Appl Microbiol Biotechnol (2007) 75:117–124
DOI 10.1007/s00253-006-0806-7

Multimerization and fusion expression of bovine lactoferricin derivative LfcinB15-W4,10 in *Escherichia coli*

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Received: 16 November 2006 / Revised: 8 December 2006 / Accepted: 8 December 2006 / Published online: 16 January 2007

Abstract Antimicrobial peptides are promising candidates for therapeutic and industrial application owing to their broad spectrum. In this work, a cost-effective method for expression of a potent antimicrobial peptide, bovine lactoferricin derivative LfcinB15-W4,10, has been developed. The oligonucleotide encoding the peptide was linked to generate different oligomeric oligonucleotide segments containing from one to nine but eight tandem copies which was inserted individually to the *E. coli* expression vector pET32a. The thioredoxin fusion peptides were successfully expressed and detected with different molecular weight on SDS gel, respectively. Among the monomer and other multimeric peptides, the tetramer was expressed at the highest level. After purification, more than 10 mg of tetramer with 99% purity was obtained from 1 l culture and exhibited similar antimicrobial activity as synthetic LfcinB15-W4,10 monomer. The expression system in this study provides a potential production method for lactoferricin derivatives and other antimicrobial peptides in research and industrial applications.

Keywords Antimicrobial peptides · Bovine lactoferricin · Multimerization · Fusion expression · *Escherichia coli*

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Introduction

In the past decades many kinds of antimicrobial peptides have been discovered and studied. These peptides exist abundantly throughout all living nature and presumably form a cornerstone of innate and adaptive immunity (van't Hof et al. 2001). The bactericidal effect of many antimicrobial peptides is thought to be the result of actions on the cytoplasmic membrane of susceptible bacteria, such as the formation of pores (Matsuzaki et al. 1998), destabilization of the bilayer (Marassi et al. 1999), thinning of the membrane (Heller et al. 2000), or depolarization of the membrane (Ulvatne et al. 2001). Owing to their broad spectrum of antimicrobial activity, antimicrobial peptides are promising candidates for therapeutic and industrial purpose (Koczulla and Bals 2003; Reddy et al. 2004).

Bovine lactoferrin (LFB) is a multifunctional glycoprotein with antimicrobial property which comes from its highly basic N-terminal region. Based on the common features of antimicrobial peptides, bovine lactoferricin (Phe 17 to Phe 41, LfcinB) (Bellamy et al. 1992) and lactoferrampin (Trp 268 to Arg 284, LfampinB) (van der Kraan et al. 2004) were identified in the N1-domain of LFB. LfcinB is a 25 amino acids (AAs) peptide possessing potent antimicrobial activity against various bacteria (Shin et al. 1998; Vorland et al. 1999), fungi (Bellamy et al. 1993), protozoa (Isamida et al. 1998), and viruses (Andersen et al. 2003; McCann et al. 2003). In addition to the broad-spectrum of LfcinB, the combined therapy of LfcinB with other clinical medicines has been more effective in the inhibitory experiment of various infections (Diarra et al. 2002; Wakabayashi et al. 2002). Recently, NMR spectroscopy showed that the structure of the LfcinB changed

drastically when it was released from the intact protein (Hwang and Vogel 1998; Schibli and Vogel 2000). In solution, the LfcinB looses the α-helical portion seen in LFB and adopts a twisted β-sheet structure. An intriguing property of the solution structure of LfcinB is that the structure becomes markedly amphipathic. Nearly all of the hydrophobic groups line upon one face of the peptide, while the opposite face contains most of the basic residues. In addition, LfcinB contains a disulfide bond between the cysteine residues in positions 19 and 36 (Bellamy et al. 1992). It is interesting to note that Kang et al. (1996) have shown that the shorter derivatives of bovine lactoferricin without the disulfide bond also exhibit antimicrobial activity.

The structure–activity relation of LfcinB and its derivatives has been extensively studied. Rekdal et al. (1999) have chemically synthesized a 15-residue derivative (LfcinB15) of bovine lactoferricin consisting of residues 17–31 of the natural bovine lactoferrin. It has only minor loss of antimicrobial activity compared with the native peptide LfcinB. The studies of indole analogs have shown that tryptophan residues have a preference for the water–phospholipid interface region when interacting with artificial phospholipid membrane (Yau et al. 1998). Trp is thought to function as an anchor in membrane proteins (Schiffer et al. 1992), and it is also suggested to act as a key factor that pulls antimicrobial peptides across phospholipid membranes (Wimley and White 2000). The aromatic group of Trp has been suggested to be the cause of its interfacial preference (Yau et al. 1998), and the indole has been suggested to interact with both hydrophobic and hydrophilic constituents of the membrane due to its amphipathic nature (Yau et al. 1998). In the performance of Trp substitutions on the counterproductive AAs in LfcinB15, a LfcinB15 derivative with improved activity was generated by replacing the residues Arg 4 and Met 10 with Trp (Strøm et al. 2002), this new LfcinB15 derivative was named as LfcinB15-W4,10 in this paper.

Recombinant expression of the antimicrobial peptides represents a potential approach for their production considering the cost associated with chemical synthesis. Some of antimicrobial peptides such as cecropin (Sun et al. 1999) and adenoregulin (Cao et al. 2005) have been successfully expressed. The expression of bovine lactoferricin in our lab and others (Feng et al. 2006; Kim et al. 2006) encouraged us to express the recombinant LfcinB15-W4,10 as a potential alternative to traditional antibiotics. Our aim was to overexpress this small peptide in prokaryotic system as efficiently as possible and minimize the cost of the production. We designed tandem multimeric peptides for expression because they are expected to be more facilitative for large-scale production than monomer peptide due to their difference in molecular weight for detection, purification and even structure stability. We report in this study the successful high level expression of active oligomeric LfcinB15-W4,10 in *E. coli* BL21 (DE3). This method could be also applicable to other recombinant peptides.

Materials and methods

Strains, plasmids, and reagents

Escherichia coli (*E. coli*) DH5α (Invitrogen, Beijing) was used for the propagation of plasmids, and *E. coli* BL21 (DE3) (TIANGEN, Beijing) was used for the expression of peptides. Plasmid pET32a(+) (Novagen, USA) was chosen for expression. *E. coli* strains were grown in Luria–Bertani (LB) or 2YT medium at 37°C and ampicillin was added for the selection of the recombinant strains. T4 DNA ligase (Promega, Beijing, China), restriction and modifying enzymes (NEB, Beijing, China) were used for construction of recombinant vector. Ni-NTA His Bind Resin (Novagen, USA) and thrombin (Sigma, USA) were used for the purification and cleavage of fusion peptides, respectively. *Staphylococcus aureus* ATCC 25923 (*S. aureus*) was kept in our laboratory. The monomer peptide LfcinB15-W4,10 was synthesized by Shanghai Invitrogen Biotechnol (China) using an automatic synthesizer and analyzed by HPLC. The lyophilized peptides were stored at −20°C and dissolved on the day of use. Other recombinant DNA techniques were performed as described by Sambrook and Russell (2002).

Construction of the genes encoding the oligomeric peptides

For the construction of the gene encoding the peptide LfcinB15-W4,10, two complementary 54 nt deoxyoligonucleotides A (forward) (5′-CCGATGTTCAAATGCTGG CGTTGGCAGTGGCGTTGGAAAAAACTGG GTGCGATG-3′) and B (reverse) (5′-TCGGCATCGCACC CAGTTTTTTCCAACGCCACTGCCAACGCCAG CATTTGAACA-3′) were synthesized (Sangon, Shanghai) based on the AA sequences (FKCW RWQWRWKKLGA) according to the codon usage of *E. coli*. A methionine coding sequence ATG and its reverse sequence CAT were introduced into oligo A and B, respectively. And the asymmetric cohesive ends 5′-CCGA-3′ and 5′-TCGG-3′ were introduced at the end of oligo A and B, respectively. The Met was added to facilitate the formation of monomer by CNBr degradation. The two oligos were phosphorylated and annealed. Then the genes were self-ligated in the correct orientation with the asymmetric cohesive ends in the presence of T4 DNA ligase at 16°C for 36 h. Additional T4 DNA ligase, ATP, and ligation buffer were added every 12 h. The self-ligation mixture was analyzed by 2% agarose gel. Two pairs of adaptor were synthesized, pair I, oligo C (F) (5′-TCGTAGGATCC-3′) and oligo D (R) (5′-TCGGG

GATCCTACGA-3′) contained a *Bam*HI site (GGATCC) and a cohesive end TCGG, pair II, oligo E (F) (5′-CCGAG TCAAGCTTAGC-3′) and oligo F (R) (5′-GCTAAGCTT GAC-3′) contained a *Hin*dIII site (AAGCTT) and a cohesive end CCGA. Adaptors were ligated at the ends of the oligomeric genes of LfcinB15-W4,10, respectively. Ligation mixture was incubated at 16°C for 12 h and then purified with QIAquick Gel Extraction Kit (QIAGEN, Germany). Thus, a series of coding genes of oligomers with forward and reverse adaptors were obtained. DNA fragments were digested with *Bam*HI and *Hin*dIII, respectively, and then ligated into the pET32a opened by the same enzymes to generate the expression vector pET32a-(LfcinB15-W4,10)$_n$, where n is the number of the LfcinB15-W4,10 gene copy. This vector encoded a 109 AAs thioredoxin and a thrombin site at the N-terminus and a hexa-histidine tag at the C-terminus of the target peptide. Positive transformants were screened by colony PCR using S-Tag (5′-CGAACGCCAGCACATGGACA-3′) and T7 terminator primers (5′-GCTAGTTATTGCTCAGCGG-3′) and identified by sequencing.

Expression of fusion oligomeric peptides

The constructs pET32a-(LfcinB15-W4,10)$_n$ were transformed into *E. coli* BL21 (DE3) for peptide expression. The *E. coli* harboring the constructs pET32a-(LfcinB15-W4,10)$_n$ were inoculated to 2YT medium (Tryptone 16 g l^{-1}, yeast extract 10 g l^{-1}, NaCl 5 g l^{-1}) supplemented with ampicillin (100 μg ml^{-1}), respectively, and grown at 37°C with shaking for 9 to 12 h. Each culture was then diluted at ratio of 1:100 into 50 ml fresh 2YT medium containing ampicillin (100 μg ml^{-1}) and grown at 37°C for 2.5 h. When the optical density at 600 nm reached about 0.4, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. The cells were cultured for another 4 h at 30°C and then harvested by centrifugation at 12,000 rpm for 5 min. The cells were washed and resuspended in phosphate-buffer saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3), and then mixed with 5× SDS-PAGE loading buffer and boiled for 5 min. The supernatant was analyzed by 12% SDS-PAGE after centrifugation at 12,000 rpm for 10 min, and the protein bands were detected with Coomassie brilliant blue R-250. Recombinant was inoculated into separate flask containing 30 ml 2YT and the conditions of expression as cell concentration, IPTG concentration and cultivation time were optimized.

Purification of the fusion peptides

The cell pellet harvested by centrifugation after IPTG induction was washed twice with PBS, and then resuspended in BugBuster protein extraction reagent (5 ml g^{-1}) (Novagen, USA) with Benzonase Nuclease (1 μl ml^{-1}) (Novagen, USA) and Lysozyme (60 kU g^{-1}) (Genview, USA) followed by an incubation at room temperature for 30 min. The mixture was then centrifuged at 12,000 rpm for 30 min at 4°C to separate the soluble supernatant and the insoluble fraction.

The insoluble inclusion body fraction harvested from 1 l culture containing the fusion protein of interest was washed with washing buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl) and then resuspended in 40 ml binding buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 8 M Urea, 10 mM imidazole). After centrifugation at 15,000 rpm for 20 min at 4°C, the solubilized fusion proteins were applied to the Ni^{2+}-NTA His·Bind resin preequilibrated with four bed volumes of binding buffer. After washing with binding buffer to baseline absorbance, the fusion peptides were eluted with four bed volumes of elution buffer containing 20, 50, 100, 150, 250, and 500 mM imidazole, respectively, at a flow rate of 2 ml min^{-1}. All of the fractions was collected and applied to 12% SDS-PAGE. The purified fusion protein was stored at 4°C.

Purification of LfcinB15-W4,10 oligomer

The purified fusion peptides were dialyzed three times against the 500 ml of thrombin reaction buffer (50 mM Tris–Cl, pH 7.5, 150 mM NaCl, 2.5 mM $CaCl_2$) and digested by thrombin at a final concentration of 5 U mg^{-1} at 25°C for 48 h with stirring according to the manufacture's instruction, and the products were analyzed by 12% SDS-PAGE.

For further purification of LfcinB15-W4,10 oligomer, the LfcinB15-W4,10 oligomer band was extracted from SDS-PAGE gel and electro-eluted in Tris–Gly running buffer (25 mM Tris–Cl, 200 mM Glycine) containing 0.1% SDS at 100 V in GeBAflex-tube for 2 h, then the polarity was reversed for 2 min. Five microliters of eluted fraction was analyzed on SDS-PAGE Gel and stained with Coomassie brilliant blue R-250. The purified recombinant oligomer was dialyzed against PBS buffer and quantified by Bradford assay.

Assay of antimicrobial activity

The antimicrobial activity of the recombinant LfcinB15-W4,10 oligomer was analyzed by inhibition zone assay. The minimal inhibitory concentration (MIC) of the LfcinB15-W4,10 monomer and tetramer against different microorganisms were also determined contrastively. For inhibition zone assay, *S. aureus* ATCC 25923 was grown overnight at 37°C in Mueller–Hinton broth (MHB) medium. Five hundred microliters of this culture was inoculated

into 50 ml of fresh MHB medium and incubated for additional 2 or 3 h at 37°C to OD_{500}=0.4 (1.44×10^9 CFUs ml^{-1}). A 400 μl of cell suspension was inoculated into 50 ml of prewarmed (42°C) MHB medium containing 1.0% (*w*/*v*) agar. After rapidly dispersing the bacteria, the medium was poured into the Petri dish (900×15 mm) to form a uniform layer about 1 mm deep. After gelation, Oxford cups were put on the surface of the medium carefully. Fifty microliters of PBS buffer, chemically synthesized LfcinB15-W4,10 (200 μg ml^{-1}) and the recombinant tetramer (300 μg ml^{-1}) were added to the central of Oxford cup, respectively. The plate was then incubated at 37°C for 24 h and assayed.

For MIC determination, the microtiter broth dilution method was modified for cationic antimicrobial peptides carried out in the sterile 96-well microtiter plates (Hancock 2001). Briefly, *S. aureus* ATCC 25923 was inoculate to 5 ml of MHB and grown overnight at 37°C with shaking (180 rpm). Bacterial culture was diluted into MHB to give $2–7\times10^5$ CFUs ml^{-1}. A 100 μl of cell suspension was dispensed in each well of 96-well plate from column 1 to column 11, and 100 μl of MHB (sterility control and blank for the plate scanner) was dispensed in column 12. Then 11 μl of 10 × test peptide oligomer was added in each well from column 1 with final concentration of 64 μg ml^{-1} to column 10 with final concentration of 0.125 μg ml^{-1} (column 11 is a control for bacteria alone). The plate was incubated at 37°C for 18–24 h and the absorption was recorded at 590 nm. MIC was taken as the lowest concentration of peptide that reduces growth by more than 50% contrast to column 11.

Results

Gene construction

Chemical synthesized LfcinB15-W4,10 is a cationic antimicrobial peptide of 15 AAs derived from bovine lactoferricin, in which the Arg 4, Met 10 were replaced by Trp. Its antimicrobial activity is 13-fold higher than that of full length LfcinB (Ulvatne et al. 2001; Strøm et al. 2002). To obtain the recombinant LfcinB15-W4,10 peptide, we construct an effective *E. coli* expression system. Because the molecular weight of LfcinB15-W4,10 is only 2, 079 Da, it will be very difficult to express, detect, and purify such a small molecule. To overcome these difficulties, we decided to express the peptide as the fusion oligomer to increase the molecular weight. Furthermore, the N-terminal fused thioredoxin can also increase the expression level, solubility, and stability of the peptide; the hexa-histidine tag will facilitate the purification. A Met residue was introduced at the N- and C-terminal of the each monomer of the oligomer, which will facilitate the degradation of oligomer to monomer by CNBr. The gene of the LfcinB15-W4,10 was designed and synthesized as described in "Materials and methods" and registered in GenBank with accession no. DQ251730. To produce tandem oligomeric peptide, the oligonucleotide encoding the peptide LfcinB15-W4,10 was self-ligated with the asymmetric cohesive ends 5′-CCGA/5′-TCGG by T4 DNA ligase after phosphorylation and annealing. Concatemeric oligomers gene of LfcinB15-W4,10 were constructed successfully as shown in Fig. 1a and purified (Tian et al. 2006a). The generated oligomeric genes were then ligated with forward and reverse adaptor, respectively, digested with *Bam*HI and *Hin*dIII and then ligated into pET32a to construct the recombinant expression vector pET32a-$(LfcinB15\text{-}W4,10)_n$. The positive clones were verified by colony PCR (Fig. 1b) and confirmed by sequencing analysis. In this study, we have successfully obtained the constructs contained the thioredoxin fused LfcinB-W4,10 from monomer to nonamer but octamer ($LfcinB15\text{-}W4,10_{1–7,9}$) encoding DNA sequence.

Expression of fusion oligomeric peptides

E. coli BL21 (DE3) containing either empty pET32a or pET32a-$(LfcinB15\text{-}W4,10)_n$ was cultured, respectively, induced by IPTG and analyzed by SDS-PAGE as described in "Materials and methods". After IPTG induction, the

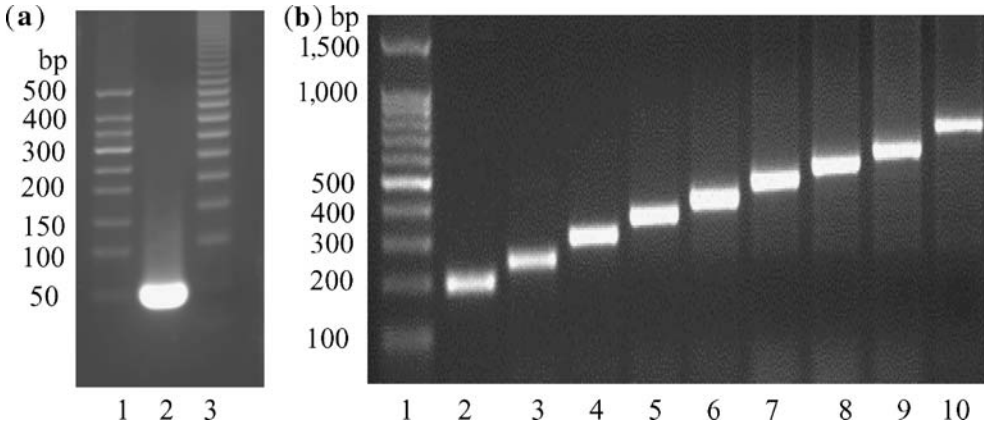

Fig. 1 Single copy and ligation products of LfcinB15-W4,10 gene (**a**) and the PCR products of recombinants (**b**). **a** *Lane 1* 50-bp ladder; *lane 2* synthesized LfcinB15-W4,10 gene; *lane 3* mixture of oligomeric genes with different copy; **b** *lane 1* 100-bp ladder; *lane 2* pET32a (+) as control; *lane 3* to *10* recombinants $(LfcinB15\text{-}W4,10)_{1–7,9}$

specifically induced band was detected by Coomassie brilliant blue R-250 in the total lysate of *E. coli* transformed by recombinant vectors, respectively, indicating that the thioredoxin-fused monomer (22.33 kDa), dimmer (24.31 kDa), trimer (26.29 kDa), tetramer (28.27 kDa), and hexamer (32.23 kDa) were successfully induced and translated in the *E. coli* BL21 (DE3), respectively (Fig. 2). Among the oligomers, the thioredoxin-fusioned LfcinB15-W4,10 tetramer showed the highest expression (about 45% of total cell proteins). To test the effect of thioredoxin on the solubility of the fused peptide, the solubility of thioredoxin only or the recombinant oligomeric peptides was analyzed. Thioredoxin was found in the soluble fraction; however, most of the thioredoxin-(LfcinB15-W4,10)$_n$ fusion peptides partitioned into the insoluble protein fraction (data not shown). Based on the optimization experiments, it is concluded that the best expression conditions for the fusion protein were as follows: after the culture was incubated to an OD_{600} of 0.6, IPTG was added to a final concentration of 0.1 mM and then the culture was continued to incubate for an additional 4 h at 30°C.

Purification of fusion peptides

Because the fusion tetramer showed the highest expression level, it was chosen for further purification. A 3.6-g cell pellet was harvested from 1-liter culture, and then lysed with BugBuster Reagent. The inclusion body fraction containing tetramer fusion proteins was resuspended in binding buffer. After centrifugation the supernatant was applied to Ni^{2+}-chelating chromatography. The contaminating proteins were successfully removed by 50 mM imidazole and the Trx-(LfcinB15-W4,10)$_4$ was eluted with 250 mM imidazole (Fig. 3). After purification, 73 mg fusion protein Trx-(LfcinB15-W4,10)$_4$ was obtained from 1 liter of *E. coli* culture. The purity of the fusion protein was around 86.3% (Table 1).

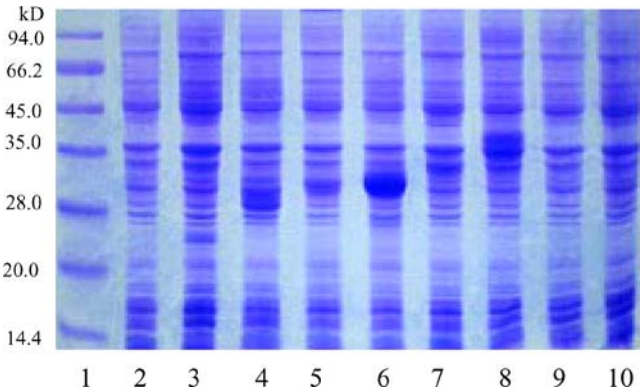

Fig. 2 SDS-PAGE analysis of expressed fusion oligomeric peptides. *Lane 1* marker; *lane 2* total proteins of induced *E. coli* BL21 (DE3) as control; *lane 3* to *10* total proteins of induced pET32a-(LfcinB15-W4,10)$_{1-7,9}$/BL21 (DE3)

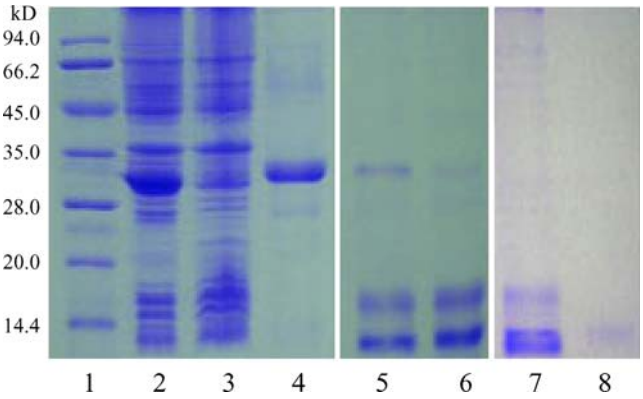

Fig. 3 Purification and cleavage of fusion protein Trx-(LfcinB15-W4,10)$_4$. *Lane 1* marker; *lane 2* total cell proteins after induction; *lane 3* total cell proteins before induction; *lane 4* recombinant fusion tetramer purified by Ni^{2+}-chelating chromatography; *lane 5* cleavage with thrombin for 24 h; *lane 6* cleavage with thrombin for 48 h; *lane 7* fusion protein Trx-tetramer after cleavage; *lane 8* electroelution product of tetramer

Release and electroelution of oligomeric peptide

To remove the thioredoxin from the tetramer of LfcinB15-W4,10, thrombin was used to specifically cleave the peptide bond between Arg and Gly in the compatible reaction buffer (Fig. 3). After cleavage, the resultant tetramer was electroeluted with MiDi GeBAflex-tube, the purity of the tetramer of LfcinB15-W4,10 was about 99% as determined by SDS-PAGE gel scanning (Fig. 3). In summary, about 10 mg of tetramer was obtained from 1 l of bacteria culture (Table 1).

Antimicrobial activity assay

The inhibitory curves of the LfcinB15-W4,10 monomer against Gram-negative (*Bacillus subtilis*, *Enterococcus Faecalis*, *Staphylococcus aureus*) and Gram-positive microorganisms (*E.coli* BL21(DE3), K88, ER2566) and tetramer against *S. aureus* were determined as showed in Fig. 4. Inhibition zone assay showed that the recombinant tetramer

Table 1 Purification and characterization of recombinant tetramer LfcinB15-W4,10 from 1 l of culture

Fusion protein Trx-tetramer		Purified recombinant tetramer		MIC against *S. aureus* (μg/ml)	
Yield (mg)	Purity (%)	Yield (mg)	Purity (%)	Synthesized monomer (Tian et al. 2006b)	Recombinant tetramer (this paper)
73.0	86.3	10.0	99.0	4	16

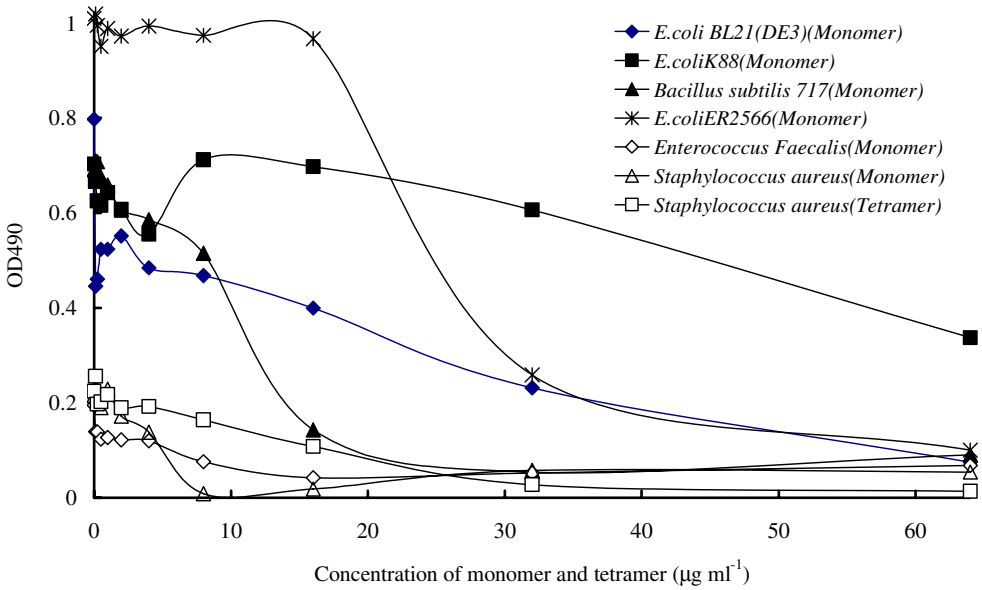

Fig. 4 Inhibitory curve of synthetic LfcinB15W-4,10 monomer against different microorganisms and tetramer against *Staphylococcus aureus* ATCC 25923

displayed antimicrobial activity against *S. aureus* obviously (Fig. 5). The MIC against *S. aureus* ATCC 25923 for the synthetic monomer LfcinB15-W4,10 and recombinant tetramer was 4 μg ml^{-1} (Tian et al. 2006b) and 16 μg ml^{-1}, respectively (Table 1). Besides, the MIC of synthetic monomer against *E. coli* BL21(DE3), K88, ER2566, *B. subtilis* 717 and *E. faecalis* was 64, 64, 32, 16 and 8 μg ml^{-1}, respectively (Fig. 4).

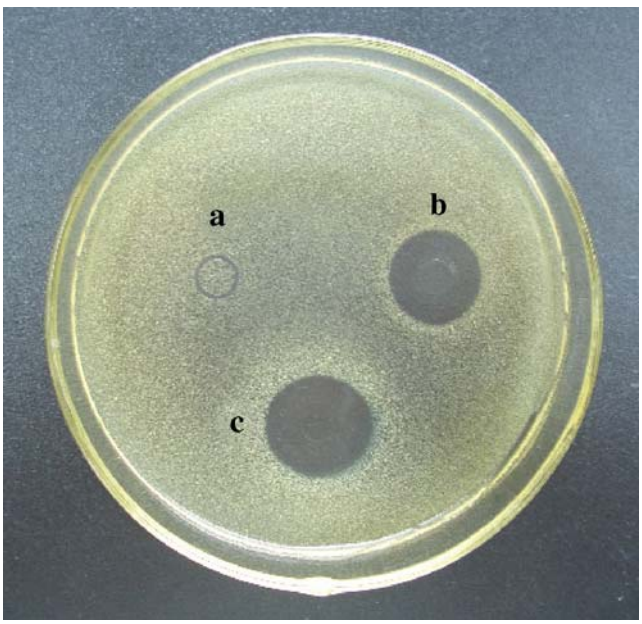

Fig. 5 The antimicrobial activity assay of recombinant LfcinB15-W4,10 tetramer against *Staphylococcus aureus* ATCC 25923. *A* 50 μl PBS as control; *B* 50 μl synthetic monomer of LfcinB15-W4,10 (200 μg ml^{-1}) as control; *C* 50 μl recombinant tetramer of LfcinB15-W4,10 (300 μg ml^{-1})

Discussion

The expression of antimicrobial peptides in *E. coli* has been suffering from two major problems—firstly, the expressed peptides are toxic to host bacteria, and secondly, the high content of positive charged AA residues makes them very sensitive to proteases, which are abundant in host cells (Skosyrev et al. 2003). To reduce the toxicity to the host, various systems for the expression of heterologous peptides fused to an affinity partner in *E. coli* have been described (LaVallie and McCoy 1995; Arnau et al. 2006). Zhang et al. (1998) reported that anionic property of fusion partner is indispensable in some recombinant antimicrobial peptide expression. In another study, biologically active bacteriocin was produced by fusion to an intein–chitin-binding domain gene (Ingham et al. 2005). Another common strategy is to express multimer of the peptide. Many experiments have been carried out to express various peptides as multimers in *E. coli* towards mass production (Jonasson et al. 2000; Raingeaud et al. 1996; Shen 1984; Stahl et al. 1990), so the expression of multimer would be another efficient way for cost-effective production. Concatemeric antimicrobial peptide buforin II with varying degrees of repetition was stably expressed in *E. coli* (Lee et al. 1999), and the high-level expression of peptide antibiotic hPAB-β was explored with the gene of multimeric hPAB-β in *E. coli* (Park et al. 1998).

LfcinB15-W4,10 is a 15 AA peptide with high antimicrobial activity and an amphipathic, cationic molecule with highly basic nature that is imparted by four tryptophan, three lysine, and two arginine residues. As far as we know, there is no report on the expression of LfcinB15 and LfcinB15-W4,10. In the present study, we have successfully expressed and purified an active LfcinB15-W4,10

peptide in *E. coli* system by adopting the multimer expressing strategy for the first time, in which, the thioredoxins fused LfcinB15-W4,10 oligomer with different copy number were expressed in *E. coli* BL21 (DE3) (Fig. 2). The great advantage of this method is that the multimerization of the peptide gene is in an efficiently and tightly controlled way, which provides a method to generate any size of desired multimers. In the multimerization, the clones containing different numbers of LfcinB15-W4,10 gene (from monomer to heptamer and nonamer) were successfully constructed and verified. Although we originally thought that the largest multimer would produce more proteins, however the LfcinB15-W4,10 tetramer had the highest expression level. This might result from the basic nature of peptides, which can inhibit transcription and translation through the interaction with DNA or RNA (Miller et al. 1989). Although the tetramer could not be cleaved efficiently to monomer by CNBr as expected, the thioredoxin-fused tetramer was efficiently cleaved by thrombin yielding a recombinant tetramer which exhibited a similar antimicrobial activity as that of the synthetic monomer peptide. However, other feasible and effective cleavage methods should be investigated further (Arnau et al. 2006).

The thioredoxin-fused LfcinB15-W4,10 was expressed in a high level by taking advantage of the commercially available vector pET32a,. There are three possible explanations. (1) The pI value of fused thioredoxin is approximately 5.2 which is lower than that of LfcinB15-W4,10 (pI=11.11), this difference could reduce the net charge and mask the toxicity of the mature basic peptides in a charge-dependent manner. (2) Vector pET32a provides an efficient one-step purification of the fusion protein by Ni^{2+}-metal chelating chromatography for the existence of the hexahistidine tag. (3) Thrombin cleaves the fusion protein with high specificity. It is known that most of the recombinant antimicrobial peptides have been expressed in *E. coli* as inclusion bodies to protect the host cells from the toxic effects of the recombinant peptides (Lee et al. 2000). In contrast to expression of nonrecombinant vector system, most of the fusion LfcinB15-W4,10 was in insoluble form in this study. The reasons could relate to a number of factors. Aberrant folding is known to cause insolubility of the recombinant proteins (Mitraki and King 1989). It is possible that the peptide LfcinB15-W4,10 altered the folding process of thioredoxin and decreased the solubility of thioredoxin to form the inclusion bodies. Alternatively, because the antimicrobial peptide LfcinB15 acts on membranes (Vorland et al. 1999), it is possible that the fused recombinant peptide may interact with the membrane component and result in the formation of the insoluble fraction. It is interesting that the treatment with denaturant to recombinant fusion peptide did not destroy its antimicrobial activity. The antimicrobial activity of tetramers is only a little bit lower than that of synthesized LfcinB15-W4,10, and the activity may be increased in monomer format.

It would be very interesting and attractive if the following aspects could be focused in future work, (1) to compare and reveal the differences of half-life span, antibacterial activity, and other characteristics among recombinant monomer, dimer, trimer, tetramer, and multimer of LfcinB15-W4,10; (2) to try to develop secretion expression system for this type of peptides; and (3) to probe and explain further the structure and function relationship of this type of peptides.

Acknowledgement This study was supported by two Chinese National Hi-Tech R & D Programs ("863" Programs) (Approving Nos. 2001AA246041 and 2004AA246040) and Beijing Natural Science Foundation (Approving No.415062031).

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