC–MS Loading Buffer, then centrifuged at 13,000× g for 15 min after vortex oscillation. ALF*Pm10* protein was identified by liquid chromatography mass spectrometer (LC–MS, ABSCIEX 5600, USA).

Antibacterial Activity Analysis of ALF*Pm10*

Testing bacteria including *Bacillus* sp. T2, *Aeromonas hydrophila*, *Bacillus*, *Streptococcus agalactiae*, *Vibrio parahaemolyticus* and *Vibrio harveyi* which stored at −80 °C were cultured in LB broth with shaking at 37 ℃, 200 rpm/min overnight for 12–16 h. Afterward the cultured bacterial solution was inoculated into fresh LB both at the ratio of 1:100 with shaking at 37 ℃, 200 r/min. Until OD_{600} reached 0.3, the bacterial solution was diluted by 1000 times using fresh LB broth, then the 50 μL/well diluted cultures were mixed with ALF*Pm10* LBD into sterile 96-well plate and cultivated at 37℃. The final concentration of ALF*Pm10* LBD was 128 μM. The OD_{600} was determined every 3 h, and the bacterial growth curve was drawn after 15 h to analyze the antibacterial activity of ALF*Pm10* LBD. The final concentration of positive control was 500 μg/mL ampicillin; the negative control was added with balance buffer. The experiment was repeated three times.

Statistical Analysis

Data in figures were shown as the average of at least three biological replicates with standard deviation. T-test (GraphPad Prism 5.0/8.0) was performed for statistically analysis, and P-value of < 0.05 were considered as statistical significance.

Results

Identification of ALF*Pm10*

After analysis of transcriptome data (SRA: PRJNA473435) with NT, NR, KOG, KEGG, Swissprot and antimicrobial peptides database, a new ALF gene was obtained and named ALF*Pm10*.

As shown in Fig. 1a, ALF*Pm10* nucleotide sequence was determined by RT-PCR, with a total length of 369 bp and encoding 123 amino acid residues, including a signal peptide containing 25 amino acid residues and a mature peptide containing 98 amino acid residues. A LBD domain containing two conserved Cysteines was located between 55 and 77th amino acid residues. The relative molecular weight of ALF*Pm10* mature peptide was 11.079 kDa and the isoelectric point (PI) was 9.04. As shown in Fig. 1b, the secondary structure of ALF*Pm10* contained one α-helix structure at its N-terminal, three β-sheet structures in the middle part and two α-helix structures at its C-terminal.

After the alignment of amino acid sequences of ALF*Pm10* by Blastp, 31 ALFs from several common shrimps were obtained, such as *Penaeus chinensis*, *Penaeus vannamei*, *Penaeus monodon* and *Penaeus japonicus*. ALF*Pm10* shared 89% similarity with ALF1 from *Penaeus chinensis,* 83% similarity with ALF1 from *Penaeus vannamei*, 76% similarity with ALFD1 from *Penaeus japonicus*, and had low similarity with other ALFs. As shown in Fig. 2, the phylogenetic tree showed that ALF*Pm10* from *P. monodon* and ALF1 from *P. chinensis* were distributed in one branch, indicating that their genetic relationship is closer.

Vector Construction and PCR Verification

According to the codon preference of *E. coli*, the gene sequence of His-SUMO-ALF*Pm*10 was successfully obtained by chemical synthesis method, and finally connected to *Nde*I and *Sac*I of pColdIV vector to obtain the recombinant plasmid pColdIV-SUMO-ALF*Pm*10 (Fig. 3a). The recombinant construct pColdIV-SUMO-ALF*Pm*10 was verified by PCR using the universal primers pColdIV-F and pColdIV-R of pColdIV vector. The PCR result showed that the recombinant vector was successfully constructed (Fig. 3b).

Expression and Soluble Analysis of SUMO-ALF*Pm*10

The recombinant construct pColdIV-SUMO-ALF*Pm*10 was successfully transferred into *E.coli* BL21 (DE3). It was shown that the fusion protein ALF*Pm*10 can be normally expressed after induction with 1.0 mM IPTG for 6 h, and its relative molecular weight was about 25 kDa. Most of the target protein existed in the supernatant, indicating that the fusion protein ALF*Pm*10 is a soluble protein (Fig. 4).

Optimization the Expression Conditions of SUMO-ALF*Pm10*

Induced by 1.0 mM IPTG, the expression of fusion protein ALF*Pm*10 increased depending on time, and reached the maximum at 9 h (Fig. 5a). Under the condition of induction with different final concentrations of IPTG for 9 h, the expression of SUMO-ALF*Pm*10 with 0.4 mM IPTG was at the highest level. Therefore, by optimizing the expression conditions, it could be determined that SUMO-ALF*Pm*10 can express at high level with the induction of 0.4 mM IPTG for 9 h (Fig. 5b).

Separation and Purification of SUMO-ALF*Pm*10

After filtering the supernatant of fusion protein ALF*Pm*10, it was separated and purified by affinity chromatography. As shown in Fig. 6, the collected fusion protein solution has a clear strip near 25 kDa, indicating that high purity target protein can be collected with 500 mM imidazole. The collected target protein solution was dialyzed at 4 ℃ for

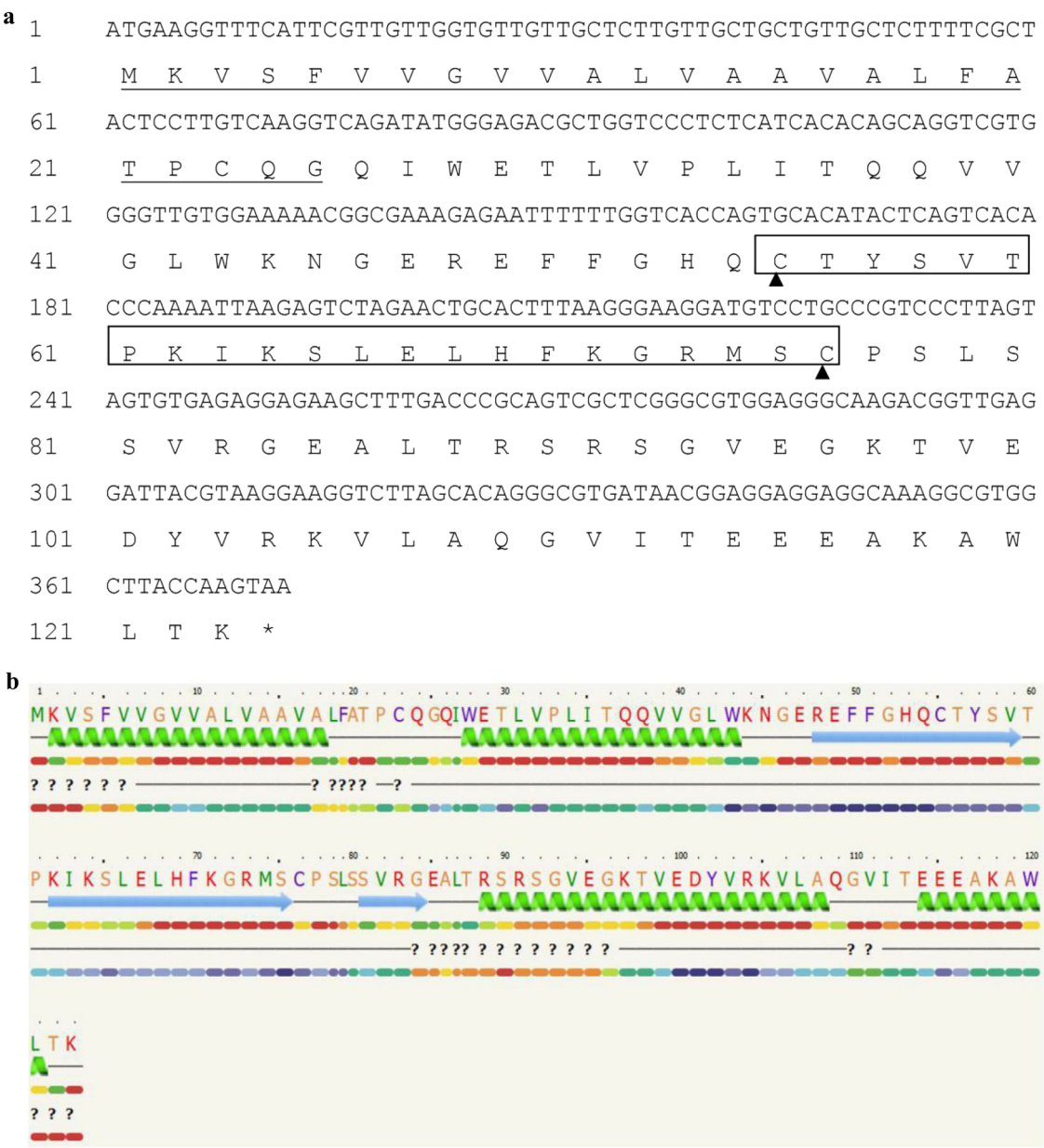

Fig. 1 Nucleotide and amino acid sequence of ALFPm10 from *P. monodon* (**a**) and Secondary structure of ALF*Pm10* from *P. monodon* (**b**). (Green: α-helix structure, Blue: β-sheet structure) (Color figure online)

16 h to remove imidazole, and the concentration of SUMO-ALF*Pm*10 was 335 μg/mL according to the BSA standard curve.

Acquisition and Mass Spectrometry Identification of Tag-Free ALF*Pm10*

SUMO enzyme was a protease with high activity. It can only distinguish the tertiary structure of SUMO protein and can cut accurately at the C-terminal of double Glycine (Gly) residue. Hence, the target protein can be separated from SUMO protein efficiently and specifically without any excess amino acids (Fig. 7a).

According to the instructions of SUMO enzyme, 50 μg ALF*Pm10* with SUMO fusion label was digested by 0.5 μg SUMO enzyme and analyzed by 4–20% SDS-PAGE. The molecular weight of ALF*Pm10* without SUMO fusion label was about 11 kDa, which was consistent with the expected size (Fig. 7b). The ALF*Pm10* free from SUMO was identified by LC–MS. A total of two peptide segments were detected, and the amino acid coverage was as high as 56%,

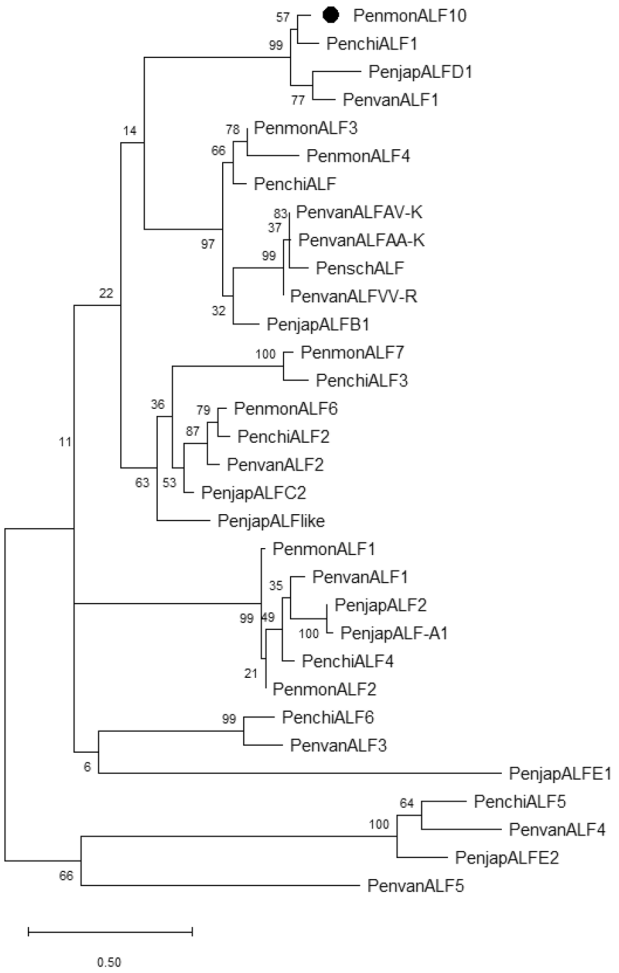

Fig. 2 Phylogenetic tree of ALF*Pm10* from *P. monodon. Penaeus chinensis* (*Penchi*ALF: AHN13886.1; *Penchi*ALF1: AFU61124.1; *PenchiALF2*: AFU61125.1; *Penchi*ALF3:: AFU61126.1; *Penchi*ALF4: AFU61127.1; *Penchi*ALF5: AFU61128.1; *PenchiALF6*: AFU61129.1); *Penaeus japonicas* (*Penjap*ALF2: BAH22585.1; *Penjap*ALF-A1: ANA91278.1; *Penjap*ALFB1: ASR74829.1; *Penjap*ALFC2: AME17862.1; *Penjap*ALFD1: AME17863.1; *Penjap*ALFE1: ASR74830.1; *Penjap*ALFE2: ASR74831.1; *Penjap*ALFlike: BAE92940.1); *P. monodon* (*Penmon*ALF1: ABP73290.1; *Penmon*ALF2: ABP73291.1; *Penmon*ALF3: AEW91477.1; *Penmon*ALF4: ABP73293.1; *Penmon*ALF5: CF415871.1; *Penmon*ALF6: AER45468.1; *Penmon*ALF7: ANP92039.2); *Penaeus vannamei* (*Penvan*ALF1: AVP74301.1; *Penvan*ALF1-: AHG99284.1; *Penvan*ALF2: AVP74302.1; *Penvan*ALF3: AVP74303.1; *Penvan*ALF4: AVP74304.1; *Penvan*ALF5: AVP74305.1; *Penvan*ALFAA-K: ABB22833.1; *Penvan*ALFAV-K: ACT21197.1; *Penvan*ALFAV-R: ABB22832.1); *Penaeus schmitti* (*Pensch*ALF: ABJ90465.1)

indicating that ALF*Pm10* were obtained through prokaryotic expression and SUMO enzyme digestion (Fig. 7c).

Antibacterial Activity Analysis of ALF*Pm10*

The antibacterial activities of ALF*Pm10* on six kinds of bacteria, including *Bacillus* sp. T2*, Aeromonas hydrophila, Bacillus, Streptococcus agalactiae, Vibrio parahaemolyticus* and *Vibrio harveyi* were studied. Results showed that 24 μM ALF*Pm10* owned obvious antibacterial activity against six bacteria including *Aeromonas hydrophila*, *Bacillus*, *Streptococcus agalactiae*, *Vibrio parahaemolyticus* within 12 h, while showed moderate inhibitory effect on *Bacillus* sp. T2 and *Vibrio harveyi* (Fig. 8). Moreover, the inhibitory effect decreased quickly after 12 h.

Discussion

In this study, a new antibacterial peptide ALF gene was obtained from the transcriptome analysis which are from the *P. monodon* infected by *Vibrio parahaemolyticus*. It was reported that the secondary structure of ALFs consists of multiple α-helix and β-sheet structures, and contains a LBD domain composed of 22 amino acids (Somboonwiwat et al. 2008). By bioinformatics analysis, the structural characteristics of the antibacterial peptide ALF*Pm10* are similar to the reported ALF, indicating that the novel discovered antibacterial peptide belongs to ALFs family. Phylogenetic tree shows that ALF*Pm10* has high homology with ALF1 of *Penaeus chinensis*, and ALF of *Penaeus chinensis* has inhibitory activity against Gram-positive bacteria and a few viruses (Li et al. 2014). Thus, it is speculated that ALF*Pm10* might have similar functions.

Due to its small molecular weight and antibacterial effect, AMPs have a certain killing effect on prokaryotic expressing host bacteria, so it is difficult to obtain a large number of expression. The expression of ALF*Pm10* without SUMO protein was failed, since ALF*Pm10* showed the inhibition of the growth of *E.coli*. As a molecular chaperone, SUMO can improve the expression of protein, promote the correct folding of protein and enhance the soluble expression. And its molecular weight is small, easy to be cut and will not affect the activity of protein (Li 2009). Therefore, SUMO fusion technology is selected for expression in this study, but the AMP generally has only 15–50 amino acids, and the presence of several exogenous amino acids will also affect its function (LI et al. 2020). Therefore, the labeled SUMO fusion protein was excised by SUMO enzyme to obtain ALF*Pm10* without exogenous amino acids.

During prokaryotic expression, induction time and IPTG concentration have a great impact on protein

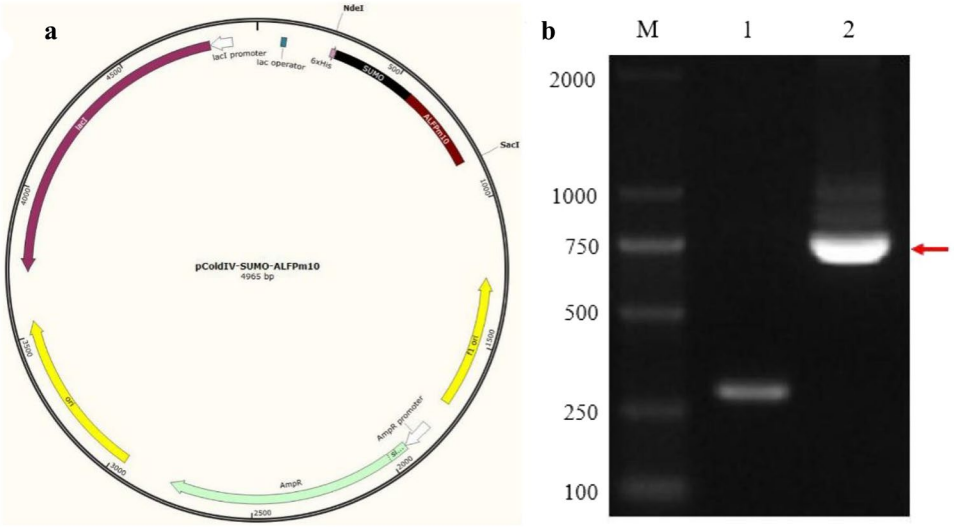

Fig. 3 The sketch map of the ALF*Pm10* plasmid (**a**) and PCR verification of recombinant plasmid (**b**). M: DL 2000 marker; 1: negative control, pColdIV plasmid; 2: PCR analysis of pColdIV-SUMO-ALF*Pm*10

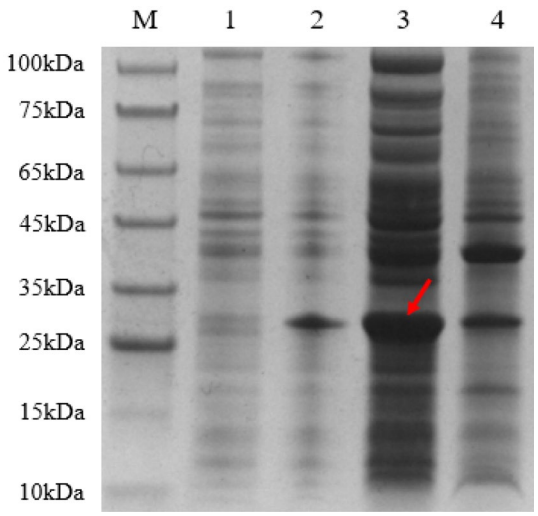

Fig. 4 Expression and soluble analysis of SUMO-ALF*Pm*10. M: protein marker; 1: without IPTG induction; 2: induction with 1.0 mM IPTG for 6 h; 3: the supernatant after cell disruption; 4: the sediment after cell disruption

expression (Wang et al. 2019). It is because after protein expression for a certain period of time, its expression will not change much, or even decrease. The host bacteria autolyse after reaching the death stage, release the protease in the body, and degrade part of the induced protein should be responsible for it (Peng et al. 2015). IPTG is an inducer with certain toxicity. If its concentration is too high, it will be toxic to the host bacteria and affect its growth and metabolism. If its concentration is too low, it will affect the expression of protein, or even no expression (Peng et al. 2015). Therefore, this study optimizes the induction time and IPTG concentration by fixing other conditions, so as to obtain the optimized conditions of SUMO-ALF*Pm10*.

Using nickel column affinity chromatography to purify SUMO-ALF*Pm10* is because the histidine exposed on the surface of the protein can specifically bind to the nickel ion on the resin, and it has the advantages of strong adsorption, suitable purification conditions and no effect on protein activity (Wang et al. 2019). Due to the different pI value, secondary structure and biological activity of each protein, the concentration of imidazole in the eluent used to elute the target protein is various. It is necessary to constantly explore and select the best imidazole concentration, which can not only ensure the removal of more miscellaneous proteins, but also smoothly obtain the target protein with higher purity. Therefore, in this study, Firstly, low concentration imidazole (100 mM) was used to remove weak binding protein, and then medium concentration imidazole (200 mM) was used to further remove impurities. Finally,

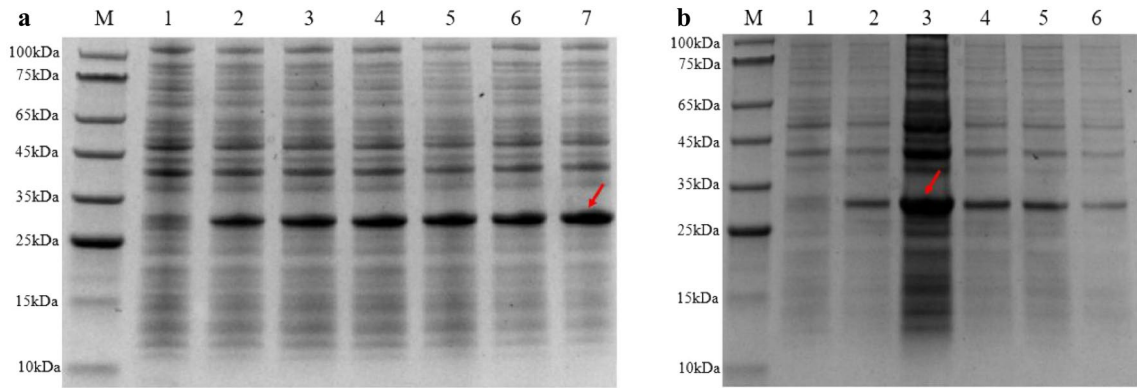

Fig. 5 Optimizing the expression of SUMO-ALF*Pm10*. **a** Optimization of the inducing time for SUMO-ALF*Pm10*. M: protein marker; 1: induction without IPTG; 2–7: induction with 1.0 mM IPTG for 1.5 h, 3 h, 4.5 h, 6 h, 7.5 h, 9 h. **b** Optimization of the IPTG concentration for SUMO-ALF*Pm10*. M: protein marker; 1: induction without IPTG; 2–6: induction with 0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM,1.0 mM IPTG for 9 h

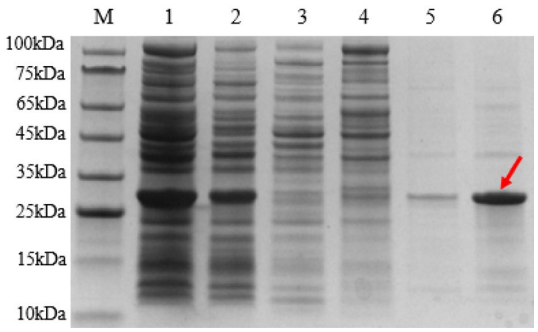

Fig. 6 Purification of SUMO-ALF*Pm10* by affinity chromatography. M: protein marker; 1: sample, which is the supernatant after ultrasonication; 2 and 3: flowthrough, which was loading effluent; 4: Removing impurity substance by Washing Buffer; 5: eluting SUMO-ALF*Pm10* with Elution Buffer 1; 6: eluting SUMO-ALF*Pm10* with Elution Buffer 2

high concentration imidazole (500 mM) was needed to completely collect the target protein.

It is reported that ALF*Pm3* found in *P. monodon* was recombined and expressed against Gram-positive bacteria *Bacillus megaterium,* Gram-negative bacteria *Vibrio harveyi* and fungi *Fusarium oxysporum*. And the minimum inhibitory concentration against various bacteria is 0.19–100 μM indicating its broad-spectrum antibacterial activity (Somboonwiwat et al. 2005), ALF*Pm6* was found in *P. monodon*, and its LPS-BD cyclic peptide has inhibitory effect on Gram-negative bacteria and Gram-positive bacteria (Kamsaeng et al. 2017). In this study, the microbial growth curve was measured by optical density at 600 nm to perform the antibacterial activity.

Compared with the other ALFs, ALF*Pm10* also contained LBD conserved domain. It was analyzed that

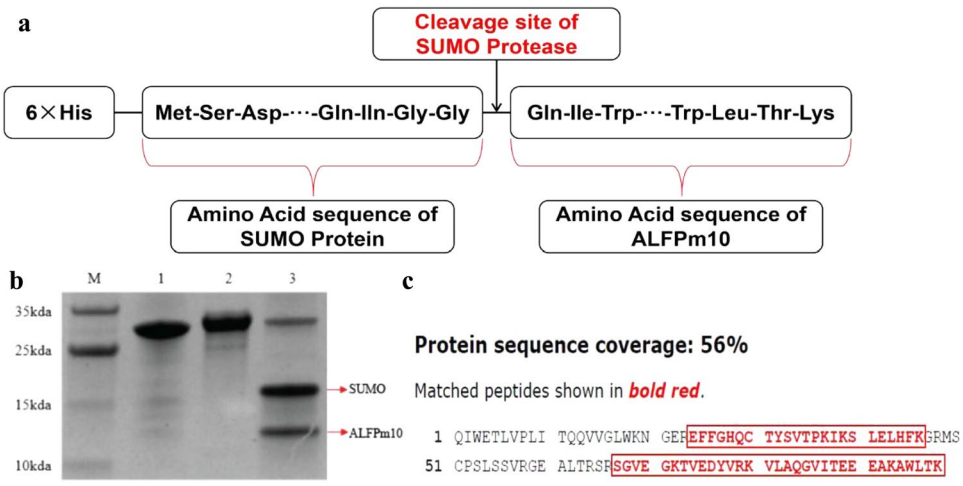

Fig. 7 **a** Schematic diagram of SUMO enzyme cutting site. **b** SUMO enzyme digestion of SUMO-ALF*Pm10*. M: protein marker; 1: SUMO-ALF*Pm10*; 2: SUMO enzyme; 3: ALF*Pm10*. **c** Mass spectrum identification of ALF*Pm10*

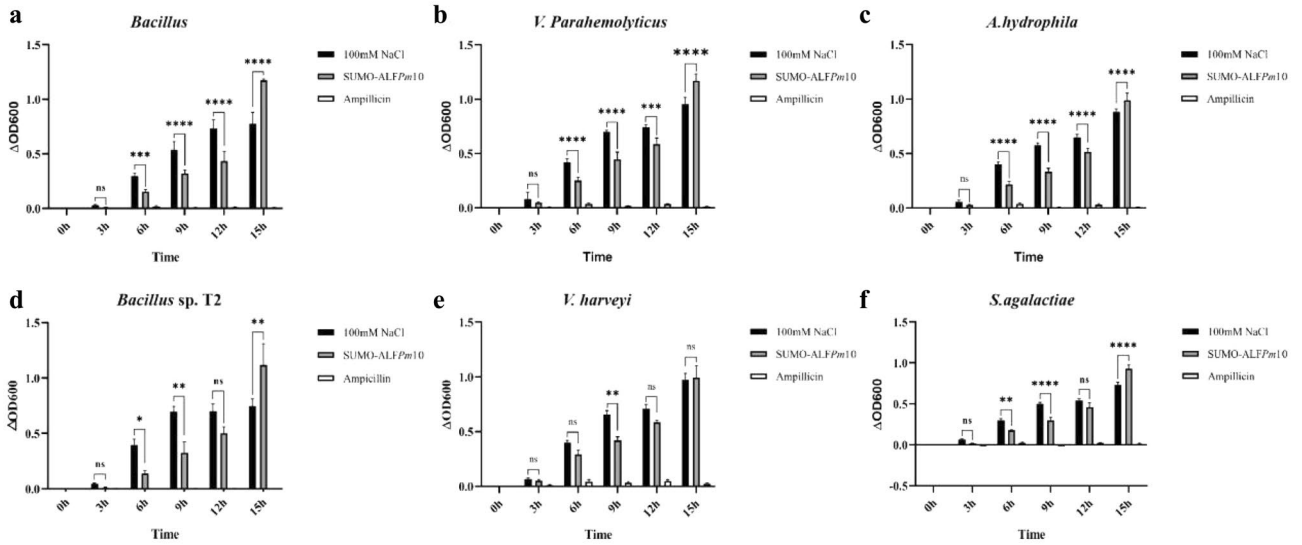

Fig. 8 The growth curve of testing bacteria under SUMO-ALF*Pm10*. **a** *Bacillus*; **b** *V. Parahemolyticus*; **c** *A.hydrophila*; **d** *Bacillus* sp. T2*;* **e** *V.harveyi;* **f** *S.agalactiae.* The concentration of SUMO-ALF*Pm10* was 24 μM and ampicilin was 500 μg/mL

ALF*Pm10* in 24 μM had a certain inhibitory effect on six testing bacteria within 12 h. Bacteria such as *Vibrio parahaemolyticus* and *Vibrio harveyi* caused fatal disease in aquaculture. Hence, our results showed its application potential in aquaculture.

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Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

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