



Improved Antimicrobial Activity of Bovine Lactoferrin Peptide (LFcinB) Based on Rational Design

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Abstract

Bovine lactoferrin peptide (LFcinB), as an antimicrobial peptide, is expected to be an alternative of antibiotics owing to its broad-spectrum antimicrobial activity and specific mechanism. However, the weak antimicrobial activity, high hemolysis, and poor stability of LFcinB limited its applications in the field of biomedicine, food and agriculture. In order to improve the antimicrobial activity of LFcinB, five mutants were designed rationally, of which mutant LF4 (M10W/P16R/A24L) showed highest antimicrobial activity. The bioinformatics analysis indicated that the improved antimicrobial activity of LF4 was related to its increased cations, higher amphiphilicity and the extension of the β -sheet in the structure. These studies will highlight the important role of bioinformatic tools in designing ideal biopeptides and lay a foundation for further development of antimicrobial peptides.

Keywords Bovine lactoferrin peptide · Actimicrobial activity · Rational design · Bioinformatics analysis

1 Introduction

Antimicrobial resistance (AMR) is a critical health challenge, which causes substantial economic burden, morbidity and death globally [1]. The emergence and rapid spread of multidrug-resistant (MDR) bacteria poses a huge threat to human health due to the misuse of antibiotics with 10 million people expected to die from antibiotic resistance by 2050 [2]. In 2019, WHO identified 32 antibiotics in clinical development, of which six were classified as innovative [3]. Therefore, it is urgent to develop novel antimicrobial drugs to replace antibiotics. Antimicrobial peptides (AMP) have broad-spectrum antibacterial activity, strong antibacterial ability and less likely to develop resistance, which are the potential alternatives to antibiotics [4, 5]. However, natural antimicrobial peptides usually have low antimicrobial activity, stability and high hemolytic activity, which greatly limits the applications of antimicrobial peptides [6].

Lactoferrin, as a multifunctional protein (including bacterial clearance and pathogen opsonization), has been well-studied in over the past decades [7–10]. Bovine lactoferrin peptide (LFcinB), the digest product of bovine lactoferrin (BLf) catalyzed by pepsin, is a peptide containing 25 amino acid residues (Residue No. 17–41, Mw: 3.1 kDa) near the N terminal of BLf [11–17]. In the intact BLf, the peptide fragment of LFcinB folds into an α -helical conformation, the conformation transforms into a twisted, antiparallel β -sheet structure when released from BLf [18]. In the mature peptide LFcinB, the hydrophobic surface is made up of the residues F1, C3, W6, W8, P16, I18, and C20 and resulted in amphiphilicity (the physicochemical property for antimicrobial effects) due to the high proportion of basic residues [19]. Owing to the small molecular weight and broad-spectrum antimicrobial activity, LFcinB has a wide range of applications in food preservation, biomedicine and feed additives [20, 21].

In this study, we constructed three expression systems (pET32a-LFe, pET32a-LFt and pET28a-SUMO-LF) to express and prepare the peptide LFcinB. Then, five mutant peptides were rationally designed using bioinformatic tools (Table S3), of which mutant LF4 (M10W/P16R/A24L) had improved antimicrobial activity compared with LFcinB. The stability and physicochemical properties of LFcinB and LF4 were analyzed and compared. Lastly, the potential

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explanations for the improved antimicrobial activity of LF4 were systematically discussed.

2 Materials and Methods

2.1 Materials

Escherichia coli BL21(DE3) was used as expression host strain. Plasmid pET32a and pET28a were utilized as expression vectors. PrimeSTAR Max Premix (2x) for PCR amplification (catalog number: R045A) and endonuclease DpnI (catalog number: 1235 S) were purchased from TaKaRa Co., Ltd (Japan). 2x Taq Master Mix (catalog number: P111-01) was purchased from Vazyme Biotech Co., Ltd (Nanjing, China). IPTG (catalog number: A100487), Ampicillin (catalog number: A610029) and Kanamycin (catalog number: A600286) were purchased from Sangon Biotech Co., Ltd (Shanghai, China). Enterokinase (catalog number: E8350) and thrombin (catalog number: T8021) were purchased from Solarbio Co., Ltd. (Beijing, China). SUMO protease (catalog number: PE007) was purchased from Novoprotein Scientific Inc (Shanghai, China). Easy II Protein Quantitative Kit (BCA) (catalog number: DQ111-01) was purchased from Transgen Biotech Co., Ltd (Beijing, China). Ni-NTA resins (catalog number: 70666-3) was purchased from Novagen Co., Ltd (America).

2.2 Construction of Recombinant Plasmids

The primers used in this work were listed in Table S1-S2. The 25-amino acid protein sequence of LFcinB (FKCRRWQWRMKKLGAPSITCVRRAF) was downloaded from NCBI and transformed into nucleotide sequence based on the codon preference in *E. coli*. The LFcinB encoding gene were RF-cloned (restriction-free) into expressing vector pET32a and pET28a to produce three recombinant plasmids with different protease cleavage sites, namely pET32a-LFe (enterokinase), pET32a-LFt (thrombin) and pET28a-SUMO-LF (SUMO protease) [22] (Fig. S1). Mutant plasmids (pET28a-SUMO-LF1, pET28a-SUMO-LF2, pET28a-SUMO-LF3, pET28a-SUMO-LF4 and pET28a-SUMO-LF5) were also constructed according to above experimental procedures.

The PCR product was transformed into competent *E. coli* BL21 (DE3) after digestion with DpnI (37°C, 1 h). The single colony in screening plate was picked up for verification by PCR with T7 universal primers and DNA sequencing.

2.3 Protein Expression, Purification and Digestion

The engineering *E. coli* BL21 (DE3) cells harboring recombinant plasmid were grown overnight at 37°C and 200 rpm in LB medium containing 50 mg/L kanamycin or 100 mg/L ampicillin. The overnight culture was transferred into fresh medium at a ratio of 1 to 100, and incubated at 37°C and 200 rpm.

Once the OD₆₀₀ reached a value of 0.6–0.8, varied concentration of IPTG (0, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mM) was added to induce protein expression, and the culture was incubated at different temperature (16, 20, 25, 30 and 37°C) and 200 rpm for an additional 16 h [23]. The cells were harvested by centrifugation (7,000 rpm, 5 min) under 4°C, and resuspended with buffer (25 mM Tris-HCl, pH8.0). The cells were lysed by ultrasonication and induced proteins were purified by Ni-NTA resins (Novagen) according to the manufacturer's instructions. Then, purified protein was qualified by SDS-PAGE analysis and quantified using Easy II Protein Quantitative Kit (BCA) (TransGen Biotech). Lastly, to obtain antimicrobial peptides for further activity assay, three kinds of purified proteins was further digested by enterokinase, thrombin and SUMO proteases (1 U/50 µg fusion protein) at 25°C, respectively.

2.4 Antimicrobial Activity Assay

Gram-negative *E. coli* MG1655 and Gram-positive *Staphylococcus aureus* ATCC 25,923 were used as indicator strains for antimicrobial activity assays [24]. Single colony was transferred into fresh Mueller-Hinton Broth (MHB) medium and incubated at 37°C and 200 rpm until the cells grow in logarithmic growth phase. The cells were diluted to 5×10^5 CFU/mL with MHB medium. Briefly, 50 µL MHB medium containing different concentration of peptide LFcinB or its mutants (0, 1.25, 2.5, 5, 10, 20 and 40 µM) was co-incubated with 50 µL diluted cells in 96 microplates and incubated at 37°C and 200 rpm for 16 h. The optical density was monitored at 600 nm. Each group had three replicates. The inhibition rate (%) can be solved according to the following equation:

$$\text{Inhibition rate} = \frac{\text{Control group's } OD_{600} - \text{Test group's } OD_{600}}{\text{Control group's } OD_{600}} \times 100\%$$

The minimum inhibitory concentration (MIC) are defined as the lowest concentration of antimicrobial agent that inhibits the visible growth of microorganism [25]. Besides MIC₉₅, MIC₅₀ can be also specified according to the 95% and 50% inhibition.

2.5 Effect of Temperature, pH, Salts and Protease on Antimicrobial Activity

To evaluate the thermostability [26, 27], antimicrobial peptides (20 μ M) were incubated at different temperatures (4, 20, 40, 60, 80 and 100°C) for 1 h, and then tested for antimicrobial activity against *E. coli* MG1655 as indicator strain.

To evaluate the stability at different pH [26, 28, 29], antimicrobial peptides were adjusted with Tris-glycine buffer (pH2.0, 100 mM), Sodium acetate buffer (pH4.0, 100 mM), Phosphate buffer (pH6.0, 100 mM), Tris-HCl buffer (pH8.0, 100 mM) and Glycine-NaOH (pH10.0, 100 mM) to different pH (2.0, 4.0, 6.0, 8.0 and 10.0), respectively and incubated at 37°C for 4 h. The treated samples were neutralized to pH7.0 with 1 N HCl or 1 N NaOH solution, and then tested for antimicrobial activity against *E. coli* MG1655 as indicator strain.

To evaluate the stability at physiological concentrations of salts [30, 31], antimicrobial peptides were added into different concentrations of salts (150 mM NaCl, 4.5 mM KCl, 6 mM NH_4Cl , 8 mM ZnCl_2 , 1 mM MgCl_2 and 2.5 mM CaCl_2) and incubated at 37°C for 4 h. Then the samples were tested for antimicrobial activity against *E. coli* MG1655 as indicator strain.

To evaluate the stability under proteases [32, 33], antimicrobial peptides were treated with following proteases: Pepsin (pH2.0), Trypsin (pH6.0), Proteinase K (pH7.0) and Papain (pH8.0). All samples were incubated at 37°C for 4 h and were neutralized to pH7.0, and then tested for antimicrobial activity against *E. coli* MG1655 as indicator strain.

2.6 Hemolysis Determination

50 μ L antimicrobial peptide LFcinB or LF4 of different concentrations (5, 10, 20 μ M) were added to the blood plates by Oxford cup method [34], and then the plates were incubated at 37°C for 16 h [35]. Sterile Tris-HCl buffer (50 mM, pH8.0) and 0.1% Triton-100 were regarded as negative and positive control, respectively. By this method, we qualitatively assess biocompatibility by area size.

2.7 Bioinformatic Analysis

CAMP_{R3} (<http://www.camp3.bicnirrh.res.in/>) is a collection of antimicrobial peptides [36], which was utilized to design antimicrobial peptide rationally (Table S3) and predict antimicrobial activity (Table S4). Physicochemical properties of antimicrobial peptide (such as Molecular weight, Charge, GRAVY, Theoretical pI and Instability index) were analyzed by ProtParam online tool (<https://web.expasy.org/prot-param/>) [37–39]. The amphipathic distribution was calculated by ProtScale (<https://web.expasy.org/protscale/>) [40].

PeptideCutter website (https://web.expasy.org/peptide_cutter/) was used to predict possible cleavage sites cleaved by proteases in a given protein sequence [40]. Homology modeling was performed by EasyModeller software [41] to generate the structures of mutants.

3 Results

3.1 Expression of Fused Protein in *E. coli* BL21 (DE3)

In order to obtain the fused LFcinB from *E. coli* BL21 (DE3), we firstly designed three recombinant plasmids (including pET32a-LFe, pET32a-LFt and pET28a-SUMO-LF) for expressing fusion proteins TrxA-LFe, TrxA-LFt and SUMO-LF respectively. The inducible protein bands of TrxA-LFe, TrxA-LFt and SUMO-LF were distributed in the range of 17–25 kDa (Fig. 1A), 11–17 kDa (Fig. 1B) and 17–25 kDa (Fig. 1C) respectively, which were well in accordance with the theoretical molecular weight (TrxA-LFe: 20.2 kDa, TrxA-LFt: 17.2 kDa, SUMO-LF: 18.44 kDa).

For heterologous expression in *E. coli*, temperature and IPTG concentration are the key factors affecting soluble expression [42]. Too high temperature or IPTG concentration tends to lead to too fast transcription, and inclusion bodies are easily produced when proteins have no enough time to fold into the correct configuration [42]. Low temperature or IPTG concentration can significantly reduce the transcription level of bacteria, so that the protein has enough time to fold properly, but the expression level will also be decreased [42]. Different proteins have different folding efficiency, which requires us to optimize the expression temperature and IPTG concentration for inducing the three fusion proteins (Fig. S2). As the temperature increased from 16°C to 30°C, the expression of TrxA-LFe gradually increased (Fig. S2A). Inclusion bodies were found at 37°C (Fig. S2A), indicating that the excessive temperature was not conducive to the soluble expression of fused peptide TrxA-LFe. 30°C was the best inducing temperature for expression of TrxA-LFe with the highest expression and solubility. Then, we further analyzed the effect of IPTG concentration on the expression of TrxA-LFe under 30°C. The expression of TrxA-LFe increased slightly with the increase of IPTG concentration (Fig. S2D). Since the high concentration of IPTG may affect the growth of organisms and even lead to the generation of inclusion bodies, 0.1 mM IPTG was finally chosen as the optimum induction concentration (Fig. S2D). Therefore, the optimal temperature and IPTG concentration for TrxA-LFe were 30°C and 0.1 mM. Similarly, the optimal temperature and IPTG concentration for TrxA-LFt and SUMO-LF were 25°C (Fig. S2B), 0.1 mM (Fig. S2E) and 20°C (Fig. S2C), 0.1 mM (Fig. S2F) respectively.

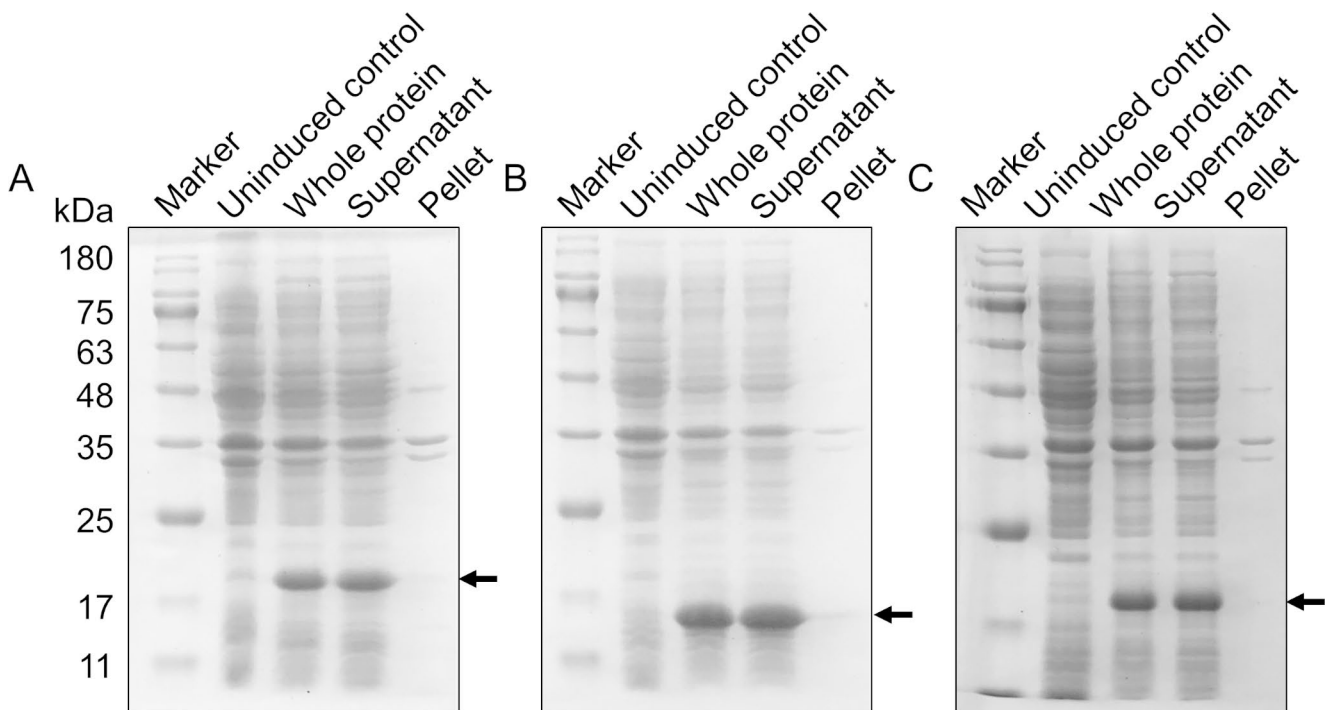


Fig. 1 Expression of recombinant fusion proteins TrxA-LFe (A), TrxA-LFt (B) and SUMO-LF (C) in *E. coli* BL21C (DE3).

3.2 Digestion of Fused Protein

The expression of three fusion proteins were induced at large scale under optimal induced conditions and purified with Ni-chelating affinity chromatography. Purified proteins were further digested by proteases according to the cleavage site in fused tag.

The digestion of TrxA-LFe by enterokinase was non-specific, with a high number of bands (Fig. 2A) appearing on SDS-PAGE. Moreover, TrxA-LFe could not completely digested within 18 h (Fig. 2A). For TrxA-LFt, it was fully cleaved within 15 h and released the N-terminal TrxA tag and C-terminal LFcinB peptide (Fig. 2B). Fusion protein SUMO-LF and N-terminal SUMO tag were similar in molecular weight (18.4 kDa and 15.3 kDa respectively). As shown in Fig. 2C, after 3 h of digestion, only SUMO tag and C-terminal LFcinB peptide were detected on SDS-PAGE, declaring the completely digestion of SUMO-LF. The Tricine SDS-PAGE is commonly used to separate proteins/peptides in the mass range 1–100 kDa [43]. LFcinB peptides released from different fusion proteins, were distributed in the range of 3.3–6.5 kDa using Tricine SDS-PAGE with 10% acrylamide gels (Fig. 2D–F) and exceed slightly the theoretical molecular weight (3.3 kDa), which might result from the high proportion of polar amino acids (52%) (reported as a factor to affect protein migration rate in electrophoresis) in LFcinB.

3.3 Rational Design of LFcinB

In order to improve antimicrobial activity, the amino acid sequence of LFcinB was analyzed using “Rational Design of Antimicrobial Peptides” service of CAMP_{R3} database. The result showed that the positions 2, 10, 16, 19 and 24 were engineering targets due to frequent substitution with high AMP probability. Therefore, five mutants were designed rationally, namely LF1–LF5 (Table S3), of which LF3, LF4 and LF5 peptides were predicted to possess improved activity (Table S4).

3.4 Antimicrobial Activities of LFcinB and its Variants

All five designed fusion proteins (LFcinB mutants fused with SUMO tag) were expressed in *E. coli* BL21 (DE3) and purified (Fig. 3A) as well as their parent LFcinB. The SDS-PAGE result implied that the mutagenesis did not affect the expression of fusion protein in *E. coli*. Five mutant peptides were obtained by SUMO protease-mediated hydrolysis (Fig. 3B).

Taking *E. coli* MG1655 and *S. aureus* ATCC 25,923 as indicator strains, antimicrobial activity of five mutant peptides were measured quantitatively and compared with LFcinB. The growth of *E. coli* MG1655 was almost inhibited by all mutant peptides at concentration of 20 μ M (Fig. 3C). Mutant LF4 (M10W/P16R/A24L) possesses

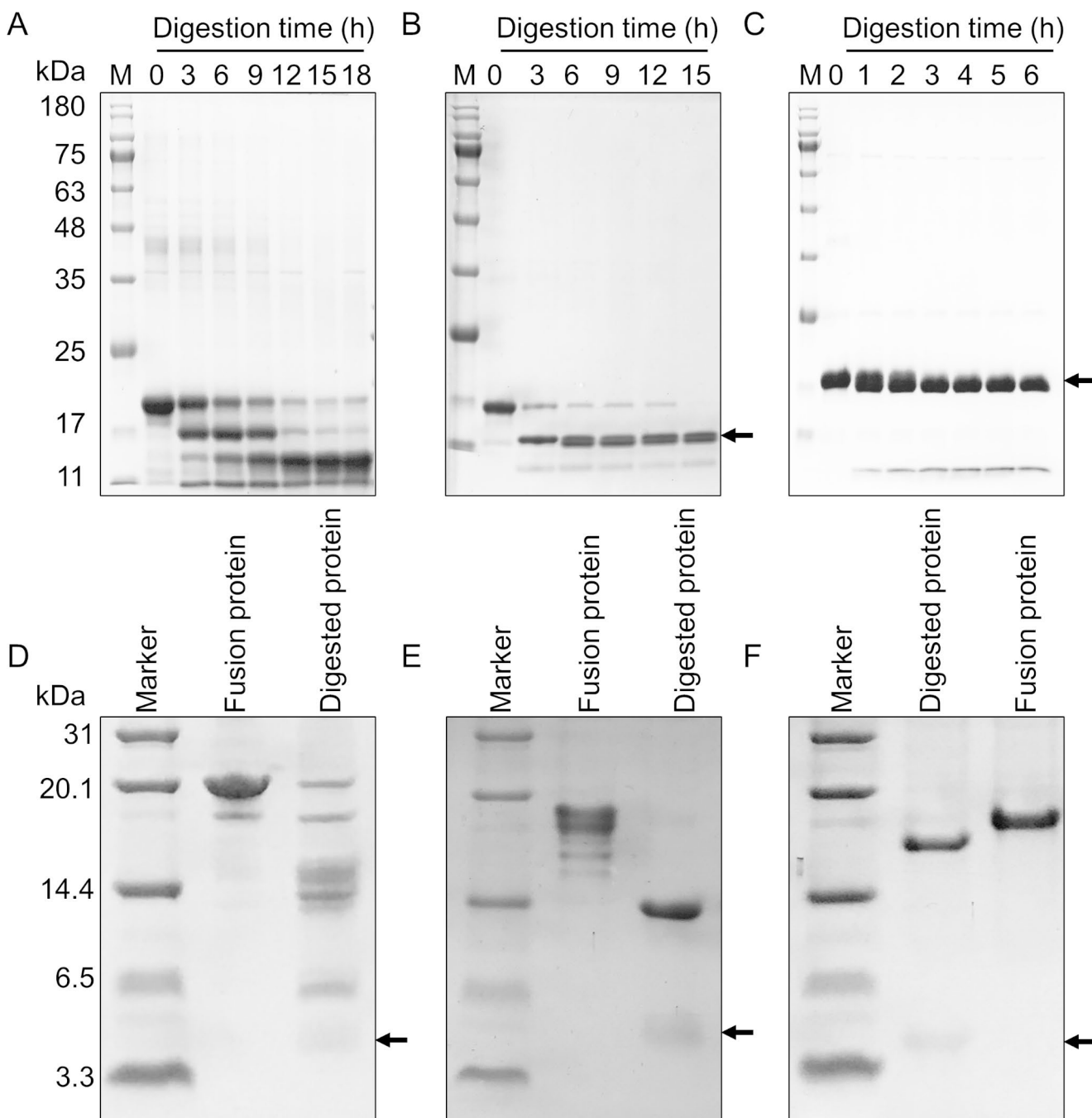


Fig. 2 LFcinB peptides obtained by enzymatic cleavage. SDS-PAGE analysis for digested fusion proteins TrxA-LFe (A), TrxA-LFt (B) and SUMO-LF (C). Tricine-SDS-PAGE analysis for digested fusion proteins TrxA-LFe (D), TrxA-LFt (E) and SUMO-LF (F)

the best inhibitory effects on *E. coli* MG1655 at different concentrations (Fig. 3C). The MIC_{50} of LFcinB and its mutants LF1-LF5 against *E. coli* MG1655 was 3.68 μ M, 4.31 μ M, 4.98 μ M, 3.27 μ M, 2.38 μ M and 6.72 μ M, respectively (Table 1). And the MIC_{95} of LFcinB and its mutants LF1-LF5 against *E. coli* MG1655 was 9.32 μ M, 14.00 μ M, 14.10 μ M, 8.26 μ M, 7.98 μ M and 21.10 μ M, respectively (Table 1). Compared to LFcinB, the MIC_{50} and MIC_{95} of

the best mutant LF4 against *E. coli* MG1655 decreased by 35.33% and 14.38%, respectively.

Similarly, peptide LF4 possessed the best inhibitory effects on *S. aureus* ATCC 25,923 (Fig. 3D). The MIC_{50} of LFcinB and its mutants LF1-LF5 against *S. aureus* ATCC 25,923 was 6.23 μ M, 6.20 μ M, 5.07 μ M, 4.54 μ M, 3.96 μ M and 5.94 μ M, respectively (Table 1). And the MIC_{95} of LFcinB and its mutants LF1-LF5 against *S. aureus* ATCC 25,923 was 46.10 μ M, 45.30 μ M, 36.40 μ M, 28.20

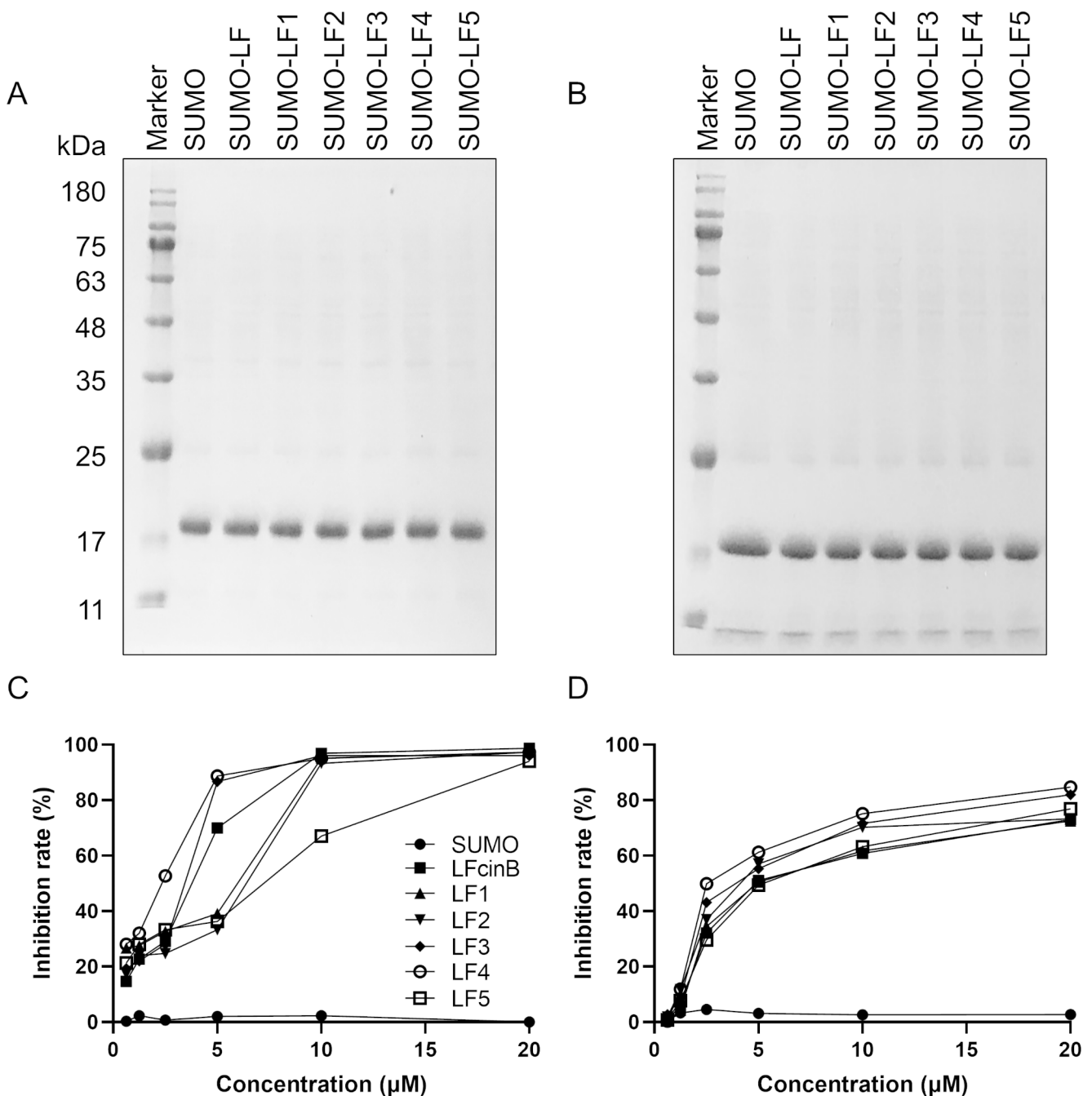


Fig. 3 Antimicrobial activities of five LFcInB mutants. (A) Purification of fusion proteins. (B) SDS-PAGE analysis for digested fusion proteins. (C) Inhibitory activities of mutants against *E. coli* MG1655. (D) Inhibitory activities of mutants against *S. aureus* ATCC 25,923

μM , 22.77 μM and 40.59 μM , respectively. Compared to LFcInB, the MIC_{50} and MIC_{95} of LF4 against *S. aureus* ATCC 25,923 decreased by 36.44% and 50.61%, respectively (Table 1).

In summary, the designed peptide LF4 showed an improved antimicrobial activity than its parent LFcInB with the lowest MIC_{50} and MIC_{95} against *E. coli* MG1655 and *S. aureus* ATCC 25,923.

3.5 Stability Comparison at Various Temperatures, pH Level, salt Environment and Proteases

In the thermostability test, both LFcInB and LF4 had good thermostability at 4–40°C (Fig. 4A) maintaining >80% inhibition rate. As the treating temperature increased, the residual inhibition rate decreased and maintained about 40% at 100°C. As a whole, LF4 possessed comparable thermostability with LFcInB (Fig. 4A).

Table 1 MIC₅₀ and MIC₉₅ of LFcinB and mutants against *E. coli* MG1655 and *S. aureus* ATCC 25,923

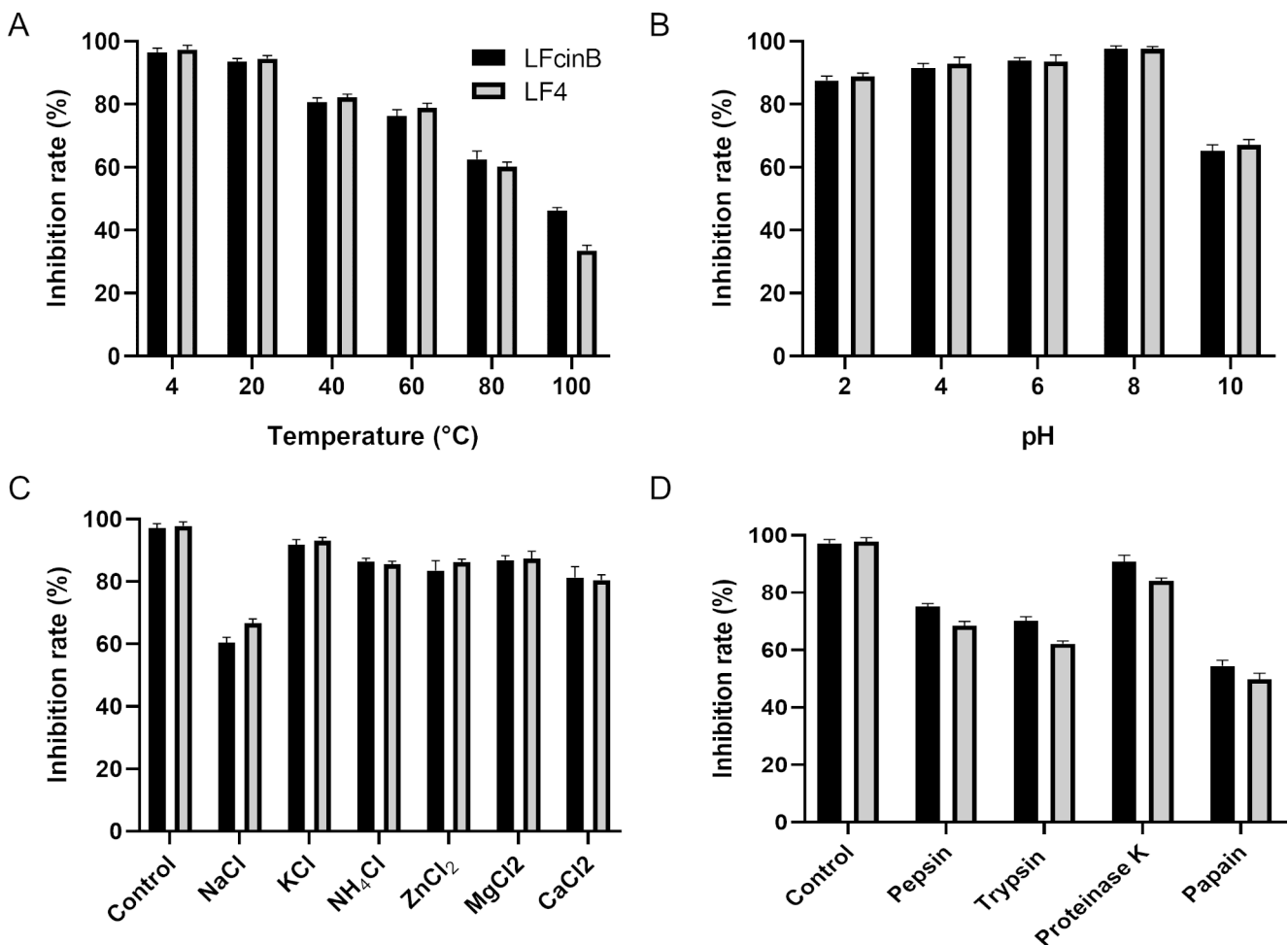
Peptides	<i>E. coli</i> MG1655		<i>S. aureus</i> ATCC 25,923	
	MIC ₅₀	MIC ₉₅	MIC ₅₀	MIC ₉₅
LFcinB	3.68	9.32	6.23	46.10
LF1	4.31	14.00	6.20	45.30
LF2	4.98	14.10	5.07	36.40
LF3	3.27	8.26	4.54	28.20
LF4	2.38	7.98	3.96	22.77
LF5	6.72	21.10	5.94	40.59

LFcinB and mutant LF4 showed little differences in pH stability (Fig. 4B). Both of LFcinB and LF4 showed good stability in the range of pH2.0–8.0 with >80% inhibition rate against *E. coli* MG1655 and reached the highest value at pH8.0 (nearly 100%) (Fig. 4B). However, both two peptides only maintained about 60% inhibition rate at pH10.0, suggesting that LFcinB and LF4 were tolerant to acid and sensitive to alkali.

Salt concentration is an important factor affecting the activity of antimicrobial peptides [31, 44, 45]. Mutagenesis

made little difference on the stability of peptides in salt environment. Compared to control group, the inhibition activity of LFcinB and LF4 exhibited slight decrease and maintained >80% inhibition rate against *E. coli* MG1655 after incubation with 4.5 mM KCl, 6 mM NH₄Cl, 8 mM ZnCl₂, 1 mM MgCl₂ and 2.5 mM CaCl₂ for 4 h (Fig. 4C). However, LFcinB and LF4 were both sensitive to NaCl and their inhibition rate decreased by 35–40% after the treatment with 150 mM NaCl (Fig. 4C).

Poor proteolytic resistance has been a main obstacle for the clinical application of antimicrobial peptides [32, 33]. Therefore, we tested the antimicrobial activities of LFcinB and LF4 after the incubation with different proteases (including Pepsin, Trypsin, Protease K and Papain). As shown in Fig. 4D, the two peptides (LFcinB and LF4) maintained effective bactericidal activities after 4 h of incubation with Proteinase K. In contrast, the inhibition rate was inactivated partially by Pepsin, Trypsin and Papain.

**Fig. 4** The thermostabilities (A), pH stabilities (B), salts stabilities (C) and proteases stabilities (D) of antimicrobial peptide LFcinB and mutant LF4

3.6 Biocompatibility Analysis

The hemolytic activities of LFcInB and LF4 were further assessed experimentally. Both LFcInB and LF4 formed hemolytic circles at the concentration of 10 μ M and 20 μ M (Fig. S3). When the concentration of antimicrobial peptide was reduced to 5 μ M, hemolytic circle was generated only with LFcInB, indicating that the hemolytic activity of mutant LF4 was lower than its parent LFcInB.

4 Discussion

4.1 Preparation of Peptide LFcInB

In order to obtain peptide LFcInB or its variants, peptide were expressed fused with different solubility-promoting tags in *E. coli* BL21 (DE3). The target peptide can be prepared from the digestion of fused protein (including TrxA-LFe, TrxA-LFt and SUMO-LF) by corresponding protease (including enterokinase, thrombin and SUMO proteases). Therefore, 3 recombinant plasmids (including pET32a-LFe, pET32a-LFt and pET28a-SUMO-LF) were constructed for expressing fusion proteins TrxA-LFe, TrxA-LFt and SUMO-LF, respectively. Due to the solubility-promoting effect of TrxA tag and SUMO tag [46, 47], almost all fusion proteins appeared in supernatant (Fig. 1), indicating that all designed fusion proteins were successfully expressed in soluble form in *E. coli* BL21 (DE3). After the condition optimization, the optimal inducing temperature (30°C, 25°C and 20°C respectively) and IPTG concentration (0.1 mM for all proteins) for expressing fused proteins TrxA-LFe, TrxA-LFt and SUMO-LF were determined.

Compared with fused proteins TrxA-LFe and TrxA-LFt, SUMO-LF can be specifically cleaved fully by SUMO proteases in 3–4 h (Fig. 2). Therefore, pET28a-SUMO expression system was selected for further experiments.

4.2 Rational Design to Improve Antimicrobial Activities of LFcInB

Bovine lactoferrin peptide LFcInB was regarded as a promising alternative of antibiotics owing to its broad-spectrum antimicrobial activity and specific mechanism. However, the weak antimicrobial activity, high hemolysis and poor stability of LFcInB limited its applications in the field of biomedicine, food and agriculture. Protein engineering is an effective way to rationally design target protein or peptide to meet the needs of application.

The antimicrobial activity of antimicrobial peptide is mainly influenced by the net positive charge [48, 49], hydrophobicity [48, 49], amino acid composition [50] and

the secondary structure [5, 51]. Compared to porcine lactoferricin (LFP-20), its analogs (LF-2, LF-4, and LF-6), in which residues Cys were replaced with Trp, exhibited 4- to 64-fold improved antimicrobial activity [52], suggesting that hydrophobic Trp plays an important role in increased antimicrobial activity. For bovine lactoferricin (11 residues, named BLFC), the mutants (B3, B5 and B7) with double Arg at the N-terminal had better activity than other mutants (B4 and B6) with double Lys at the N-terminal [53]. Substitution with hydrophobic Leu in antimicrobial peptide is also a promising work [54, 55].

Based on above findings, LF1(L13W), LF2(A24L), LF3(K2R/M10W/T19R), LF4(M10W/P16R/A24L) and LF5(K2R/W10R/A24R) were rationally designed using bioinformatic tools (Table S3). The antimicrobial activity of LFcInB and mutants were determined and compared. Mutant LF4 was the best mutant with the highest antimicrobial activity against *E. coli* MG1655 and *S. aureus* ATCC 25,923 reflected in the lowest MIC₅₀ and MIC₉₅ value (Fig. 3C, D).

Overall, the inhibitory activities of LFcInB and its variants against *S. aureus* ATCC 25,923 (Fig. 3D) were weaker than that against *E. coli* MG1655 (Fig. 3C). This difference in antibacterial activity against different microorganisms might be explained that Gram-positive bacterium *S. aureus* ATCC 25,923 contains peptidoglycan and phosphopeptidic acid in the cell wall, which inhibits the binding of peptides [56].

4.3 Stability of LFcInB and Mutant LF4

In order to compare the stability of bioactive peptides, LFcInB and its mutant LF4 were subjected to stability analysis under given condition with various temperature, pH level, salts environment and proteases. *E. coli* MG1655 was taken as indicator strain.

Instability index is used to predict the stability of the target protein according to the weight of the stability of each dipeptide unit [38]. Generally speaking, both of LFcInB and mutant LF4 were unstable, which accorded with instability index (II) (Table S5). A protein whose instability index (II) is smaller than 40 is regarded as stable. Both two peptides were predicted to be unstable with 77.92 and 103 of instability index (II) (Table S5), respectively. Their instability might be related to the difficulty to form stable structure with short length of amino acid sequence [57].

To form a hydrophobic surface, LFcInB and LF4 contain > 20% aromatic residues in their structure, which is the reason for the sensitive to Pepsin. Trypsin and Papain prefer to cleave the carboxyl terminal of basic residues (Lys and Arg) [58] that happen to be abundant in LFcInB and LF4. The potential protease cleavage sites of LFcInB and

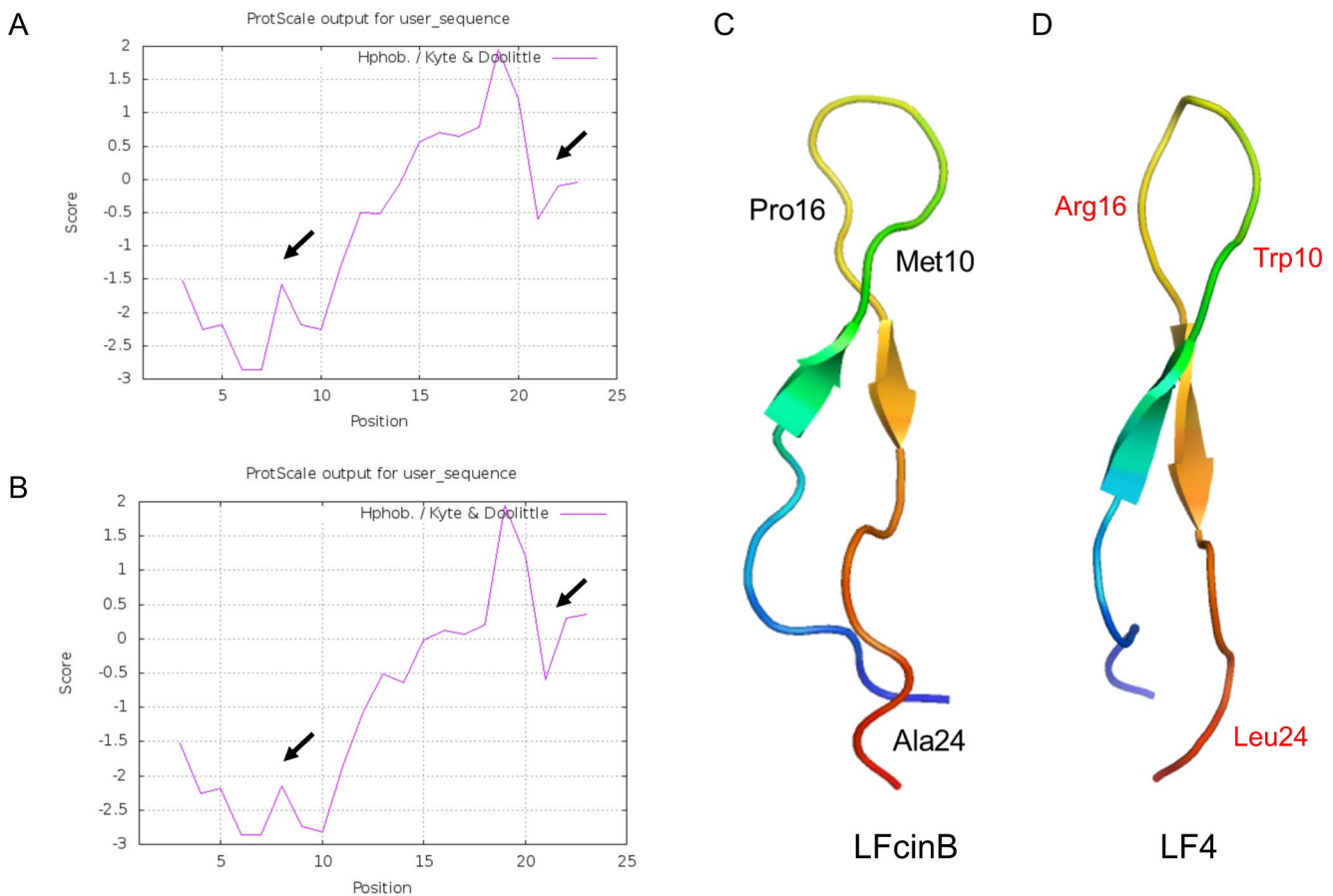


Fig. 5 Analysis of amphipathic and structural properties for LFcinB and LF4. The amphipathic distribution of antimicrobial peptide LFcinB (A) and mutant LF4 (B). Ribbon representation of the crys-

tal structure (PDB: 1LFC) of LFcinB (C) and predicted structure by EasyModeller of mutant LF4 (D). The mutated residues are labeled in the structures

mutant LF4 were predicted with PeptideCutter tool (Table S6). After the mutation, LF4 had one more cleavage site for Pepsin, Trypsin and Proteinase K than LFcinB (Table S6), which was consistent with the poorer tolerance of LF4 towards tested proteases than LFcinB (Fig. 4D).

4.4 Enhanced Biocompatibility of LF4

High biocompatibility of antimicrobial peptide is required for the clinical applications [59]. The hemolytic activities of LFcinB and LF4 were further assessed experimentally and compared in Fig. S3. For LFcinB, there is no apparent differences in the formed circles between the concentration of 20, 10, and 5 μM (Fig. S3). When the concentration of peptide achieved 5 μM , hemolytic circle only appeared in LFcinB (Fig. S3), indicating that the mutant LF4 had a better biosafety with lower hemolytic activity than LFcinB.

4.5 Mechanism for the Improved Antimicrobial Activity of LF4

4.5.1 More net Positive Charge Endowed LF4 Higher Antimicrobial Activity

ProtParam was used for the physicochemical characterization of the antimicrobial peptide LFcinB and mutant LF4 [37–39]. LF4 had more positive charge (+9) than that of LFcinB (+8) (Table S5). The net positive charge of bioactive peptides is reported to be positively correlated with antimicrobial activity [48, 60]. Therefore, the increased antimicrobial activity of LF4 might be closely correlated with its more positive charge.

4.5.2 Better Amphiphilicity Promoted the Interact Between LF4 and cell Membrane

ProtScale is employed to analyze hydrophobicity of each residue in a given protein sequence. The residue with a minus/positive hydrophobicity value is regarded as hydrophilic/

hydrophobic amino acid. Both of LFcInB and LF4 displayed typical amphiphilic character with hydrophilic N-terminal region and hydrophobic C-terminal region (Fig. 5A, B). Furthermore, after the mutagenesis, N-terminal hydrophilicity and C-terminal hydrophobicity of LF4 exceeded slightly that of LFcInB (indicated by the arrows in Fig. 5A, B) and endowed LF4 with better amphiphilicity, which may promote LF4 to interact with and disrupt the cell membrane of *E. coli* MG1655 [61].

4.5.3 Longer β -sheet made for the Binding of LF4 to cell Membrane

The three-dimensional structure of LF4 was modelled and optimized by EasyModeller software using LFcInB crystal structure (PDB: 1LFC) as the template. Similar to LFcInB [62], LF4 folded into an antiparallel β -sheet structure (Fig. 5C, D). It's worth noting that the β -sheet was longer in LF4 (2 sheets, 8 residues) than that in LFcInB. It was known that the β -sheet can facilitate the binding of antimicrobial peptide to negatively charged cell membrane [63]. Therefore, we speculated that the increase of antibacterial activity of LF4 may be related to its extension of β -sheet structure.

To sum up, it was the increased cations, higher amphiphilicity and the extension of β -sheet in 3D structure that resulted in the improvement in the antimicrobial activity of LF4.

5 Conclusion

In this study, we firstly determined the preparation method of peptide LFcInB including aspects of expression vector, fused tag, expression condition, purification and cleavage. Then, in order to improve the antimicrobial activity of LFcInB, five LFcInB-derived mutant peptides (LF1-LF5) were designed rationally with bioinformatic tools. The antimicrobial activity assay showed that mutant LF4 (M10W/P16R/A24L) possessed highest antimicrobial activity against *E. coli* MG1655 and *S. aureus* ATCC 25,923. The mutagenesis made little difference on the stability of antimicrobial peptide in various temperature, pH level and salty condition. LFcInB and LF4 had good thermostability under temperature below 40°C and showed strong tolerance to acid and sensitivity to NaCl and alkaline environment. However, the tolerance of LF4 to proteases (Pepsin, Trypsin, Proteinase K and Papain) were poorer than that of LFcInB which was consistent with the more cleavage sites in LF4. Lastly, the bioinformatics analysis indicated that the improvement in the antimicrobial activity of LF4 may be related to the increased cations, higher amphiphilicity and the extension of β -sheet in 3D structure.

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Author Contributions J. L. conceived the study; X. L. collected the data; X. L. and X. H. analyzed the data; X. H. and B. S. drafted the manuscript; All authors edited and approved the final version of the manuscript.

Data Availability Data will be made available on request.

Declarations

Competing Interests The authors declare that they have no conflict of interest.

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