Biometals (2010) 23:523–530
DOI 10.1007/s10534-010-9294-4

Cloning, expression and characterization of Kunming mice lactoferrin and its N-lobe

Jiarong Wang · Zigang Tian · Da Teng · Yalin Yang · Jiancheng Hu · Jianhua Wang

Received: 28 December 2009 / Accepted: 23 January 2010 / Published online: 4 February 2010

Abstract The lactoferrin cDNA of Kunming mice was isolated by reverse transcription polymerase chain reaction and cloned into vector pET28a(+). Its deduced amino acid sequence was analyzed and compared with lactoferrin of other species. Its secondary and tertiary structure are predicted and modeled by bioinformatics tools online. Then recombinant Kunming mice lactoferrin and its N-lobe were both expressed successfully in the *Escherichia coli* BL21(DE3) in the form of inclusion bodies. After purification with Ni–NTA His-Bind resin, the yield of recombinant lactoferrin was 17 mg l^{-1} with purity of 92.1%, and that of lactoferrin N-lobe was 20 mg l^{-1} with purity of 98.5%. The inhibition efficiency of refolded lactoferrin N-lobe against *Staphylococcus aureus* ATCC 25923 reaches 48.6% at the concentration of 25 μmol l^{-1}. However, the refolded lactoferrin (12.5 μmol l^{-1}) didn't display obvious inhibition activity in the test. The expression of recombinant Kunming mice lactoferrin and its N-lobe will be helpful for the study of lactoferrin on structure, function and application in a mouse model system.

Keywords Expression · Lactoferrin · N-lobe · Kunming mice · *Escherichia coli*

Introduction

Lactoferrin (LF) is a mammalian iron-binding glycoprotein with a molecular mass of about 78 kDa, belongs to the transferrin family. It is generally expressed and secreted by glandular epithelial cells and is found in milk, saliva, tears and intestinal secretions. LF exists widely among human, bovine, goat, porcine, murine and other species (Teng 2002; Baker and Baker 2009). In recent decades, except for regulation of iron metabolism, several other functions of LF have been revealed such as protection against microbial infection, anti-inflammatory and antitumor activity, immunomodulatory activity. Especially, the antimicrobial activity is a new route for exploring LF in pharmacy, food, feed and other industrial purpose.

Jiarong Wang and Zigang Tian contributed equally to this work.

J. Wang · Z. Tian · D. Teng · Y. Yang · J. Wang
Key Laboratory of Feed Biotechnology, Ministry of Agriculture, 100081 Beijing, China

Z. Tian · D. Teng · Y. Yang · J. Wang (✉)
Gene Engineering Laboratory, Feed Research Institute, Chinese Academy of Agricultural Sciences, 12 Zhongguancun Nandajie St., 100081 Haidian District, Beijing, People's Republic of China
e-mail: jhwangfribio@yahoo.com.cn; wangjianhua@mail.caas.net.cn

J. Wang · J. Hu
College of Life Science, Lanzhou University, 730000 Lanzhou, Gansu, China

LF is a single polypeptide consisting of two homologous lobes designated the N-lobe and C-lobe, and each lobe can reversibly bind one ferric ion with the synergistic binding of a carbonate anion (Moore et al. 1997). Structure and sequence analysis indicate that the two lobes might have arisen by gene duplication in the process of evolution (Lambert et al. 2005). Separation of the N-lobe and C-lobe is necessary for studying the structure–function relationship of LF (Rahman et al. 2009), but it is difficult to achieve in native LF by proteolysis or chemical cleavage (Shimazaki et al. 1993; Brines and Brock 1983). Several expression systems including bacteria, yeast, fungi, insect and mammary bioreactor are used to study recombinant mammalian proteins, especially *Escherichia coli* (*E.coli*) and *Pichia pastoris* systems.

Luo et al. (2007) have expressed recombinant bovine LF N-terminal peptide in *E.coli,* and the product displayed obvious antimicrobial activity. Bovine LF derivates lactoferricin (LfcinB; Feng et al. 2006), LfcinB15 (Tian et al. 2007) and lactoferrampin (LfampinB; Yang et al. 2009) were expressed in *E. coli* system and displayed obvious antibacterial activity. Choi et al. (2008) expressed recombinant human lactoferrin in glycoengineered *Pichia pastoris* to evaluate the effects of terminal N-glycan structures on immune responses. The coding sequence of bovine LF C-lobe has been expressed and characterized successfully in *Rhodococcus erythropolis* (Kim et al. 2006). The attempt of using a baculovirus insect cell system to provide recombinant mouse LF was described (Suzuki and Lonnerdal 2004). And Ward et al. (1997) have expressed and characterized recombinant murine LF in *Aspergillus awamori* system.

The reproduction cycle of murine is shorter than that of other mammalians, so it is convenient to use murine as an animal model for LF studies. The secreted lactoferrin in mouse milk is 0.1–1 g l^{-1} (Teng 2002). In the present study, the Kunming mice which are the widely used experiment mouse in China are chosen as the initial material. The cDNA of Kunming mice LF (KmLF) and its N-lobe (KmLFN) were evaluated by means of bioinformatics online. Both coding sequence were cloned into expression vector pET28a(+), and then both recombinant proteins were expressed, purified and characterized.

Materials and methods

Strains, plasmids and reagents

The plasmid pET28a(+) (Novagen, USA) vector was used as expression vector. *E. coli* DH5α and BL21(DE3) (stored at our laboratory) were used as subcloning and expression hosts respectively. Mammary tissue of Kunming mice were excised from the mammary gland of lactating mouse (from Institute of Genetics and Developmental Biology, Chinese Academy of Sciences). The rabbit polyclonal antibody was prepared in Beijing Wanger Biotechnology Inc. against native bovine LF (bLF) from Nanjing Tianchun Trading Co. Ltd. T4 DNA ligase (TransGen, Beijing), DNA restriction and modifying enzymes (NEB, Beijing) were used for construction of the recombinant vector. Ni–NTA His Bind Resin (Novagen, USA) was used for purification of the recombinant protein. The bioinformatics tools: DNAMAN 5.2.2, ClustalX 1.83, MolMol 2 K.2 and Swiss-PdbViewer 4.0 were obtained from website. The secondary structure prediction was performed on the website of Network Protein Sequence @nalysis (NPS@; Deleage et al. 1997). The tertiary structure was predicted by SWISS-MODEL (Arnold et al. 2006).

Cloning of the Kunming LF cDNA

Total RNA was isolated from the mammary gland of lactating mouse using TRNzol-A^+ reagent (TIANGEN, Beijing). The single-strand cDNA was synthesized in two steps. Firstly, about 1.5 μg of total RNA was annealed with specific downstream primer (mLF-R: 5′-TATGAATTCAGTGTTTTCACTGGGTAAGA-3′, the sequence underlined is the *Eco*R I restriction site) at 70°C for 5 min and cooled on ice. Avian myeloblastosis virus (AMV) reverse transcriptase (TaKaRa, Japan) was added to the final concentration of 5 U μl^{-1}. The mixture was incubated at 42°C for 60 min, then at 5°C for 5 min. The full-length coding sequence of KmLF was amplified by primers mLF-F: 5′-CCAGGATCCAGAGACATGAGGCTGCTCA-3′ (the sequence underlined is the *Bam*H I restriction site) and mLF-R. The PCR reaction mixture 25 μl consisted of 5 μl of above cDNA template, 12.5 μl twofold selective PCR buffer, 4 μl $MgCl_2$ (25 mmol l^{-1}), 2 μl dNTP analog mixture (10 mmol l^{-1}

each), 0.5 μl mLF-F primer (20 mmol l^{-1}), 0.5 μl mLF-R primer (20 μmol l^{-1}) and 0.5 μl AMV-optimized Taq (5 U μl^{-1}). After pre-denaturation at 94°C for 5 min, it was run for 35 cycles: denaturing at 94°C for 50 s, annealing at 50°C for 50 s, and extension at 72°C for 3 min, followed by a final extension for 10 min at 72°C. The product was analyzed by 0.8% agarose electrophoresis and DNA sequencing. Each experiment was repeated three times.

Sequence analysis of KmLF

The amino acid sequence deduced from KmLF cDNA by DNAMAN 5.2.2 was aligned and compared with mouse LF sequence (NCBI accession no. BAA13633) by ClustalX 1.83. Meanwhile, the relative sequences of LfcinB (Bellamy et al. 1992; Munoz and Marcos 2006) and LfampinB (Haney et al. 2007) were searched in KmLF, and their physical properties were calculated by ProtParam (Gasteiger et al. 2005) and HydroMCalc (Tossi et al. 2002). The deduced amino acid sequence was also submitted to NPS@ (Deleage et al. 1997) for secondary structure prediction. The conformational states of amino acid residues are set to four states (helix, sheet, turn, and coil), and the predictions were combined into a consensus result. The tertiary structure was predicted by SWISS-MODEL (Arnold et al. 2006). The evaluation structure was displayed by MolMol 2 K.2. Particularly, the surface charge distribution was modeled and analyzed by Swiss-PdbViewer 4.0.

Construction of expression vectors for KmLF and KmLFN

The purified PCR product was digested by *Bam*H I/*Eco*R I and inserted into pET28a(+). The constructed plasmid was named as pET28-KmLF. *E. coli* DH5α competent cells were transformed by pET28-KmLF and screened in LB agar medium containing kanamycin (50 mg l^{-1}).

The coding sequence of KmLFN (1–333, without signal sequence) was amplified using one pairs of primers KmLFN-F: 5′-AGCTCACATATGAAGGC AACAACTGTTCGA-3′ and KmLFN-R: 5′-AGGCT GAATTCTCAATTCAGGTTCTGTATGGA-5′ (the sequence underlined are the *Nde* I and *Eco*R I restriction sites, respectively). The PCR product was inserted into pET28a(+) to construct vector pET28-KmLFN. *E. coli* DH5α was transformed and screened by colony PCR and DNA sequencing.

Expression and purification of KmLF and KmLFN in E. coli BL21(DE3)

The expression vectors pET28-KmLF and pET28-KmLFN was introduced into *E. coli* BL21(DE3). The transformants were grown in LB medium with kanamycin (50 mg l^{-1}) at 37°C overnight. Then 500 μl culture was inoculated into 50 ml fresh 2YT medium containing kanamycin (50 mg l^{-1}) and grown at 37°C. When the optical density at 600 nm reached about 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mmol l^{-1}. After further growth at 37°C for 4 h, the cells were harvested through centrifugation at 10,000 rpm for 3 min. The SDS-PAGE (12% separation gel; 5% stacking gel) was performed to detect the expression of the recombinant KmLF and KmLFN.

The cell pellet from 1 l culture was washed and resuspended in 80 ml PBS buffer (137 mmol l^{-1} NaCl, 2.7 mmol l^{-1} KCl, 10 mmol l^{-1} Na_2HPO_4, 1.76 mmol l^{-1} KH_2PO_4, pH 7.3) containing lysozyme (1 g l^{-1}), followed by an incubation on ice for 30 min. Then the cells were lysed by sonication (2 × 5 min, 150 W, 30% duty cycles). The mixture was centrifuged at 12,000 rpm for 15 min at 4°C. The insoluble fraction was washed twice with PBS and dissolved in 6 ml binding buffer (500 mmol l^{-1} NaCl, 20 mmol l^{-1} Tris-HCl, 8 mol l^{-1} Urea, pH 7.9). After centrifugation at 12,000 rpm for 15 min at 4°C, the soluble fraction was applied to Ni–NTA His-Bind resin pre-equilibrated with four volumes of binding buffer. After washing with binding buffer to baseline absorbance, the resin was eluted with four volumes of elution buffers (500 mmol l^{-1} NaCl, 20 mmol l^{-1} Tris-HCl, 8 mol l^{-1} Urea, pH 7.9) containing 5, 10, 20, 60, 160, 300, 1000 mmol l^{-1} imidazole respectively at a flow rate of 2 ml min^{-1}. All the fractions were collected and applied to 12% SDS-PAGE, and the percentage of target band in the lane was assayed by program Gel-Pro analyzer.

Western blotting analysis

The purified His-tagged-KmLF and His-tagged-KmLFN were applied to SDS-PAGE and electrically

transferred onto polyvinylidene fluoride (PVDF) membrane using an electro-transfer system DYCZ-40B (Beijing Liuyi Instrument Factory). The PVDF membrane was blocked with 5% BSA in TBST buffer (150 mmol l^{-1} NaCl, 0.1% Tween 20, 20 mmol l^{-1} Tris-Cl, pH 7.6) at room temperature for 2 h, and then incubated with a 1:5,000 dilution of rabbit polyclonal antiserum against bLF in TBST buffer with 1% BSA for 1 h. After washing four times with TBST for 10 min, the PVDF membrane was incubated with a 1: 8,000 dilution of goat anti-rabbit antibody conjugated to alkaline phosphatase in TBST buffer with 1% BSA. The PVDF membrane was washed four times with TBST buffer for 10 min, and then covered by BCIP/NBT reaction solution prepared freshly by mixing 50 µl NBT solutions and 50 µl BCIP solutions with 10 ml substrate buffer (TIANGEN, Beijing). Finally, the system was incubated in dark with occasional agitation for 5–10 min, washed with dH_2O and the results were recorded.

Refolding by stepwise dialysis

To obtain soluble and biologically active protein, the purified His-tagged-KmLF (0.5 g l^{-1}) and His-tagged-KmLFN (1 g l^{-1}) were refolded by dialysis according to the method of Kim et al. (2006) with slightly modifications. The 10 ml solution were dialyzed against 1 l refolding buffers (100 mmol l^{-1} sodium phosphate, 10 mmol l^{-1} Tris-Cl, 10% glycerol, pH 8.0) at 4°C. The urea concentration of the dialysis buffer was changed every 24 h, and it was gradually decreased from 8 to 0 mol l^{-1} in step of 2 mol l^{-1}. Then the proteins were further dialyzed against dH_2O for 24 h at 4°C. The soluble fraction after centrifugation was lyophilized and used for antimicrobial activity analysis.

Antimicrobial activity assay

In brief, *Staphylococcus aureus* (*S. aureus*) ATCC 25923 was grown at 37°C in Mueller–Hinton Broth (MHB), and incubated to $OD_{600} = 0.4$ (1.44×10^9 CFUs ml^{-1}). A 50 µl of cultures were incubated at 37°C for 1 h with 100 µl of sterile dH_2O (as negative control), bLF (12.5 µmol l^{-1}, as positive control 1), ampicillin (25 µmol l^{-1}, as positive control 2), KmLF (12.5 µmol l^{-1}) and KmLFN (25 µmol l^{-1}) respectively. Then these reaction mixtures were serially diluted and plated in MHB plates respectively. The plates were incubated at 37°C until colonies form and the quantity of colony were counted and compared. Each sample was repeated three times.

Results

Cloning and the sequence analysis of KmLF

The KmLF cDNA (2,124 bp) cloned in this study encodes a mature protein of 688 amino acids with a signal peptide of 19 amino acids, and have been registered to GenBank with the accession number of FJ538998.1. The sequence identity of signal peptide and mature peptide between KmLF and house mouse LF (GenBank no. BAA13633) are 100 and 99%, respectively. Only six amino acids Arg6, Met63, Ser359, Ala381, Glu420 and Ala615 in mature protein of KmLF are different from that of house mouse LF (Table 1). Besides, the sequence identity between KmLF and human LF is 71%, while the sequence identity between KmLF and bovine LF is 64%. The relative sequences of LfcinB and LfampinB in KmLF sequence are KmLF 16–40 and KmLF 267–285, respectively. The net positive charge of peptide KmLF 16–40 and KmLF 267–285 are +4 and +2, which are lower than that of LfcinB (+8) and LfampinB (+5). The hydrophobicity of KmLF 16–40 (−2.82) is stronger than that of LfcinB (−1.48), but that of KmLF 267–285 (−2.8) is weaker than LfampinB (−3.2).

The predicted secondary structure generated from the NPS@ Web server showed that, the sequence predominantly appeared to be α-helical structure joined by β-sheet. In brief, 168, 113 and 393 amino acids adopt α-helix, β-sheet and coil structure respectively. The homology modeling result (visualized by MolMol 2 K.2) showed that KmLF appears to be two globular lobes (Fig. 1a), representing N-terminal (residues 1–333) and C-terminal (residues 343–688) lobes. The two lobes are connected by a 9 residues α-helix (334–342). In each lobe, α-helix and β-sheet referred to N1 and N2, or C1 and C2 domain. The KmLF has high pI (8.91), and the distribution of surface charge

Table 1 Difference of amino acids between mouse LF (NCBI accession no. BAA13633) and KmLF in this study

No.	Mutation location	Codon in mouse LF	Amino acid in mouse LF	Codon in KmLF	Amino acid in KmLF
1	74	CAA	Gln6	CGA	Arg6
2	244	TTG	Leu63	ATG	Met63
3	729	GAC	Asp224	GAT	Asp224
4	1,075	ACA	Thr359	TCA	Ser359
5	1,199	GTC	Val381	GCC	Ala381
6	1,346	GGA	Gly420	GAA	Glu420
7	1,791	AAA	Lys578	AAG	Lys578
8	1,901	GTT	Val615	GCT	Ala615

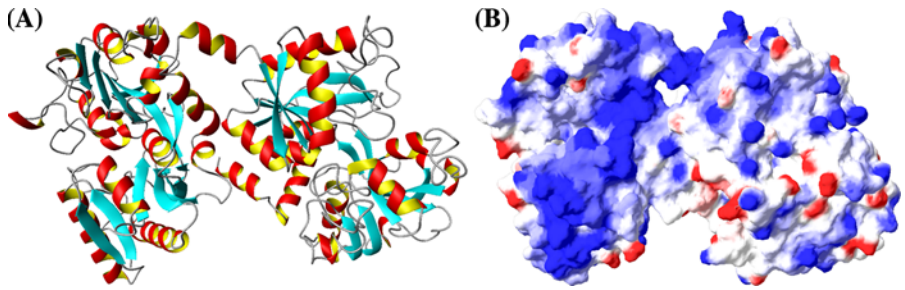

Fig. 1 KmLF tertiary structure. **a** Homology modeling result displayed by program MolMol 2 K.2, presented as a *ribbon diagram*. *Light blue* is *β*-strands and *red* and *yellow* are *α*-helix. **b** A charge distribution on the KmLF surface in the same orientation displayed by Swiss-PdbViewer 4.0. The net positive, neutral and negative charges are shown in *blue*, *white* and *red*, respectively

displayed by Swiss-PdbViewer 4.0 is highly uneven (Fig. 1b). The net positive charge of N-lobe (+14) is notably higher than that of C-lobe (+7). Besides, there are two notable regions with concentrations of positive charge: the N-terminus (residues 1–68) and the intermediate region (310–342) which is close to the connecting helix (Fig. 1b).

Construction of expression vectors for KmLF and KmLFN

The sequence coding KmLF was directly introduced into pET28a(+) by *Bam*H I/*Eco*R I to construct vector pET28-KmLF. Then the coding fragment of KmLFN was amplified and subcloned into pET28a(+) by *Nde* I/*Eco*R I generating pET28-KmLFN. The fragments generated by restriction digestion from pET28-KmLF (by *Bam*H I and *Eco*R I) and pET28-KmLFN (by *Nde* I and *Eco*R I) were about 2.14 and 1.02 kb which were consistent with theoretical size (Fig. 2). The sequence accuracy of both vectors was further determined by DNA sequencing.

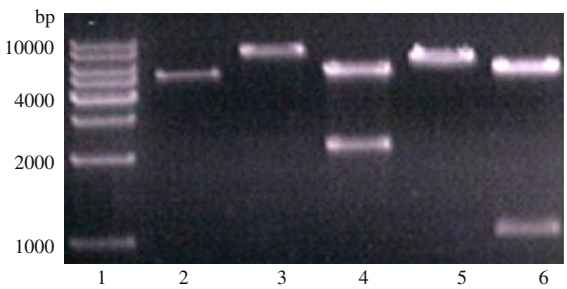

Fig. 2 Restriction analysis of constructed vectors. *Lane 1*: DNA Marker; *Lane 2*: *Eco*R I digested pET28a(+); *Lane 3*: *Eco*R I digested pET28-KmLF; *Lane 4*: *Bam*H I and *Eco*R I digested pET28-KmLF; *Lane 5*: *Eco*R I digested pET28-KmLFN; *Lane 6*: *Nde* I and *Eco*R I digested pET28-KmLFN

Expression and purification of recombinant KmLF and KmLFN

The molecular weight of expressed His-tagged-KmLF and His-tagged-KmLFN in SDS-PAGE(Fig. 3a) were about 80 and 38 kDa (Fig. 3a), which were close to the calculated size 81.84 and 38.83 kDa, respectively. In purification of His-tagged-KmLF, the contaminated

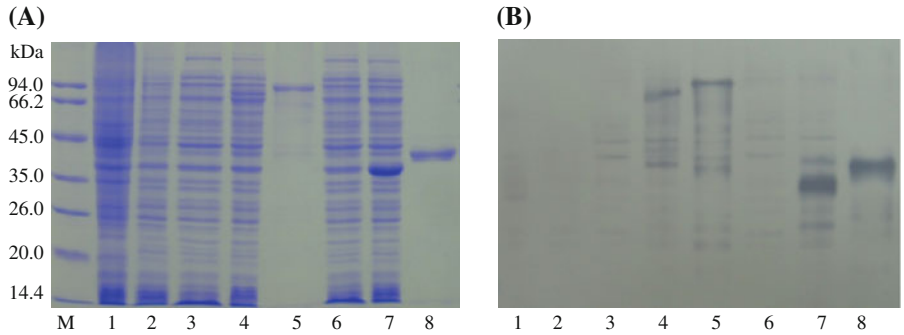

Fig. 3 SDS-PAGE **a** and western blotting **b** analysis of expressed and purified KmLF and KmLFN. *M* Protein marker; *Lane 1*: total proteins of induced *E. coli* BL21(DE3); *Lane 2*: total proteins of induced *E. coli* BL21(DE3) containing pET28a(+); *Lane 3*: total proteins before induction of *E. coli* BL21(DE3) containing pET28-KmLF; *Lane 4*: total proteins after induction of *E. coli* BL21(DE3) containing pET28-KmLF; *Lane 5*: purified KmLF; *Lane 6*: total proteins before induction of *E. coli* BL21(DE3) containing pET28-KmLFN; *Lane 7*: total proteins after induction of *E. coli* BL21(DE3) containing pET28-KmLFN; *Lane 8*: purified KmLFN

Table 2 Viability of *S. aureus* ATCC 25923 after incubation with bLF, KmLF and KmLFN at 37°C for 1 h

Sample	dH_2O	bLF (12.5 μmol l^{-1})	KmLF (12.5 μmol l^{-1})	KmLFN (25 μmol l^{-1})	Ampicillin (25 μmol l^{-1})
CFUs ml^{-1}	1.4×10^7	2.62×10^6	1.38×10^7	7.2×10^6	6.87×10^6

proteins were removed successfully when the concentration of imidazole was 60 mmol l^{-1}, and His-tagged-KmLF was eluted at the concentration of 1 mol l^{-1} (Fig. 3a). It is slightly different that the contaminants in purification of His-tagged-KmLFN were removed at 40 mmol l^{-1} imidazole, and the His-tagged-KmLFN was eluted when imidazole was 500 mmol l^{-1}. The identification of these two induced protein bands was also confirmed by western blotting (Fig. 3b). Conclusively, the expression level of recombinant His-tagged-KmLF was 17 mg l^{-1} with purity of 92.1%, and that of His-tagged-KmLFN was 20 mg l^{-1} with purity of 98.5%.

Refolding by stepwise dialysis

The isolated inclusion body was refolded by stepwise dialysis against refolding buffers. In order to improve the efficiency of renaturation, other two dialysis buffers were applied: buffer A (0.1 mmol l^{-1} $FeCl_3$, 500 mmol l^{-1} NaCl, 50 mmol l^{-1} NaH_2PO_4, pH8.0) and buffer B (500 mmol l^{-1} NaCl, 50 mmol l^{-1} NaH_2PO_4, 1% Triton X-100, 10% glycerol pH8.0). However, with regard to the yield of the soluble fractions, the same result was obtained in three buffers. Yield of the His-tagged-KmLF and His-tagged-KmLFN were estimated as 60 and 90 μg l^{-1}.

Antibacterial activity assay

Compared with the negative control sterile dH_2O (1.4×10^7 CFUs ml^{-1}), the viability of *S. aureus* ATCC 25923 was markedly reduced (about 48.6% reduction, Table 2) by incubation with 25 μmol l^{-1} His-tagged-KmLFN (7.2×10^6 CFUs ml^{-1}). Accordingly, the His-tagged-KmLF (12.5 μmol l^{-1}) didn't display obvious antimicrobial activity against *S. aureus* ATCC 25923. As the positive control, the survival of *S. aureus* ATCC 25923 treated by 25 μmol l^{-1} ampicillin and 12.5 μmol l^{-1} bLF were 6.87×10^6 and 2.62×10^6 CFUs ml^{-1} (Table 2), respectively.

Discussion

Lactoferrin is an iron binding protein belonging to the superfamily of transferrins (Wally and Buchanan 2007). Current sequence databases contain LF sequences from nine species: human, mouse, cow,

horse, pig, goat, sheep, buffalo and camel (Teng 2002; Baker and Baker 2009).

In this study, the results of Blast revealed that there are only six amino acids (Table 1) in mature protein of KmLF different from house mouse LF (NCBI accession no. BAA13633). This difference might be caused by natural genomic polymorphism. The sequence identity of LF between Kunming mice and Bovine is 64%, which probably contributed to the recognition of anti-bLF antibody against KmLF in the western blotting assay (Fig. 3b). The results of secondary structure prediction according as DSC, PHD and MLRC method demonstrated that 24.42, 16.42 and 57.12% of 688 amino acids adopts α-helix, β-sheet and coil conformation. The homology modeling in SWISS-MODEL (Fig. 1a) is similar to the structure of human LF (Anderson et al. 1987; Wally and Buchanan 2007) and bovine LF (Moore et al. 1997). In each lobe, α-helix and β-sheet refer to N1 and N2, or C1 and C2 domain, encloses a deep cleft where it may be the iron binding site like other species LF (Baker and Baker 2009).

Antimicrobial activity is one of the various biological functions of LF, and the mechanism investigation is always the key point of research. Some studies concluded that iron binding activity and restricting iron metabolism are the foundation of inhibiting the growth of microbe (Caraher et al. 2007; Zarember et al. 2007). Besides, some reports demonstrated that the ability to bind to other macromolecules (proteins, DNA) (Baker and Baker 2009; Nevinskii et al. 2009) may also play important role in the many functions. The modeled distribution of surface charge (Fig. 1b) shown that the net positive charge of N-lobe (+14) is higher than that of C-lobe (+7). It means that the separated N-lobe has the higher activity to bind to anionic molecules than C-lobe. So coding sequence of N-lobe was separately subcloned and expressed in *E.coli* system, and the recombinant KmLFN has higher inhibition activity against *S. aureus* than intact KmLF in this study. The relative sequences of LfcinB and LfampinB in KmLF are KmLF 16–40 and KmLF 267–285. And KmLF 16–40 and KmLF 267–285 have high strong hydrophobicity (−2.82 and −2.8) which is the important properties to interact with the lipid bilayer of cell membrane. Whether the KmLF 16–40 and KmLF 267–285 have the similar antimicrobial spectrum as LfcinB and LfampinB is still need to be confirmed.

In the present study, the purified His-tagged-KmLF and His-tagged-KmLFN were visualized as single band with approximately 87.3 and 40.9 kDa, respectively (Fig. 3a). It is noticeable that the molecular weight of purified His-tagged-KmLF and His-tagged-KmLFN were a little larger than the unpurified proteins band (Fig. 3a). This difference might be resulted from complete and total denaturation of the proteins because of 8 mol l^{-1} urea in the purification, especially in combination with SDS-PAGE. When the protein was completely linearized in this way, it will tend to run more slowly through the gel (Schägger 2006). The several lower bands in the lane (Fig. 3b) might be caused by the degradation of the expressed full-length KmLF or antibody cross reaction.

The antibacterial activity against *S. aureus* ATCC 25923 of recombinant N-lobe was slightly stronger than that of intact LF after refolding. This was most likely attributable to the difference in refolding efficiency of inclusion body. The intact KmLF has 744 amino acids, but its N-lobe only has 353 amino acids, so the recombinant N-lobe is easier to be refolded in correct conformation. The intact KmLF didn't display obvious antimicrobial activity at the concentration of 12.5 μmol l^{-1}. And the antimicrobial activity of N-lobe of KmLF (25 μmol l^{-1}) was almost equal to 25 μmol l^{-1} ampicillin. The *E. coli* system doesn't have the process of post-translation modification such as glycosylation, so the recombinant KmLF and KmLFN are short of carbohydrate side-chains compared with the native LF and its N-lobe. The influence of glycosylation on the antimicrobial activity of LF should be further studied based on expression in eukaryotic system or isolation of native KmLF (Choi et al. 2003; 2008).

Lactoferrin is expected to be used as a natural antimicrobial material in general and clinical drugs. The expression of KmLF and KmLFN in *E. coli* system in this work will facilitate the study of lactoferrin on structure, function and application in a mouse model system.

Acknowledgments This study is supported by National Natural Science Foundation of China (no. 30972125; 30771574; 30810303084), Beijing Natural Science Foundation (no. 5062031; no. 5093030).

References

Anderson BF, Baker HM, Dodson EJ, Norris GE, Rumball SV, Waters JM, Baker EN (1987) Structure of human lactoferrin at 3.2-A resolution. Proc Natl Acad Sci USA 84:1769–1773

Arnold K, Bordoli L, Kopp J, Schwede T (2006) The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics 22:195–201

Baker EN, Baker HM (2009) A structural framework for understanding the multifunctional character of lactoferrin. Biochimie 91:3–10

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 73:472–479

Brines RD, Brock JH (1983) The effect of trypsin and chymotrypsin on the in vitro antimicrobial and iron-binding properties of lactoferrin in human milk and bovine colostrum. Unusual resistance of human apolactoferrin to proteolytic digestion. Biochim Biophys Acta 759:229–235

Caraher EM, Gumulapurapu K, Taggart CC, Murphy P, McClean S, Callaghan M (2007) The effect of recombinant human lactoferrin on growth and the antibiotic susceptibility of the cystic fibrosis pathogen *Burkholderia cepacia* complex when cultured planktonically or as biofilms. J Antimicrob Chemother 60:546–554

Choi BK, Bobrowicz P, Davidson RC, Hamilton SR, Kung DH, Li H, Miele RG, Nett JH, Wildt S, Gerngross TU (2003) Use of combinatorial genetic libraries to humanize N-linked glycosylation in the yeast *Pichia pastoris*. Proc Natl Acad Sci U S A 100:5022–5027

Choi BK, Actor JK, Rios S, d'anjou M, Stadheim TA, Warburton S, Giaccone E, Cukan M, Li H, Kull A, Sharkey N, Gollnick P, Kocieba M, Artym J, Zimecki M, Kruzel ML, Wildt S (2008) Recombinant human lactoferrin expressed in glycoengineered *Pichia pastoris*: effect of terminal N-acetylneuraminic acid on in vitro secondary humoral immune response. Glycoconj J 25:581–593

Deleage G, Blanchet C, Geourjon C (1997) Protein structure prediction. Implications for the biologist. Biochimie 79:681–686

Feng XJ, Wang JH, Shan AS, Teng D, Yang YL, Yao Y, Yang GP, Shao YC, Liu S, Zhang F (2006) Fusion expression of bovine lactoferricin in *Escherichia coli*. Protein Expr Purif 47:110–117

Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, Bairoch A (2005) Protein identification and analysis tools on the ExPASy server. In: Walker JM (ed) The proteomics protocols handbook. Humana Press, Totowa, pp 571–607

Haney EF, Lau F, Vogel HJ (2007) Solution structures and model membrane interactions of lactoferrampin, an antimicrobial peptide derived from bovine lactoferrin. Biochim Biophys Acta 1768:2355–2364

Kim WS, Shimazaki K, Tamura T (2006) Expression of bovine lactoferrin C-lobe in *Rhodococcus erythropolis* and its purification and characterization. Biosci Biotechnol Biochem 70:2641–2645

Lambert LA, Perri H, Halbrooks PJ, Mason AB (2005) Evolution of the transferrin family: conservation of residues associated with iron and anion binding. Comp Biochem Physiol B Biochem Mol Biol 142:129–141

Luo H, Chen S, Ren F, Guo H, Lin S, Xu W (2007) In vitro reconstitution of antimicrobial pathogen activity by expressed recombinant bovine lactoferrin N-terminal peptide in *Escherichia coli*. J Dairy Res 74:233–238

Moore SA, Anderson BF, Groom CR, Haridas M, Baker EN (1997) Three-dimensional structure of diferric bovine lactoferrin at 2.8 A resolution. J Mol Biol 274:222–236

Munoz A, Marcos JF (2006) Activity and mode of action against fungal phytopathogens of bovine lactoferricin-derived peptides. J Appl Microbiol 101:1199–1207

Nevinskii AG, Soboleva SE, Tuzikov FV, Buneva VN, Nevinsky GA (2009) DNA, oligosaccharides, and mononucleotides stimulate oligomerization of human lactoferrin. J Mol Recognit 22:330–342

Rahman M, Kim WS, Kumura H, Shimazaki K (2009) Bovine lactoferrin region responsible for binding to bifidobacterial cell surface proteins. Biotechnol Lett 31:863–868

Schägger H (2006) Tricine-SDS-PAGE. Nat Protoc 1:16–22

Shimazaki K, Tanaka T, Kon H, Oota K, Kawaguchi A, Maki Y, Sato T (1993) Separation and characterization of the C-terminal half molecule of bovine lactoferrin. J Dairy Sci 76:946–955

Suzuki YA, Lonnerdal B (2004) Baculovirus expression of mouse lactoferrin receptor and tissue distribution in the mouse. Biometals 17:301–309

Teng CT (2002) Lactoferrin gene expression and regulation: an overview. Biochem Cell Biol 80:7–16

Tian ZG, Teng D, Yang YL, Luo J, Feng XJ, Fan Y, Zhang F, Wang JH (2007) Multimerization and fusion expression of bovine lactoferricin derivative LfcinB15–W4, 10 in *Escherichia coli*. Appl Microbiol Biotechnol 75:117–124

Tossi A, Sandri L, Giangaspero A (2002) New consensus hydrophobicity scale extended to non-proteinogenic amino acids. In: Benedetti E, Pedone C (eds) Peptides 2002: Proceedings of the twenty-seventh European peptide symposium. Edizioni Ziino, Napoli, pp 416–417

Wally J, Buchanan SK (2007) A structural comparison of human serum transferrin and human lactoferrin. Biometals 20:249–262

Ward PP, Chu H, Zhou X, Conneely OM (1997) Expression and characterization of recombinant murine lactoferrin. Gene 204:171–176

Yang YL, Tian ZG, Teng D, Zhang J, Wang JR, Wang JH (2009) High-level production of a candidacidal peptide lactoferrampin in *Escherichia coli* by fusion expression. J Biotechnol 139:326–331

Zarember KA, Sugui JA, Chang YC, Kwon-Chung KJ, Gallin JI (2007) Human polymorphonuclear leukocytes inhibit *Aspergillus fumigatus* conidial growth by lactoferrin-mediated iron depletion. J Immunol 178:6367–6373