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Contribution of bovine lactoferrin inter-lobe region to iron binding stability and antimicrobial activity against *Staphylococcus aureus*

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Abstract The investigation of the recombinant bovine lactoferrin-derived antimicrobial protein (rBLfA) demonstrates that the inter-lobe region of bovine lactoferrin contributes to iron binding stability and antimicrobial activity against *Staphylococcus aureus*. rBLfA containing N-lobe (amino acid residues 1–333) and inter-lobe region (residues 334–344) was expressed in *Pichia pastoris* at shaking flask and fermentor level. The recombinant intact bovine lactoferrin (rBLf) and N-lobe (rBLfN) were expressed in the same system as control. The physical–chemical parameters of rBLfA, rBLfN and rBLf including amino acid residues, molecular weight, isoelectric point, net positive charge and instability index were computed and compared. The simulated tertiary structure and the calculated surface net charge showed that rBLfA maintained original structure and exhibited a higher cationic feature than rBLf and rBLfN. The three proteins showed different iron binding stability and antimicrobial activity. rBLfA released iron in the pH range of 7.0–3.5, whereas rBLfN lost its iron over the pH range of 7.0–4.0 and iron release from rBLf occurred in the pH range of 5.5–3.0. However, the minimum inhibition concentration of rBLfA against *S. aureus* ATCC25923 was 6.5 μmol/L, compared with 12.5 and 25 μmol/L that of rBLfN and rBLf, respectively. These results revealed that *S. aureus* was more sensitive to rBLfA than rBLfN and rBLf. It appeared that the strong cationic character of inter-lobe region related positively to the higher anti-*S. aureus* activity.

Keywords Bovine lactoferrin · Inter-lobe region · Iron binding · Antimicrobial activity · Recombinant expression

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Introduction

Bovine lactoferrin (BLf) is a non-heme iron binding glycoprotein belonging to the transferrin family (Metz-Boutigue et al. 1984). It is considered as the first line defense protein involved in protection against microbial infection and prevention of systemic inflammation (Lonnerdal 2009; Choi et al. 2008). BLf consists of a single-chain polypeptide of 689 amino acid residues and folds into two homologous globular lobes (Moore et al. 1997), which are N-lobe (residues 1–333) and C-lobe (residues 345–689). Each lobe could tightly and reversibly bind a

single Fe^{3+} ion accompanied by one synergistically bound CO_3^{2-} ion. There is a 3-turn α-helix of 11 residues (334–344) named inter-lobe region between two lobes.

In addition to the iron binding property and receptor-mediated systemic immune regulation, strongly cationic nature of BLf has been considered contributing to its broad-spectrum antimicrobial activities (Bellamy et al. 1992). BLf's bactericidal function is partially due to the direct interaction of the positive charge regions with anionic molecules on some bacterial, viral, fungal and parasite surfaces, causing an increase in the membrane's permeability and ensuing damage to the bacteria (Haversen et al. 2010). The N-terminus, along the outside of the first helix and the inter-lobe region are the three regions with notable concentration of positive charges (Baker and Baker 2009). The first two regions are both located at BLf N-lobe which contains functional domains (lactoferricin and lactoferrampin) associated with the bactericidal activity. In vitro expressions of recombinant BLf N-lobe have been developed for the structures, functions, and applications studies (Nakamura et al. 2001; Tanaka et al. 2003). However, investigations in the structural and functional properties of the inter-lobe region (residues 334–344) have not been closely concerned. The helical linker at inter-lobe region is also one of the regions with notable concentration of positive charges, which seems likely to reinforce the interaction between BLf and anionic constituents on the surface of pathogen cells.

In the present study, a recombinant BLf-derived antimicrobial molecule (rBLfA) that including N-lobe (residues 1–333) and inter-lobe connecting region α-helix (residues 334–344) was expressed both at shaking flask and fermentor level by *Pichia pastoris* X-33. In order to investigate inter-lobe region's contributions to iron binding and antimicrobial activity, rBLfA was compared with the intact protein (rBLf) and N-lobe (rBLfN) that were expressed in the same system.

Materials and methods

Sequence analysis

The structure homology-modeling of the peptides BLfA (residues 1–344), BLfN (residues 1–333), and BLf (residues 1–689) was made by SwissModel Alignment Mode (Kiefer et al. 2009). The preliminary model for BLfA was submitted to the PMDB (Protein Model DataBase, http://www.caspur.it/PMDB/). Physico-chemical parameters including amino acid residues, molecular weight, isoelectric point (p*I*), net positive charge and instability index of the peptides were calculated by ExPAsy ProtParam (Wilkins et al. 1999). N-glycosylation sites (Asn-X-Ser/Thr) in BLfA were predicted by the NetNglyc 1.0 server.

Cloning of the BLf cDNA

Mammary tissue samples of Holstein cow were obtained from Beijing Sanyuan Green Lotus Dairy Center, frozen in liquid nitrogen immediately and stored at −80°C. Total RNA extracted using Trizol reagent (Invitrogen, USA), M-MLV reverse transcriptase (Promega, USA) and one pair of random primers were used to synthesize the single-stranded cDNA encoding BLf, then its mature cDNA sequence was amplified by the polymerase chain reaction (PCR) under the primers P1 and P2 (Table 1) using single-stranded cDNA as the template. PCR products

Table 1 Sequences of synthesized oligonucleotide primers used in this work

Primer name	Sequence (5′ → 3′)
P1	gTCCCATggCCCCgAggAAAAACgTTCgATggTgTA
P2	ACgTCgACCCCTCgTCAggAAggCgCAg
PF-BLfA	ggAATTCgCCCCgAggAAAAACg
PR-BLfA	gCTCTAgAgCCCTggTgTACCgCgCCTTCA
PF-BLfN	ggAATTCgCCCCgAggAAAAACg
PR-BLfN	gCTCTAgAgCTTCCCTgAggTTCTTCAAggTggTC
PF-BLf	TCCCCgCggAgCCCCgAggAAAAACg
PR-BLf	gCTCTAgAgCCCTCgTCAggAAggCgCAg

* The restriction enzyme sites were underlined

were ligated to the pMD18-T vectors (TaKaRa, Dalian) to get pMDBLf using T4 DNA ligase (TransGen Biotech, Beijing). Then the pMDBLf were introduced into *Escherichia coli* DH5α competent cells. Transformants were determined by colony PCR and DNA sequencing.

Construction and transformation of the expression vectors

The cDNA sequences of BLfA, BLfN and BLf were amplified from pMDBLf using three pairs of primers PF/R-BLfA, PF/R-BLfN and PF/R-BLf (Table 1). PCR products were cloned into the *Pichia pastoris* expression vector pPICZα A to get three recombinant vectors pPICBLfA, pPICBLfN and pPICBLf, respectively. They were verified by PCR and DNA sequencing. These recombinant vectors linearized by *Pme*I were, respectively, introduced into *P. pastoris* X-33 at the *AOX*1 locus (*MUT*^{s}) by electroporation according to Cregg (2007). The positive recombinants were chosen by PCR.

Expression of rBLfA, rBLfN, and rBLf at shaking flask and fermentor level

A 100 μL of the transformation mix was plated on YPDS (yeast extract peptone dextrose medium) plates with 100, 200, 300, 800, and 1000 μg/mL zeocin and incubated at 29°C until colonies appeared. A single colony of recombinant protein-derived *P. pastoris* strain was inoculated into a 1 L shake-flask containing 100 mL BMGY medium and cultured at 29°C and 250 rpm in a shaker until $OD_{600\ nm}$ of 2–6 was reached. Cells were harvested by centrifugation at 3,250*g* and 4°C for 10 min and incubated in 25 mL BMMY medium. Methanol was added every 24 h into the medium to maintain the concentration at 0.5% (v/v). The supernatant was collected by centrifugation at 10,000*g* and 4°C for 10 min for further assays. Next expression at fermentor level was performed according to *Pichia Protocols* (Cregg 2007). Over night culture was inoculated into 200 mL fresh BMGY medium, and cultivated at 29°C and 250 rpm until $OD_{600\ nm}$ of 3–6 was reached. 200 mL seed culture was inoculated into a fermentor (BIOSTAT B plus, Satorius Stedim Biotech) containing 1.8 L basal salts medium (BSM) and 200 mL 45% (w/v) glucose. The pH of the medium was adjusted and controlled at 5.0 with the addition of ammonium hydroxide and phosphoric acid. The temperature was controlled at 29°C, and dissolved oxygen (DO) level was set at 35% by adjusting agitation rate. Methanol was supplied from 1 to 6 mL/Lh within first 6 h after the glucose was exhausted. Supernatant sample was taken out for cell wet weight, production of extracellular proteins and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Each sample (20 μL) was mixed with 4 μL 6× loading buffer and loaded to a 12% SDS–polyacrylamide gel. The proteins were visualized by Coomassie brilliant blue (CBB) R250 staining, and the percentage of target band in the lane was assayed by Electrophoresis band concentration determination program of Quantity One software (Bio-rad). The protein yield was evaluated by Bradford protein assay kit (TIANGEN Biotech, Beijing),

Purification of rBLfA, rBLfN and rBLf

The supernatant culture (100 mL) of the recombinant *P. pastoris* was ultrafiltrated with 20 mL binding buffer (50 mmol/L NaH_2PO_4, pH 8.0; 300 mmol/L NaCl; 10 mmol/L imidazole), and then loaded to Ni-NTA His-Bind resin (Merck KgaA EMD, USA) pre-equilibrated with binding buffer. After washing with binding buffer to baseline absorbance, the resin was eluted with four volumes of elute buffer (50 mmol/L NaH_2PO_4, pH 8.0; 300 mmol/L NaCl) containing 20, 60, 160, 300, 500, 1000 mmol/L imidazole, respectively, at a flow rate of 2 mL/min. All the fractions were collected and loaded to 12% SDS–PAGE.

Deglycosylation and Western blotting of rBLfA and rBLfN

Denatured 20 μg of rBLfA and rBLFN was separately incubated in glycoprotein denaturing buffer (0.5% SDS, 0.04 mol DTT) at 100°C for 10 min. About 1/10 volume of 10× G5 buffer (0.5 mol sodium citrate, pH 5.5) and 1 μL endoglycosidase H (Endo H) (10,000 units, New England Biolabs, USA) was added into the reaction system and incubated at 37°C for 1 h. Samples were incubated at 100°C for 10 min in nonreducing loading buffer and then analyzed by 12% SDS–PAGE and Western blotting detection (Mukhtar et al. 2001). Native BLf (nBLf) purchased from Nanjing Tianchun Trading Co. Ltd

was as an antigen. The first antibody was rabbit anti-nBLf polyclonal antibody prepared in Beijing Wanger Biotechnology Inc. Samples in SDS–polyacrylamide gel were electroblotted onto a PVDF (polyvinylidene fluoride) membrane with Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad). Then membrane was incubated by the first antibody rabbit anti-BLf polyclonal antibody (dilution rate 1:5000), and followed by the second antibody alkaline phosphatase goat anti-rabbit IgG (ZSGB-BIO) (dilution rate 1:8000). Western blotting results were visualized using BCIP/NBT alkaline phosphatase color development kit (TIANGEN Biotech, Beijing).

Iron-binding and pH dependent iron release

Purified rBLfA, rBLfN and rBLf (5 micrograms each protein per mL) separately dissolved in a mixture of 0.1 mol/L sodium citrate and 0.1 mol/L sodium bicarbonate, pH 8.0 (Dong and Zhang 2006), and were completely saturated by adding appropriate amounts of a reagent containing $FeCl_3 \cdot 6H_2O$ dissolved in the same buffer for 24 h at 4°C. Saturated recombinant proteins were prepared by ultrafiltration with 0.1 mol/L citrate–phosphate buffer, pH 8.0. The iron saturation was judged by the absorbance at 465 nm (Day et al. 1992). The pH dependent dissociation studies were performed by the iron-saturated rBLfA, rBLfN and rBLf equilibration in 0.1 mol/L citrate–phosphate buffer with different pHs for 24 h at 4°C. The free Fe^{3+} was removed by ultrafiltration. The values of absorbance at 465 nm of each sample were measured.

Antimicrobial activity assay

The antimicrobial activity against *Staphyloccus aureus* ATCC 25923 was tested. The test strains were grown to exponential phase at 37°C in Brain heart infusion (BHI) broth. Cultures were washed two times and suspended in sterile saline to a concentration of $\sim 10^7$ CFU/mL. Samples of 100 μL of washed cells were incubated at 37°C for 1 h with 200 μL apo-recombinant proteins at gradient concentration of 3.13, 6.25, 12.5, 25, 50 μmol/L. The reaction mixtures were serially diluted and plated in triplicate on BHI agar. Colony counts were determined after 24 h incubation at 37°C. Time-dependent antimicrobial activities against *S. aureus* investigation were also employed to estimate the biological activities of rBLfA, rBLfN and rBLf. *S. aureus* grown to the exponential phase were collected and resuspended in BHI medium. Then 100 μL cells suspension was added into 10 mL BHI medium with 5 mg/mL rBLfA, rBLfN, rBLf, nBLf and 2 μg/mL ampicillin (positive control), respectively. The mixtures were incubated at 37°C and 220 rpm. The growth of *S. aureus* was evaluated by the determination of the $OD_{600\ nm}$.

Results

Sequence analysis

The structure homology-modeling of the peptides BLfA, BLfN, and BLf showed that three proteins were different in structural features and interactions of inter-lobe region. The structure of independent BLfA was similar to that of amino acid residues 1–344 in the intact BLf and exhibited a higher cationic surface feature than BLfN, which might increase the antimicrobial activity through the extra interaction with pathogen negative charged cell surface. The rBLfA model was submitted to the PMDB database, and the PMDB identifier was PM0075890. The amino acid residues, molecular weight, pI, and instability index calculation results were showed in Table 2. The molecular weight of BLfA is 1305.5 Da higher than that of rBLfN, and it has the most net positive charges (+16) and highest theoretical p*I* (8.82) of the three recombinant proteins. The high p*I* and the notable concentration of positive charges could form its ability of binding cell surfaces of various cell types and anionic molecules (Baker and Baker 2009). BLfA, BLfN and BLf were predicted as unstable, but BLfA is more stable than BLfN and less stable than BLf. There were five potential glycosylation sites (Asn281, 233, 368, 476, 545) in rBLf, while the first two were located in rBLfA (Asn281, 233).

BLf cDNA Cloning and expression vectors construction

The BLf cDNA was amplified by RT-PCR from total RNA extracted from a sample of bovine mammary gland. It was cloned into pMD18-T and generated the plasmid pMDBLf. Sequence analysis revealed that

Table 2 Physical-chemical parameters and structural features of BLfA, BLfN and BLf

Peptide	Amino acid residues	Molecular weight (Dalton)	p*I*	Net positive charge	Instability index	Structural features
BLfA	1–344	41971.9	8.82	16	48.01	N-lobe, inter-lobe region
BLfN	1–333	40666.4	8.75	15	48.79	N-lobe
BLf	1–689	81126.2	8.48	15	41.58	N-lobe, inter-lobe region, C-lobe

the intact of BLf cDNA was 2067 bp, and it encoded 689 amino acid residues. The cDNA sequence was registered to GenBank, and the accession number is GQ351344.

PCR and sequencing results showed that BLfA and BLfN contained 1032 bp and 999 bp open reading frame, and encoded 344 and 333 amino acid residues, respectively. BLfA, BLfN, and BLf DNA fragments were constructed into expression vector pPICZα A at the *Eco*RI/*Xba*Isites (BLfA and BLfN) or *Sac*II/*Xba*Isites (BLf), respectively. Three recombinant expression vectors pPICBLfA, pPICBLfN and pPICBLf were linearized and transformed into *P. pastoris*. The transformant for each construct with the highest expression level was selected by secreted protein SDS–PAGE.

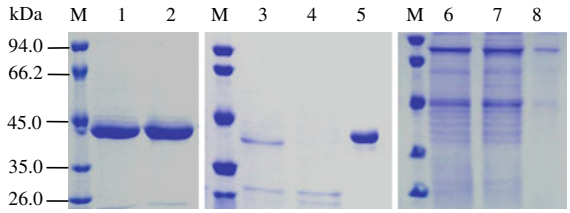

Fig. 1 Expression and purification of rBLfA, rBLfN and rBLf by *P. pastoris* at shaking flask level. *M* Protein marker (TIANGEN, Beijing); *1* rBLfA from supernatant of shaking flask culture after 24 h inducement by methanol; *2* purified rBLfA by Ni-NTA His-Bind resin; *3* rBLfN from supernatant of shaking flask culture after 24 h inducement by methanol; *4* supernatant of *P. pastoris* harboring pPICZα A; *5* purified rBLfN by His-Bind resin; *6* and *7* rBLf from supernatant of shaking flask culture after 24 h inducement by methanol; *8* purified rBLf by His-Bind Resin

Expression and purification of rBLfA, rBLfN, and rBLf

The highest yields of rBLfA, rBLfN, and rBLf in shaking flask culture were 485, 106 and 88 mg/L after induction for 48, 72 and 72 h, respectively. *P. pastoris* system was used to produce various bioactive Lf with yields of 1–100 mg/L (Choi et al. 2008; Wang et al. 2002; Chen et al. 2004; Dong and Zhang 2006; Chen et al. 2007; Paramasivam et al. 2002). The apparent molecular weight of expressed His-tagged-peptides rBLfA, rBLfN, and rBLf in SDS–PAGE were about 43.0, 41.5, and 83.0 kDa (Fig. 1), which were higher than the calculated size 41.972, 40.666, and 81.126 kDa, respectively. It might result from the glycosylation of the recombinant peptides. In purification, the background proteins were removed when the concentration of imidazle was 60 mmol/L. rBLfA and rBLfN were eluted at the imidazole concentration of 250 mmol/L, while rBLf was eluted at the concentration of 500 mmol/L. The purity of rBLfA, rBLfN and rBLf were 93.8, 94.5, and 91.2%, respectively (Fig. 1).

The fermentor level expression of rBLfA and rBLfN was also confirmed. The cell wet weight and total protein concentration exhibited an increasing tendency during the methanol induction phase. For rBLfA-derived *P. pastoris* fermentation, the maximum cell wet weight reached 336.9 g/L and the concentration of total proteins in culture supernatant was 998 mg/L. The maximum cell wet weight and total protein concentration of rBLfN-derived *P. pastoris* fermentation product was 304 g/L and 0.803 g/L, respectively. It was showed in SDS–PAGE that rBLfA and rBLfN both accounted for about 50% of the total protein in the medium (Fig. 2).

Deglycosylation analysis

After deglycosylation (Fig. 3), the apparent molecular weight of rBLfA and rBLfN was decreased from 43.0 and 41.5 kDa to 42.0 and 40.5 kDa which were close to the calculated molecular weight of His-tagged-peptides rBLfA (41.972 kDa) and rBLfN (40.666 kDa). As it was showed in Western Blotting results, the diffuse patterns did not exist after

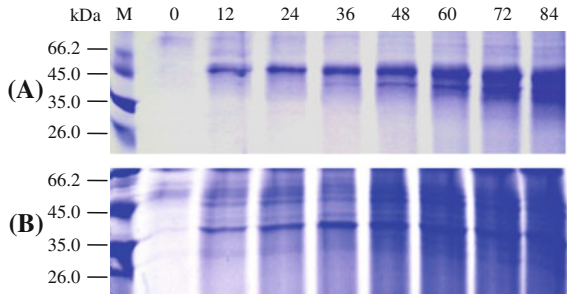

Fig. 2 Expression of rBLfA (**a**) and rBLfN (**b**) by *P. pastoris* at fermentor level. *M* Protein marker (TIANGEN, Beijing). The lane headings 12, 24, 36, 48, 60, 72 and 84 showed the induction time in hours

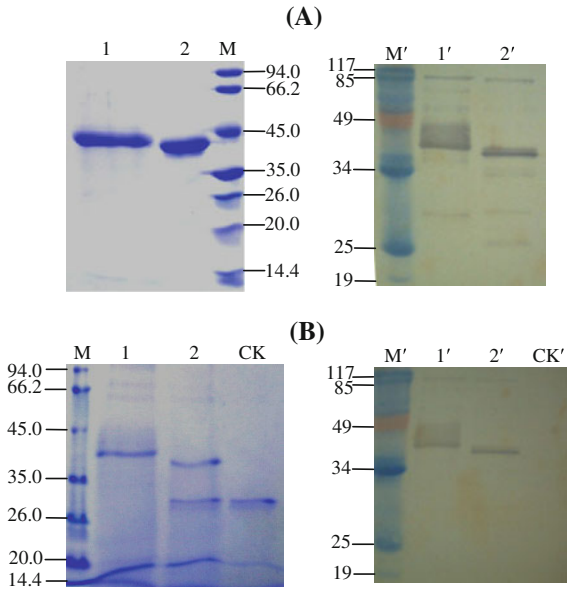

Fig. 3 SDS-PAGE and Western blotting analysis of rBLfA (**a**) and rBLfN (**b**) deglycosylation. *M* Protein Marker (kDa, TIANGEN, Beijing); *M′* Prestained protein marker (kDa, TIANGEN, Beijing); (**a**) *1* and *1′* rBLfA treated with Endo H; *2* and *2′* untreated rBLfA; *3* and *3′* deglycosylation reaction buffer; (**b**) *1* and *1′* rBLfN treated with Endo H; *2* and *2′* untreated rBLfN; *3* and *3′* negative control, double distilled water treated with Endo H

deglycosylation, demonstrating that the rBLfA and rBLfN were glycosylated by the *Pichia pastoris* cells. Bovine Lf has five potential N-glycosylation sites (Asn 233, Asn 281, Asn 368, Asn 476, and Asn 545), but only four sites (Asn 233, Asn 368, Asn 476, and Asn 545) are usually glycosylated (Baker and Baker 2009). There are two potential glycosylation sites at rBLfA (Asn 233, Asn 281). The glycosylated and unglycosylated forms of human Lf showed similar binding iron and identical affinities towards human lysozyme and bacterial lipopolysaccharide, but different susceptibilities towards tryptic proteolysis (van Berkel et al. 2002). Therefore, the degree of glycosylation might not influence the iron binding and antimicrobial activity of recombinant proteins.

Iron-binding and pH dependent iron release

The recombinant proteins were fully saturated in the iron saturation buffer until the stable $OD_{465\ nm}$ reached. The $OD_{465\ nm}$ of rBLfA, rBLfN, rBLf, and nBLf demonstrated that the three proteins were different in the degree of iron saturation after 24 h iron binding (Fig. 4, pH 8.0). And the results might reflect the iron binding ability difference among the three proteins, which was nBLf (0.482) > rBLf (0.431) > rBLfA (0.330) > rBLfN (0.322). The pH dependence of iron release from rBLfA, rBLf, rBLfN and nBLf was showed in Fig. 4. The pH dependence iron release from rBLfA occurred in the pH range of 7.0–3.5, whereas iron release from rBLfN ended at slightly higher pH (4.0). rBLf and nBLf released iron in the similar pH range (5.5–2.5) under the same conditions. These results demonstrated that rBLfA was more stable than rBLfN at low pH, but both rBLfA and rBLfN bound iron more weakly than rBLf and nBLf.

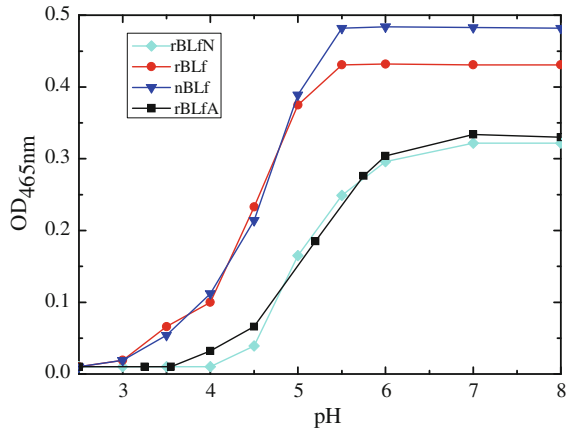

Fig. 4 The pH dependent iron release from rBLfA, rBLfN, rBLf and nBLf. Purified rBLfA, rBLfN, rBLf and nBLf (5 mg/mL, respectively) were dialyzed against Fe^{3+} release buffers at the pH values gradient for 24 h at 4°C, followed by the absorbance determination at 465 nm

Antimicrobial activity assay

The decrease in viability of *S. aureus* was showed after incubation with 6.25 μmol iron-free (apo) rBLfA, 12.5 μmol apo-rBLfN or 25 μmol apo-rBLf (Table 3). The three recombinant proteins did not exhibite antibacterial effects toward to the strain in the lower concentration. It was demonstrated that rBLfA had more efficient bactericidal effect to *S. aureus* than rBLfN and rBLf. Besides the MICs, time-dependent studies were performed to estimate the antimicrobial activity of rBLfA secreted from the *P. pastoris*. Figure 5 showed that the three recombinant proteins all had the inhibiting effects to the growth of *S. aureus* at the concentration of 5 mg/mL. rBLfA was weaker inhibitor than the rBLfN. There was a more significant growth inhibiting effects of rBLfA than that of rBLf, indicating that rBLfA was expressed by *P. pastoris* with the higher antimicrobial activity against *S. aureus.*

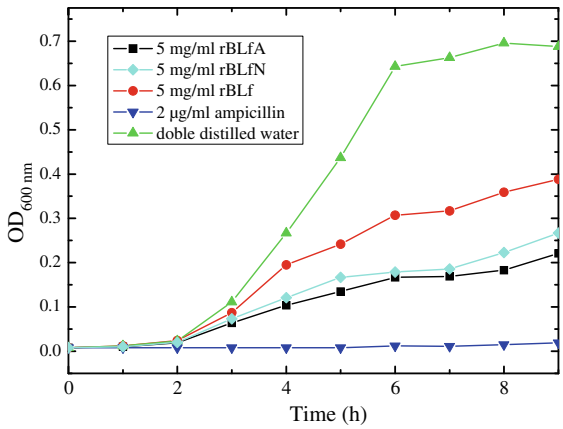

Fig. 5 Time-dependant anti-*S. aureus* activities comparison of rBLfA, rBLfN, and rBLf. *S. aureus* cells in exponential phase were inoculated in BHI medium containing purified rBLfA, rBLfN, and rBLf (5 mg/mL, respectively), at 37°C. The growth of *S. aureus* was analyzed by the determination of $OD_{600\ nm}$

Discussion

Both nBLf and rBLf release iron over the pH range of 5.5–3.0 (Fig. 4), slight higher than pH 4.0–2.5 of human Lf (Baker et al. 2002). It revealed that BLf binds iron more weakly than human Lf, and it is similar to previous investigation (Peterson et al. 2000). The pH dependence of iron retention by rBLfA is different from that of rBLfN, rBLf, and nBLf (Fig. 4). The ability of iron binding tightly and reversibly from nBLf and rBLf (Fig. 4) is the intrinsic among the transferrin family (Sandrini et al. 2010). rBLfA and rBLfN tend to release iron than bind iron at higher pH, compared with intact molecules as nBLf and rBLf (Fig. 4). These differences may result from their structural variations. Firstly, like the human Lf (Baker et al. 1994), iron release from nBLf and rBLf is considered biphasic; however, acid instability of iron binding in N-lobe (rBLfN) is higher than that of C-lobe. Secondly, the cooperative non-covalent interactions from C-lobe of intact molecule might enhance iron binding of N-lobe in acid condition (Legrand et al. 1990). Finally, a salt bridge between Arg341 within the helical inter-lobe linker and Asp390 in C-lobe could create and strengthen the interaction between the two lobes (Wally and Buchanan 2007). In addition, acid stability of rBLfA binding iron is higher than rBLfN, therefore we consider that this inter-lobe region can also stabilize iron binding structure of N-lobe even though without C-lobe, and the rigidity of the

Table 3 Viability (CFU/mL) of *Staphylococcus aureus* ATCC 25923 after incubation with varying concentrations of iron-free rBLfA, rBLfN, and rBLf for 1 h at 37°C

Sample	Treatment concentration (μmol/L)					
	0	3.13	6.25	12.5	25	50
Apo rBLfA	5.7×10^7	7.5×10^7	6.8×10^6	7.1×10^5	4.5×10^4	2.2×10^4
Apo rBLfN		2.7×10^8	5.6×10^7	2.0×10^6	1.3×10^5	6.7×10^4
Apo rBLf		1.3×10^8	5.8×10^7	9.6×10^7	2.0×10^7	6.7×10^6

Viable counts (CFU/mL) were determined by plating serial dilutions on MHB agar

Initial CFU/mL was 1.0×10^7

Control: Bacteria were incubated in saline for 1 h at 37°C

α-helical linker in inter-lobe region in intact LF molecule might be increased from cooperative non-covalent interaction and the salt bridge.

BLfA has the lower iron binding ability and the higher antimicrobial activity against *S. aureus* than rBLf (Table 3). We deduce that its ability to sequester essential iron is not determinant for the bactericidal activity. BLfA has more surface net positive charges and higher calculated pI than BLfN and BLf. The distribution of the BLf surface positive charges is highly uneven, and the inter-lobe region is one of the three regions of BLf with notable concentration of positive charges (Baker and Baker 2009; Moore et al. 1997). In N-lobe, the first two regions are N-terminus (residues 1–7) and locate along the outside of the first helix. A strong cationic N-lobe contributed into its ability to bind many different cell types and anionic molecules (Valenti and Antonini 2005), whereas the inter-lobe region's contribution to that has not been reported yet. In this work, rBLfA's higher antimicrobial activity than rBLfN is for the first time considered resulting from a direct interaction between the extra positive charges of inter-lobe region and anionic molecules on *S. aureus* surfaces. On the other hand, rBLf has the lowest anti-*S. aureus* activity among the three recombinant proteins, and this might because that its positive charge regions was folded into the internal part of the molecule or balanced by the negative region in C-lobe (Sharma et al. 2003). It appeared that α-helical linker of BLf inter-lobe region may play an important role in binding the anionic molecules on cell surface to establish its antimicrobial activity.

According to the instability index (Table 2), BLfA, BLfN and BLf were shown to be unstable, but BLfA was more stable than BLfN. The higher stability of BLfA might result from the inter-lobe interaction. In addition, BLf is more stable than BLfA probably due to the existence of the C-lobe, and the interactions between two lobes would increase this stability (Day et al. 1992).

In conclusion, the inter-lobe region is one of the characteristic structures that distinguish lactoferrin from transferrin (Wally and Buchanan 2007). Our work proves that the strong cationic character of inter-lobe region related positively to the higher anti-*S. aureus* activity. The rigidity of the inter-lobe region may independently contribute to iron binding structure of N-lobe with higher stability. This stability might be enhanced by a synergistic effect between inter-lobe region and C-lobe, therefore its detail should be focused in next work.

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