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Design and high-level expression of a hybrid antimicrobial peptide LF15-CA8 in *Escherichia coli*

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Abstract Antimicrobial peptides (AMPs) have been paid considerable attention owing to their broad-spectrum antimicrobial activity and have great potential as novel antimicrobials. In this study, a novel hybrid peptide LF15-CA8 was designed on the basis of bovine lactoferricin (LfcinB) and cecropin A. The gene segment encoding LF15-CA8 was synthesized and cloned into pGEX-4T-BH to form pGEX-4T-LC1 containing one copy of the LF15-CA8 coding region. A series of recombinant vectors containing up to six multiple-copy LF15-CA8 coding regions, i.e., pGEX-4T-LCn ($n = 1$–6), were subsequently constructed, and used for transformation in *Escherichia coli* BL21(DE3). After induction with IPTG, pGEX-4T-LC1 and pGEX-4T-LC2 transformants successfully expressed fusion proteins GST-LF15-CA8 and GST-$(LF15\text{-}CA8)_2$ in the form of inclusion bodies, respectively. The inclusion bodies were dissolved and the peptide was successfully released in 70 % formic acid in a single step. After purification, about 10.0 mg of the recombinant peptide LF15-CA8 with purity more than 97 % was obtained from 1 l of bacteria culture of pGEX-4T-LC2 transformants. LF15-CA8 caused an increase in antibacterial activity against Gram-positive bacterium (*Staphylococcus aureus* ATCC 25923) compared with the parent peptides and did not show obvious hemolytic activity against human erythrocytes in the range of effective antibacterial concentration. These results suggest that the peptide LF15-CA8 could be a promising candidate for therapeutic applications, and may lead to a cost-effective solution for the large-scale production of AMPs.

Keywords Antimicrobial peptides · LfcinB · Cecropin · Recombinant expression · Antibacterial activity

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Introduction

The effectiveness of antibiotics in controlling infectious bacterial disease and their increased availability due to successful mass production have led to widespread and often inappropriate use. As a result, multidrug antibiotic resistance of pathogenic microorganisms is an increasingly serious public health problem worldwide. There is a significant and urgent need for the development of novel classes of antimicrobial agents that could be used against antibiotic-resistant microbes [30]. Antimicrobial peptides (AMPs) are naturally occurring components of innate immunity and are found in virtually all organisms. They exhibit a diverse range of biological effects and have a similar efficiency against strains resistant and susceptible to antibiotics [35]. Most AMPs have nonspecific membrane-lytic activities, which are much more difficult for bacteria to develop resistance to than to conventional antibiotics [10]. AMPs have recently generated

considerable interest as excellent novel therapeutic agents [5, 33].

Among AMPs, bovine lactoferricin (LfcinB) is a cationic AMP and is obtained by acid-pepsin hydrolysis of bovine lactoferrin. LfcinB consists of 25 amino acid residues with a molecular weight of 3,124 Da [3]. The peptide possesses broad spectrum antimicrobial activity against various bacteria, fungi, viruses, and protozoa. LfcinB has also been found to induce apoptosis of cancer cells [21] and exert immunoregulatory effects [9].

Cecropins are a family of potent AMPs originally isolated from the hemolymph of the giant silk moth *Hyalophora cecropia* [11]. They are synthesized in the fat bodies as a preproprotein and consist of 31–39 amino acid residues. Like other polycationic peptides, cecropins possess a broad antimicrobial spectrum. At low concentrations (0.1–5 μM range), cecropins exhibit lytic antibacterial activity against several Gram-negative and Gram-positive bacteria, but not against eukaryotic cells [24].

In recent years, many efforts have been focused on developing novel AMPs with more potent activity. Researchers have done this by altering the original sequences of AMPs via several strategies, including substitution at one or more positions, truncation, and deletion. Such strategies permit the determination of those residues or domains that are important in modulating activity and can result in analogues with more potent activity than the parental peptides [13, 26]. Hybridization of two peptides with different properties is one especially effective method to obtain novel AMPs, because hybrid peptides ideally possess a more desirable combination of various parameters than their parental peptides. Several hybrid segments of cecropin A combined with segments of melittin [1, 6, 8] and magainin [18, 36] have demonstrated broader antimicrobial spectra than the parental peptide cecropin A without introducing the hemolytic activity of melittin or magainin. The broad spectrum and high antimicrobial activity suggest that these hybrid peptides may have great application potential as novel therapeutic agents. However, for the pharmaceutical realization and commercial viability of AMPs applications, one of the great obstacles is the large-scale preparation and production of active AMPs.

In the present study, a novel hybrid peptide LF15-CA8 was designed. The peptide incorporates 1–15 amino acid residues of LfcinB, in which Met at the position 10 is substituted with Trp, and 1–8 amino acid residues of cecropin A. The soft peptide (–GSG–) for linkage was introduced between them. A highly efficient protocol for bacterial expression of LF15-CA8 was provided, and the antibacterial and hemolytic activities of the peptide were determined, which should lay a good foundation for developing LF15-CA8 as a therapeutic agent.

Materials and methods

Strains, vectors, and reagents

Escherichia coli DH5α for plasmid amplification, *E. coli* BL21 (DE3) for the expression of fusion protein, and the plasmid pGEX-4T-2 for producing fusion protein with GST were generously provided by Dr. Jianhua Wang from Feed Research Institute of the Chinese Academy of Agricultural Sciences. Restriction enzymes, T4 DNA ligase, and *Taq* DNA polymerase were purchased from TaKaRa Biotechnology Co., Ltd (Dalian, China). All oligonucleotides were ordered from Sangon Biotech Co., Ltd (Shanghai, China). LfcinB, cecropin A, and LF15-CA8 were purchased from GL Biochem Ltd. (Shanghai, China), and they were synthesized by the solid-phase method with a purity of more than 98 %. All other chemical reagents were made in China and were of analytical grade.

Construction of expression vectors containing oligomeric peptides encoding sequence

A DNA fragment containing the monomeric LF15-CA8 encoding sequence was constructed using two complementary deoxyoligonucleotides (oligos) A (forward): 5′-*GAA TTCGGATCC***GACCCG**TTCAAATGCCGTCGTTGG CAGTGGCGATTGAAAAAGCTGGGTGCTGGC TCTGGCAAATGGAAGCTGTTCAAGAAAATC**GACC CG***AGATCTAAGCTT*-3′ and B (reverse): 5′-*AAGCTTAGA TCT*CGGGTCGATTTTCTTGAACAGCTTCCATTTGCC AGAGCCAGCACCCAGCTTTTTCAATCGCCACTGC CAACGACGGCATTTGAACGGGTC*GGATCCG AATTC*-3′ (restriction sites *Eco*RI (GAATTC), *Bam*HI (GGATCC), *Bgl*II (AGATCT), and *Hind*III (AAGCTT) are indicated in the oligos in italics). The Asp-Pro coding sequence GACCCG was introduced (and is highlighted in bold in the oligos). Asp-Pro moieties were added to facilitate the formation of the monomer by formic acid cleavage. Because the multiple cloning sites of pGEX-4T-2 contain the site of *Bam*HI and are not suitable for the construction of expression vectors containing multimeric genes, a DNA sequence (*AGATCT GAATTC AAGCTT* GCG GCC GCA) digested with *Bgl*II and *Not*I was connected with pGEX-4T-2 digested with *Bam*HI and *Not*I, which resulted in the plasmid pGEX-4T-BH. The two synthesized complementary oligos were annealed and ligated into the vector pGEX-4T-BH between *Eco*RI and *Hind*III sites, generating pGEX-4T-LC1. The resultant plasmid was used for transformation in *E. coli* DH5α and positive transformants were screened by colony PCR using primers P1 (5′-GGGCTGGCAAGCCAC GTTTGGTG-3′) and P2 (5′-CCGGGAGCTGCATGTGTCA GAGG-3′) and further verified by sequencing. The peptide monomer DNA fragment of pGEX-4T-LC1 was isolated

after digestion with *Eco*RI and *Bgl*II and cloned into the pGEX-4T-LC1 digested with *Eco*RI and *Bam*HI, producing pGEX-4T-LC2 carrying a dimer of the LF15-CA8 encoding sequence. These steps were repeated for construction of tandem multimers of the LF15-CA8 encoding sequence, generating pGEX-4T-LCn (n = number of LF15-CA8 coding regions, 1–6).

Induced expression of LF15-CA8 oligomeric fusion gene

Escherichia coli BL21 (DE3) cells were transformed with the recombinant expression vectors pGEX-4T-LCn, and desired clones were selected. Each transformant harboring pGEX-4T-LCn was inoculated into LB medium supplemented with ampicillin (50 μg/ml) and grown at 37 °C overnight. Each culture was then diluted 1:100 into 50 ml of fresh medium and grown at 37 °C. At OD_{600} = 0.6, IPTG was added to a final concentration of 0.3 mM for the induced expression of the GST fusion protein. The cells were cultured for 4 h and harvested by centrifugation at 4,000×*g* for 10 min. The cell pellet was washed twice with 30 ml of ice-cold lysis buffer (20 mM of Tris–HCl, 500 mM of NaCl, and 5 mM of EDTA, pH 8.0) and resuspended in 3 ml of this buffer. Cell preparations were then sonicated (20 × 3 s with 3 s on ice between each cycle) at maximum amplitude using the ultrasonic amplitude transformer Φ6 (SCIENZ-IID, Ningbo, China). Insoluble and soluble fractions were separated by centrifugation at 12,000×*g* for 10 min at 4 °C. 15 % SDS-PAGE was performed to monitor the expression of the recombinant LF15-CA8 [27].

Production and cleavage of inclusion bodies

Escherichia coli cells harboring the expression vectors pGEX-4T-LC1 and pGEX-4T-LC 2 were separately cultured in 1 l of LB medium. After induction by IPTG and lysis of cells by sonication as described above, the inclusion bodies fraction containing the fusion protein of interest was recovered by centrifugation at 12,000×*g* for 30 min at 4 °C and washed with 10 ml of PBS (20 mM of Tris–HCl, 50 mM of NaCl, 1 mM of EDTA, 0.5 % Triton X-100, pH 8.0). After washing twice with 50 ml of urea (2 M) and 50 ml of PBS, respectively, the inclusion bodies were resuspended in 50 ml of the 70 % formic acid solution and digested for 2 h at 30 °C with shaking. The reaction mixture was lyophilized and analyzed by 15 % Tricine-SDS-PAGE [28].

Purification of the recombinant peptide

The lyophilized digested product was resuspended in 0.1 % trifluoroacetic acid (TFA). After centrifugation at 10,000×*g* for 10 min, the peptide in the clear supernatant was purified by semi-preparative reversed-phase HPLC (RP-HPLC) on a C18 column (250, 4.6 mm, 5 μm, 300 Å), which was pre-equilibrated in 0.1 % TFA. The bound proteins were eluted with a linear gradient of acetonitrile (20–45 %, v/v) (at 1.5 % per min) in 0.1 % TFA. The flow rate was 1.0 ml/min and the absorbance of the eluent was monitored at 214 nm. Purification was monitored by 15 % Tricine-SDS-PAGE. The purified peptide was lyophilized and stored at −20 °C until needed. The peptide yield was determined as described by Bradford [4]. The molecular weight was determined by electrospray ionization mass spectrometry (ESI–MS) on an LTQ Orbitrap mass spectrometer (Thermo, USA). The ionization voltage was approximately 2.0 kV and the capillary temperature was set to 200 °C. Helium (5.0 purity) and nitrogen were utilized as a damping gas in the linear ion trap and in the curved linear trap, respectively.

Determination of antibacterial activities

Antibacterial activities of LfcinB, cecropin A, and the recombinant peptide LF15-CA8 against different microorganisms were tested using the microbroth dilution method [7]. The stock solutions of three peptides were serially diluted tenfold with 0.01 % acetic acid and 0.2 % bovine serum albumin. The tested bacteria growing in the log phase were diluted to 2 × 10^5–7 × 10^5 CFU/ml with MHB medium. Aliquots (100 μl) from each strain suspension were distributed into a 96-well polypropylene microtiter plate, and to each well was added 11 μl of each peptide dilution. Cultures were grown for 18 h with vigorous shaking. The absorbance was evaluated at 600 nm using a microplate reader (Bio-Rad, USA). Experiments were performed in triplicate. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of peptide that would fully inhibit bacterial growth as measured by optical density.

Hemolytic activity assay

The hemolytic activities of LF15-CA8, LfcinB, and cecropin A were determined using human red blood cells (HRBC) as described by Maher and McClean [22]. The fresh erythrocytes were prepared and suspended in PBS to a concentration of 4 % (v/v). Peptides were added and the resulting suspension was incubated for 1 h at 37 °C. The absorbance of the supernatant was measured at 414 nm with an ELISA plate reader. Controls for zero hemolysis (blank) and 100 % hemolysis consisted of HRBC suspended in PBS and 1 % Triton X-100, respectively.

Results

Construction of the expression vectors

The LF15-CA8 coding sequence was obtained by annealing of oligos A and B, in which the cleavage site of formic acid immediately was created on the N-terminal and C-terminal of the peptide. One pair of isocaudamer restriction sites, *Bam*HI (GGATCC) and *Bgl*II (AGATCT) were introduced up- and downstream of the coding sequence, respectively, to facilitate construction of different tandem expression plasmids for the peptide. Expression plasmids pGEX-4T-LCn (n = 1–6) were constructed using the isocaudamer method. Primers P1 and P2 bind at nucleotides 869–891 and 1,042–1,020 on the vector pGEX-4T-2, respectively. As shown in Fig. 1, the PCR products of pGEX-4T-LCn (n = 1–6) showed the bands of 264, 360, 456, 552, 648, and 744 bp in size, respectively, which were consistent with the expected ones.

Induced expression of fusion proteins

Escherichia coli BL21 (DE3) cells were transformed with recombinant plasmids and were induced with IPTG. *E. coli* transformed with pGEX-4T-LC1 and pGEX-4T-LC2 successfully expressed the targeted recombinant fusion proteins GST-LF15-CA8 and GST-(LF15-CA8)$_2$ with the molecular weights of 29.6 and 32.9 kDa, respectively, whereas the expression levels of transformants harboring pGEX-4T-LCn (n = 3–6) were very low and not detected by SDS-PAGE (as shown in Fig. 2). Therefore, the transformants of pGEX-4T-LC1 and pGEX-4T-LC2 were used for further expression experiments. To test the existing state of the recombinant fusion proteins in cells, the solubilities of GST only and the recombinant fusion proteins were analyzed. GST was found in the soluble fraction; however, most of the recombinant fusion proteins partitioned into the insoluble protein fractions (data not shown).

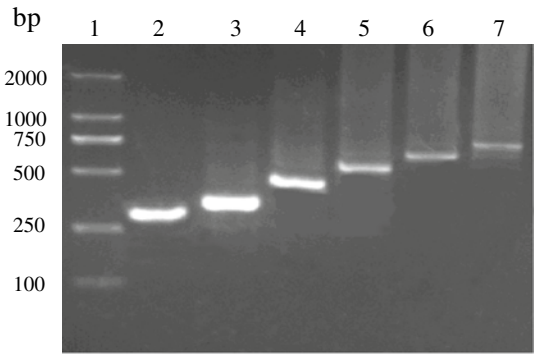

Fig. 1 Construction of the recombinant expression plasmids pGEX-4T-LCn (n = 1–6). *Lane 1* DNA marker, *lanes 2–7* PCR products of plasmids of pGEX-4T-LCn (n = 1–6), respectively

Production and digestion of inclusion bodies

After washing with 2 M of urea, approximately 90 mg of GST-LF15-CA8 and 83 mg of GST-(LF15-CA8)$_2$ inclusion bodies were obtained from 1 l of *E. coli* culture,

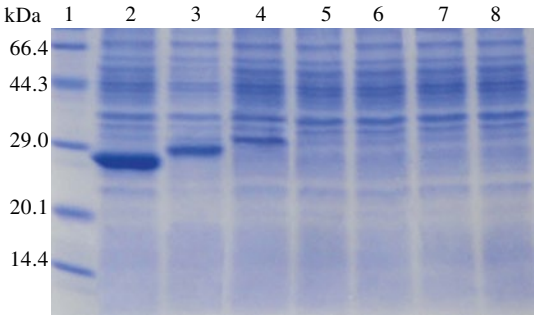

Fig. 2 SDS-PAGE analysis of expression of *E. coli* BL21 (DE3) transformed with different recombinant plasmids. *Lane 1* protein molecular weight marker, *lanes 2–8* induced expression of *E. coli* BL21(DE3) transformed with pGEX-4T-2, pGEX-4T-LCn (n = 1–6), respectively

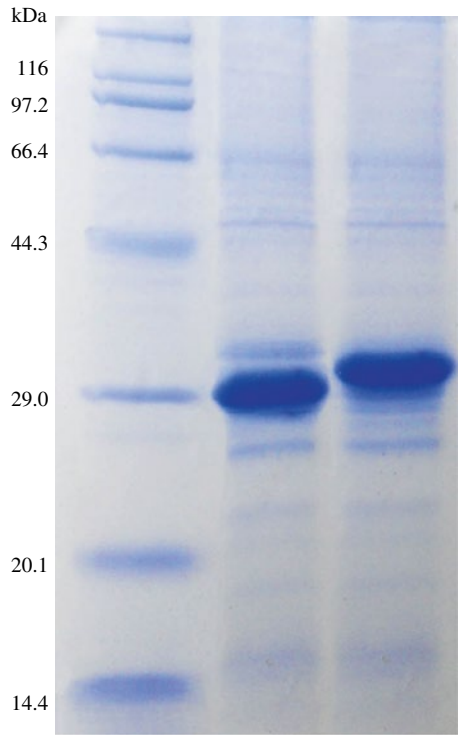

Fig. 3 Production of the inclusion bodies of fusion proteins. *Lane 1* protein molecular weight marker, *lanes 2* and *3* the inclusion bodies of GST-LF15-CA8 and GST-(LF15-CA8)$_2$ washed with 2 M of urea, respectively

respectively, with a purity of more than 85 % as determined by SDS-PAGE gel scanning (Fig. 3). To release the peptide LF15-CA8, the purified inclusion bodies of both fusion proteins were treated with 70 % formic acid at 30 °C. After incubating for 2 h, all the inclusion bodies were dissolved in the formic acid solution and successfully generated a targeted band of the peptide at about 3.5 kDa on Tricine-SDS-PAGE (Fig. 4, lanes 1 and 2).

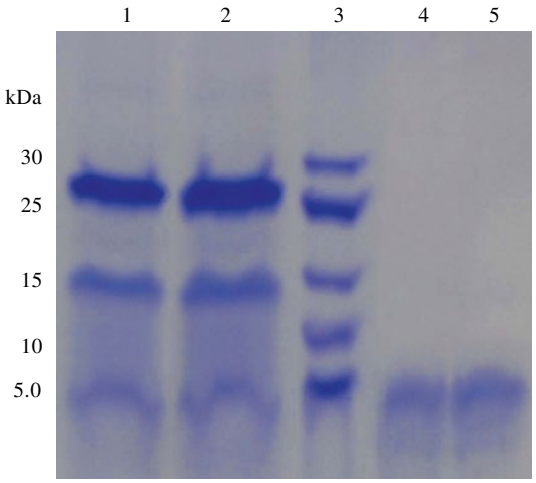

Fig. 4 Tricine-SDS-PAGE analysis of the fusion proteins cleaved by formic acid and the purified recombinant LF15-CA8. *Lanes 1* and *2* GST-LF15-CA8 and GST-$(LF15\text{-}CA8)_2$ cleaved by formic acid, respectively, *lane 3* protein molecular weight marker, *lanes 4* and *5* the purified recombinant peptide LF15-CA8 from GST-LF15-CA8 and GST-$(LF15\text{-}CA8)_2$, respectively

Purification of the recombinant peptide

The peptide LF15-CA8 was further purified from the lyophilized digested mixture using RP-HPLC. The eluted fractions were monitored by Tricine-SDS-PAGE and the target peptide eluted at around 35 % acetonitrile (data not shown). The purified peptide on Tricine-SDS-PAGE revealed a molecular weight of about 3.5 kDa (Fig. 4, lanes 4 and 5). ESI–MS analysis (Fig. 5) showed the mass to charge (*m/z*) ratio of peptide ions with different numbers of hydrogen ions. The average mass/charge of the molecular ion was 3,462.0 Da which matched the theoretical mass (3,462.2 Da) of the recombinant peptide. In summary, about 10.0 and 5.1 mg of LF15-CA8 with more than 97 % purity were obtained from 1 l of bacteria culture of pGEX-4T-LC2 and pGEX-4T-LC1, respectively.

Antibacterial activity of the peptide

Antibacterial activity assay was performed to determine the function of the recombinant LF15-CA8. The peptide MICs for several microorganisms are shown in Table 1. The peptide exhibited obvious activity against both Gram-positive bacterium (*S. aureus* ATCC 25923) and Gram-negative bacteria (*E. coli* O78, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella pullorum* CCVC 533). The MIC values of LF15-CA8 for the tested bacteria were lower than those

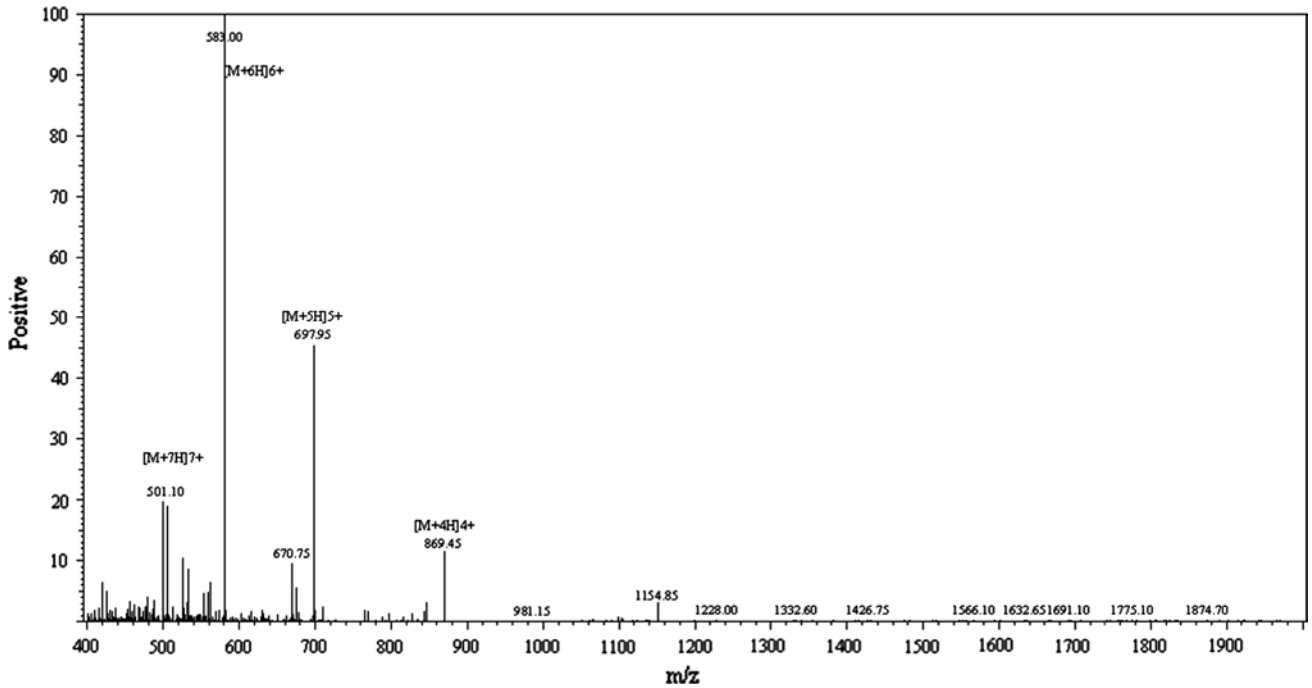

Fig. 5 Molecular weight determination of the recombinant peptide LF15-CA8 by ESI–MS

of LfcinB, which showed that LF15-CA8 had much better antibacterial activity than LfcinB. The MICs of LF15-CA8 against *E. coli* O78 and *P. aeruginosa* ATCC 27853 were higher than those of cecropin A, and the two peptides had similar activity against *S. pullorum* CCVC 533. However, cecropin A exhibited a lower or no effect on *S. aureus* ATCC 25923 (MIC > 256 mg/l), and LF15-CA8 exhibited a significantly improved activity against *S. aureus* ATCC 25923 (32 mg/l) as shown in Table 1. These results demonstrated that the LF15-CA8 peptide was functional and active after separation from the fusion protein and had improved inhibitory activity against Gram-positive bacteria compared with the parent peptides.

Hemolytic activities of the peptide

Fresh HRBC cells were used in the present study to find out whether the peptide LF15-CA8 caused membrane lysis of eukaryotic cells. As observed in Table 2, the three tested peptides all showed a certain lytic activity against HRBC and the peptide LF15-CA8 showed similar hemolytic properties to LfcinB and cecropin A. The lytic activity of LF15-CA8 was increased at higher peptide concentration. However, the percentage lysis of HRBC was very low and less than 1.5 % even at the LF15-CA8 concentration of 256 μg/ml, which was eight times higher than the lethal concentration for *E. coli*. These results suggested that there was no obvious or very low hemolytic activity for LF15-CA8 against HRBC in the range of its antibacterial concentration.

Discussion

To expedite the pharmaceutical and clinical applications of AMPs, a cost-effective and scalable method for production of active and effective AMPs is required. It is presently recognized that the recombinant DNA technique is a potential means of producing AMPs in large quantities. In recent years, many AMPs have been successfully produced using genetic engineering methods. Many host cells have been used for expression of AMPs, but *E. coli* has been considered as one of the most efficient recombinant bioreactors owing to its fast growth rate and well-established expression system [25, 34].

Procedures to express AMPs in *E. coli* have encountered difficulties associated with low levels of expression, susceptibility to proteolytic degradation, and poor recovery yields as the result of toxicity to the bacteria host and the small size of AMPs. These problems can be efficiently solved using a fusion expression strategy generally employed in the production of AMPs by an *E. coli* expression system. But the actual expression level of AMPs is not satisfactory because of the small proportion of AMPs relative to the fusion proteins, especially when large fusion partners are chosen. Expression of AMPs multimer would be an efficient way to increase the production of AMPs expressed as fusion proteins. Many experiments have been performed to express various peptides as multimers in *E. coli* to achieve mass production [14, 19, 31]. In this study, different constructs containing 1–6 LF15-CA8 coding regions were developed by the isocaudamer method. Somewhat unexpectedly and unfortunately, only transformants harboring pGEX-4T-LC1 and pGEX-4T-LC2 successfully expressed the target fusion proteins, and other constructs (pGEX-4T-LC3-6) transformants failed to produce more proteins. This result was similar to that of many other reports [16, 19, 31]. This might result from the basic nature of peptides, which can inhibit transcription and translation through the interaction with DNA or RNA [12, 23].

A lot of work on the production of AMPs by an *E. coli* expression system was carried out. However, purification requires multiple biochemical steps that are expensive and significantly reduce yield, even when production is possible. Expression of AMPs in inclusion bodies is beneficial to high expression level, facile separation, and high recovery from the cell lysate. But it will raise new challenges such as the time-consuming and low refolding rate in the following dissolving and renaturing processes of inclusion bodies [15, 37]. Generally, there are several options available to cleave a peptide from its fusion partner, utilizing both

Table 1 MICs of LF15-CA8 for the tested bacteria (mg/l)

Peptides	Tested bacteria			
	S. aureus ATCC 25923	*E. coli* O78	*P. aeruginosa* ATCC 27853	*S. pullorum* CCVC 533
LF15-CA8	32	32	64	32
LfcinB	64	128	128	128
Cecropin A	>256	16	32	32

Table 2 Percent lysis of human red blood cells at different concentrations of peptides

Peptides	Concentrations of peptides (μg/ml)					
	8	16	32	64	128	256
LF15-CA8	0.16 ± 0.04	0.30 ± 0.08	0.54 ± 0.13	0.97 ± 0.17	1.13 ± 0.23	1.32 ± 0.18
LfcinB	0.10 ± 0.07	0.19 ± 0.10	0.41 ± 0.09	0.86 ± 0.08	0.99 ± 0.17	1.30 ± 0.20
Cecropin A	0.13 ± 0.06	0.27 ± 0.09	0.48 ± 0.11	1.11 ± 0.12	1.21 ± 0.19	1.42 ± 0.22

chemical and enzymatic routes of cleavage. Several proteases, such as thrombin, factor Xa, or enterokinase, will cleave at their recognition sites, once these are introduced within the linker between the fusion partner and the target peptide. However, these enzymes are expensive and not feasible for industrial-scale purification.

Chemical cleavage reagents are economical and applicable on an industrial scale. Formic acid can recognize and cleave the acid-labile peptide bond between Asp and Pro. Several reports have introduced methods for producing AMPs in an *E. coli* expression system and the yield is attractive and competitive. Kovaleva et al. [17] employed the pET system to express PsDef1 fused to GST and achieved 1 mg of rPsDef1 per liter of bacterial culture after removing the GST moiety with factor Xa. Shen et al. [29] utilized a self-cleaving ELP system as the tool to express AMPs and obtained 0.6 mg of moricin CM4 and 1.8 mg of human β-defensins 4 from 1 l of their growth media of *E. coli*. Bang et al. [2] developed a pET expression system of *E. coli* which produced 2.10 mg of the hybrid peptide hin/MSH from 800 ml of culture medium.

In this work, the cleavage site of formic acid was introduced at both terminals of the peptide. The specific cleavage to the fusion protein was successfully achieved by formic acid and the recombinant peptide, which has the same amino acid sequence and molecular weight as predicted, was obtained after purification by RP-HPLC. Another important advantage of the protocol is that the inclusion bodies were dissolved and digested by 70 % formic acid in a single step, and the complicated and time-consuming treatment steps were left out for dissolution and renaturation of inclusion bodies. About 10.0 mg of the recombinant peptide with a purity of more than 97 % was obtained from 1 l of bacteria culture of pGEX-4T-LC2, and this yield is almost two times that of pGEX-4T-LC1, suggesting the effectiveness of the protocols developed in this study for production improvement of small peptides by the gene recombinant technology.

In recent years, hybridization methods have attracted considerable interest as an approach to obtain novel AMPs with more potent activity. Hybrid peptides can possess better combinations of various parameters than their parent peptides. The novel hybrid AMP LF15-CA8 was designed on the basis of LfcinB and cecropin A. To increase the degree of the hybrid peptide and develop individual antimicrobial activity as far as possible, a soft peptide linker (GSG) was introduced between them. Absence of the side-chain group of the Gly residue allows the free formation of an α-helix, while the Ser residue is often involved in a disordered structure [20, 32]. So, the peptide linker composed of Gly and Ser residues is helpful to keep separate spatial structure and biological function for two peptides.

The antibacterial assay demonstrated that the recombinant peptide had remarkable antibacterial activity, and improved activity against Gram-positive bacterium *S. aureus* ATCC 25923 compared with the parent peptides. No difference between the recombinant and chemically synthetic peptides was observed in antibacterial activities against the tested bacteria, in spite of the unwanted residues Asp and Pro left at the N-terminal and C-terminal, respectively, of the recombinant peptide after digestion by formic acid. In addition, LF15-CA8 has no obvious hemolytic effect on HRBC. However, further work will be required to elucidate the action mechanism, immunogenicity, and biologic function of LF15-CA8 in vivo.

Conclusions

A novel hybrid AMP was designed on the basis of LfcinB and cecropin A, and successfully expressed by an *E. coli* expression system. Dissolution and digestion of the inclusion bodies of fusion protein were achieved in one step by treatment with formic acid. The recombinant peptide was active against tested bacteria and showed higher activity against the Gram-positive bacterium *S. aureus* ATCC 25923 than the parent peptides. These results suggested that the hybrid peptide is a potential candidate for pharmaceutical and clinical development. The protocols presented in this study may be a valuable method for producing large quantities of active AMPs in *E. coli*.

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References

1. Andreu D, Ubach J, Boman A, Wåhlin B, Wade D, Merrifield RB, Boman HG (1992) Shortened cecropin A-melittin hybrids. Significant size reduction retains potent antibiotic activity. FEBS Lett 296(2):190–194
2. Bang SK, Kang CS, Han MD, Bang IS (2010) Expression of recombinant hybrid peptide Hinnavin II/aIpha-melanocyte-stimulating hormone in *Escherichia coli*: purification and characterization. J Microbiol 48(1):24–29
3. Bellamy W, Takase M, Wakabayashi H, Kawase K, Tomita M (1992) Antibacterial spectrum of lactoferricin B, a potent bactericidal peptide derived from the N-terminal region of bovine lactoferrin. J Appl Bacteriol 73(6):472–479
4. Bradford MM (1976) A rapid and sensitive method for the quantization of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
5. Brogden KA (2005) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat Rev Microbiol 3(3):238–250
6. Cao Y, Yu RQ, Liu Y, Zhou HX, Song LL, Cao Y, Qiao DR (2010) Design, recombinant expression, and antibacterial activity of the

cecropins-melittin hybrid antimicrobial peptides. Curr Microbiol 61(3):169–175
7. Chen ZJ, Wang DM, Cong YG, Wang J, Zhu JM, Yang J, Hu Z, Hu XM, Tan YL, Hu FQ, Rao XC (2011) Recombinant antimicrobial peptide hPAB-β expressed in *Pichia pastoris*, a potential agent active against methicillin-resistant *Staphylococcus aureus*. Appl Microbiol Biotechnol 89(2):281–291
8. Ferre R, Melo MN, Correia AD, Feliu L, Bardají E, Planas M, Castanho M (2009) Synergistic effects of the membrane actions of cecropin-melittin antimicrobial hybrid peptide BP100. Biophys J 96(5):1815–1827
9. Gifford JL, Hunter HN, Vogel HJ (2005) Lactoferricin: a lactoferrin-derived peptide with antimicrobial, antiviral, antitumor and immunological properties. Cell Mol Life Sci 62(22):2588–2598
10. Guani-Guerra E, Santos-Mendoza T, Lugo-Reyes SO, Teran LM (2010) Antimicrobial peptides: general overview and clinical implications in human health and disease. Clin Immunol 135(1):1–11
11. Hultmark D, Steiner H, Rasmuson T, Boman HG (1980) Insect immunity, purification and properties of three inducible bactericidal proteins from emolymph of immunized pupae of *Hyalophora cecropia*. Eur J Biochem 106(1):7–16
12. Iost I, Dreyfus M (1995) The stability of *Escherichia coli* lacZ mRNA depends upon the simultaneity of its synthesis and translation. EMBO J 14(13):3252–3261
13. Jang SA, Kim H, Lee JY, Shin JR, da Kim J, Cho JH, Kim SC (2012) Mechanism of action and specificity of antimicrobial peptides designed based on buforin IIb. Peptides 34(2):283–289
14. Jonasson P, Nygren PA, Jornvall H, Johansson BL, Wahren J, Uhlen M, Stahl S (2000) Integrated bioprocess for production of human proinsulin C-peptide via heat release of an intracellular heptameric fusion protein. J Biotechnol 76(2–3):215–226
15. Khan J, Gupta S, Chattopadhyay K, Mukhopadhaya A (2012) Refolding and functional assembly of the Vibrio cholerae porin OmpU recombinantly expressed in the cytoplasm of *Escherichia coli*. Protein Expr Purif 85(2):204–210
16. Kim JM, Jang SA, Yu BJ, Sung BH, Cho JH, Kim SC (2008) High-level expression of an antimicrobial peptide histonin as a natural form by multimerization and furin-mediated cleavage. Appl Microbiol Biotechnol 78(1):123–130
17. Kovaleva V, Krynytskyy H, Gout I, Gout R (2011) Recombinant expression, affinity purification and functional characterization of Scots pine defensin 1. Appl Microbiol Biotechnol 89(4):1093–1101
18. Lee DG, Park Y, Jin I, Hahm KS, Lee HH, Moon YH, Woo ER (2004) Structure-antiviral activity relationships of cecropin A-magainin 2 hybrid peptide and its analogues. J Pept Sci 10(5):298–303
19. Lee JH, Kim MS, Cho JH, Kim SC (2002) Enhanced expression of tandem multimers of the antimicrobial peptide buforin II in *Escherichia coli* by the DEAD-box protein and trxB mutant. Appl Microbiol Biotechnol 58(6):790–796
20. Lee M, Bang K, Kwon H, Cho S (2013) Enhanced antibacterial activity of an attacin-coleoptericin hybrid protein fused with a helical linker. Mol Biol Rep 40(6):3953–3960
21. Mader JS, Salsman J, Conrad DM, Hoskin DW (2005) Bovine lactoferricin selectively induces apoptosis in human leukemia and carcinoma cell lines. Mol Cancer Ther 4(4):612–624
22. Maher S, McClean S (2006) Investigation of the cytotoxicity of eukaryotic and prokaryotic antimicrobial peptides in intestinal epithelial cells in vitro. Biochem Pharmacol 71(9):1289–1298
23. Miller KW, Evans RJ, Eisenberg SP, Thompson RC (1989) Secretory leukocyte protease inhibitor binding to mRNA and DNA as a possible cause of toxicity to *Escherichia coli*. J Bacteriol 171(4):2166–2172
24. Moore AJ, Beazley WD, Bibby MC, Devine DA (1996) Antimicrobial activity of cecropins. J Antimicrob Chemother 37(6):1077–1089
25. Park TJ, Kim JS, Choi SS, Kim Y (2009) Cloning, expression, isotope labeling, purification, and characterization of bovine antimicrobial peptide, lactophoricin in *Escherichia coli*. Protein Expr Purif 65(1):23–29
26. Paulsen VS, Blencke HM, Benincasa M, Haug T, Eksteen JJ, Styrvold OB, Scocchi M, Stensvåg K (2013) Structure-activity relationships of the antimicrobial peptide arasin 1 and mode of action studies of the N-terminal, proline-rich region. PLoS ONE 8(1):e53326
27. Sambrook J, Fristsch EF, Maniatis T (1989) In: Sambrook J (ed) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, New York, pp 96–99
28. Schagger H, von Jagow G (1987) Tricine–sodium dodecyl sulfate–polyacrylamide gel electroporesis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 166(2):368–379
29. Shen Y, Ai HX, Song R, Liang ZN, Li JF, Zhang SQ (2010) Expression and purification of moricin CM4 and human β-defensins 4 in *Escherichia coli* using a new technology. Microbiol Res 165(8):713–718
30. Spížek J, Novotná J, Rezanka T, Demain AL (2010) Do we need new antibiotics? The search for new targets and new compounds. J Ind Microbiol Biotechnol 37(12):1241–1248
31. Tian ZG, Dong TT, Yang YL, Teng D, Wang JH (2009) Expression of antimicrobial peptide LH multimers in *Escherichia coli* C43(DE3). Appl Microbiol Biotechnol 83(1):143–149
32. Wang LN, Yu B, Han GQ, He J, Chen DW (2010) Design, expression and characterization of recombinant hybrid peptide Attacin–Thanatin in *Escherichia coli*. Mol Biol Rep 37(7):3495–3501
33. Wiesner J, Vilcinskas A (2010) Antimicrobial peptides: the ancient arm of the human immune system. Virulence 1(5):440–464
34. Wu GQ, Deng XP, Li XF, Wang XY, Wang SL, Xu HM (2011) Application of immobilized thrombin for production of S-thanatin expressed in *Escherichia coli*. Appl Microbiol Biotechnol 92(1):85–93
35. Zasloff M (2002) Antimicrobial peptides of multicellular organisms. Nature 414(6870):389–395
36. Zhang JL, Peng S, Cheng X, Wang HY (2012) Functional analysis of hybrid peptide CAMA-Syn: expression in mammalian cells and antimicrobial potential. Protein Pept Lett 19(10):1076–1081
37. Zhao PW, Cao GF (2012) Production of bioactive sheep β-defensin-1 in *Pichia pastoris*. J Ind Microbiol Biotechnol 39(12):11–17