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In vitro reconstitution of antimicrobial pathogen activity by expressed recombinant bovine lactoferrin N-terminal peptide in *Escherichia coli*

Hongxia Luo[1,2], Shangwu Chen[1], Fazheng Ren[1,*], Huiyuan Guo[1], Shaohua Lin[1] and Wentao Xu[1]

[1] Key Lab of Functional Dairy Science of Beijing and Ministry of Education of China, College of Food Science and Nutritional Engineering, China Agricultural University, Beijing, China 100083
[2] Beijing Vocational College of Agriculture, Beijing, China 102442

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Recombinant bovine lactoferrin N-terminal polypeptide (rbLF-N) *Escherichia coli* expression system was constructed and the rbLF-N antimicrobial activity was displayed by enzymatic proteolysis in this study. A 162 bp 5′-terminal fragment of bovine lactoferrin (bLF) gene from bovine liver gDNA was amplified by PCR. The DNA fragment containing exon-2 of the bLF gene was cloned into the expression vector pGEX-4T1 and the glutathione-S-transferase–rbLF-N (GST-rbLF-N) fusion protein was obtained by over-expression in *Esch. coli* BL21(DE3). After thrombin/pepsin digestion, the rbLF-N was released from the fusion protein. The recombinant peptide was separated and identified by SDS-PAGE, HPLC and LC-MS/MS analysis. A very strong anti-food-born microbial pathogen activity of the rbLF-N peptides was displayed through bio- and kinetic-assays *in vitro*. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the rbLF-N peptide for bacterial pathogens *Staphylococcus aureus, Streptococcus mutans, Esch. coli* and *Klebsiella pneumoniae* were 11·7, 11·7, 11·7, 23·4 μg and 23·4, 11·7, 11·7, 46·4 μg, respectively. This study created a new route for exploring lactoferrin peptide application in food science.

Keywords: Lactoferrin, anti-bacterial pathogen peptide, rbLF-N, *Escherichia coli*.

Milk has been shown to contain numerous biologically active substances. Lactoferrin (LF) and its polypeptides are of special interest. LF is an iron-binding glycoprotein with a molecular mass of about 80 kDa (Pierce et al. 1991). LF is especially abundant in bovine and human milk. LF is also widely present in the milk, body fluid, tears, sperm and saliva of many other animals. It can be found in secondary lysosomes from blood and neutrophils as well. LF was first isolated from bovine and human milk. The content of LF in milk varies substantially among human, cow, goat, pig, mouse, rat, rabbit, dog and guinea pig. Previous studies attributed many unique biological functions to LF, including iron-intake enhancement (Mikogami, 1995), antimicrobial (Samaranayake et al. 1997), antivirus (Dapsanse et al. 2001), antioxidant (Zullo et al. 2005), anti-inflammation and anticancer (Hayes et al. 2006). Other biological functions include roles in regulation of the body immune system (Lee et al. 1998), involvement in immune regulation, and growth promotion of osteoblast and skeleton (Naot et al. 2005). Among all its biological functions, the antimicrobial activity has been extensively investigated (Farnaud & Evans, 2003).

N-terminus of LF is the active region responsible for its biological functions. Lactoferricin (LFcin) is a fragment of polypeptide released from the N-terminus of LF after peptic cleavage at acidic condition (Tomita et al. 1991). These were initially found in bovine and human milk, and homologous LFcin was then proved to exist in LF from rat, pig and goat milk (Bellamy et al. 1992a). Bovine lactoferricin (bLFcin) contains 25 amino acids corresponding to residues 17 to 41 of bLF and human lactoferricin (hLFcin) corresponds to residues 1 to 47 of human lactoferrin (hLF) (Bellamy et al. 1992a) while caprine lactoferricin corresponds to residues 14 to 42 of goat LF (Recio & Visser, 1999). LFcin has potent antimicrobial activities against a

*For correspondence; e-mail: Renfazheng@263.net

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wide range of microorganisms including Gram-positive and Gram-negative bacteria as well as filamentous fungi. The antimicrobial activity of bLFcin is much stronger than that of LF (Farnaud & Evans, 2003). Genes encoding LF from different sources including human, pig, bovine, horse and mouse have been cloned and expressed in many hosts. Especially, hLF has been expressed in hosts such as mammals (Platenburg et al. 1994), rice (Nandi et al. 2005), potato (Chong & Langridge, 2000) and insect cells with virus (Liu et al. 2005). These studies provide useful data for applied research. However, the recombinant expression system with N-terminal polypeptide (corresponded to exon-2 of bLF gene) derived from bLF has not been established. In this study, the recombinant expression system was established with N-terminus of bLF and its antimicrobial activity was determined.

Materials and Methods

Animal materials and extraction of genomic DNA from bovine liver

Fresh liver was obtained from single tissues of *Bos taurus domesticus Gmelin* (a 4-year, bull). Genomic DNA (gDNA) was extracted from fresh bovine liver by applying the modified method of Sambrook et al. (2001).

Bacterial strains

Escherichia coli C84010, *Staphylococcus aureus* 1844, *Klebsiella pneumoniae and Streptococcus mutans* were purchased from Chinese Academy of Sciences (Beijing, China).

Primer design, synthesis and PCR amplification

Oligonucleotide primers were designed based on the cDNA sequence of bovine lactoferrin deposited in Genebank (Accession number: NM_180998, and L19982). The primer were 5′-GGATCCTGTC TGGCTGCCCC GAGG-3′ (forward) and 5′-CCGCGAATTC CCGGATACAT GCCAA-3′ (backward) with the addition of *Bam*H. and *Eco*R. restriction sites boxed. The bLF-N sequence fuses into the glutathione S-transferase (GST) C-terminal through the *Bam*H.site, just behind the thrombin site. PCR amplification was performed with gDNA as template using PCR kit (MJ Research Inc., BOSTON, USA). The PCR mixture consisted of 250 ng DNA, 0·5 μM each primer, 100 μm-dNTP, 2·5 μl 10 × PCR buffer, 2·5 U Taq DNA polymerase and ddH_2O was added to 25 μl total volume. The mixture was heated at 95 °C for 4 min and then subjected to 35 cycles of amplification (94 °C, 30 s; 55 °C, 60 s; 72 °C, 30 s) followed by a final extension at 72 °C for 10 min. The PCR products was sequenced by Shanghai Sangon Co. Ltd (Shanghai, China) to confirm the right gene sequence with 162 bp in length compared with the sequence deposited in GeneBank.

Cloning of recombinant plasmids and construction of expression vector

The amplified fragment was gel-purified, sub-cloned into a pGEM-T easy vector (Promega Corporation, USA) and transformed into *Esch. coli* TOP10. Selected clones were cultured and plasmids were prepared. The plasmids were digested with *Bam*H I and *Eco*R I (New England BioLabs, Beijing Ltd.), and cloned into a pGEX-4T1 (Amersham Biosciences) expression vector cut with the same enzymes. The ligation reaction (mixture of 1 ng target fragment, 50 ng pGEX-4T1, 10 U T_4 ligase, 1 μl 10 × buffer and ddH_2O to final 10 μl) was carried out at 16 °C for 6 h. The ligation product transformed *Esch. coli* BL21(DE3) was spread on Luria-Bertani (LB) agar plate supplemented with 100 mg ampicillin/l and a single clone was selected, cultured overnight, and then the plasmids were prepared for gel detection.

Heterologous expression of rbLF-N in Esch. coli *and its purification*

The host strain containing recombinant plasmid was grown overnight at 37 °C in LB medium (containing 100 mg ampicillin/l). The expression of fusion protein was induced by adding 0·2 mM-IPTG (isopropyl-β-D-thiogalactopyanoside) into the culture when optical density ($OD_{600\ nm}$) reached 0·6–0·8, and cells were grown at 37 °C for another 3 h with vigorous shaking. Cells were harvested by centrifugation at 2,000 ***g*** for 6 min, suspended in PBS buffer and sonicated, collecting the inclusion body pellets by centrifugation at 15,000 ***g***, 20 min at 4 °C. The inclusion body pellets were solubilized by 8 M-urea with 20 mM-DTT, the supernatant of 15,000 ***g*** was dialyzed against PBS at 4 °C for 48 h. The supernatant was loaded on to a glutathione S-Sepharose 4B affinity chromatography column (Amersham Bioscience) equilibrated with PBS to purify the glutathione S-transferase (GST) fusion protein. Fusion Protein eluted with 50 mM-Tris-HCl buffer (pH 8·0) containing 5 mM-glutathione was diluted to 5% (w/v) with water, adjusted to pH 2·5 and then digested with 0·15% (w/v) porcine pepsin (Ameresco, USA) at 37 °C for 4 h (Tomita et al. 1991). The digestion was terminated by heating at 80 °C for 10 min, centrifuging at 6000 ***g*** for 20 min, and the solution was adjusted to pH 7·0 for further application and identification.

Identification of fusion protein and rbLF-N

A 12% SDS-PAGE system was hired to analysis the rbLF-N protein fusion product (Laemmli, 1970). Coomassie brilliant Blue and Silver staining was performed according to Oakley et al. (1980). Recombinant fusion protein was digested by thrombin, and the product (rbLF-N) was purified by 10 KDa molecular weight cut membrane filtration (Millipore, USA). The protein concentration was determined by the Bradford protein assay method using bovine

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NM_180998,mRNA  ATGAAGCTCTTCGTCCCCGCCCTGCTGTCCCTTGGAGCCCTTGGACTGTG   50
   L19982,mRNA  ATGAAGCTCTTCGTCCCCGCCCTGCTGTCCCTTGGAGCCCTTGGACTGTG
    bLF Exon-2  ----------------------------------------GGACTGTG
     bLF-N,PCR  ----------------------------------------GGATCCTG

NM_180998,mRNA  TCTGGCTGCCCCGAGGAAAAACGTTCGATGGTGTACCATCTCCCAACCTG   100
   L19982,mRNA  TCTGGCTGCCCCGAGGAAAAACGTTCGATGGTGTACCATCTCCCAACCCG
    bLF Exon-2  TCTGGCTGCCCCGAGGAAAAACGTTCGATGGTGTACCATCTCCCAACCCG
    rbLF-N,PCR  TCTGGCTGCCCCGAGGAAAAACGTTCGATGGTGTACCATCTCCCAACCCG

NM_180998,mRNA  AGTGGTTCAAATGCCGCCGATGGCAGTGGAGGATGAAGAAGCTGGGTGCT   150
   L19982,mRNA  AGTGGTTCAAATGCCGCCGATGGCAGTGGAGGATGAAGAAGCTGGGTGCT
    bLF Exon-2  AGTGGTTCAAATGCCGCCGATGGCAGTGGAGGATGAAGAAGCTGGGTGCT
    rbLF-N,PCR  AGTGGTTCAAATGCCGCCGATGGCAGTGGAGGATGAAGAAGCTGGGTGCT

NM_180998,mRNA  CCCTCTATCACCTGTGTGAGGAGGGCCTTTGCCTTGGAATGTATCCGGGC   200
   L19982,mRNA  CCCTCTATCACCTGTGTGAGGAGGGCCTTTGCCTTGGAATGTATCCGGGC
    bLF Exon-2  CCCTCTATCACCTGTGTGAGGAGGGCCTTTGCCTTGGCATGTATCCGGGC
    rbLF-N,PCR  CCCTCTATCACCTGTGTGAGGAGGGCCTTTGCCTTGGCATGTATCCGGGA

NM_180998,mRNA  CATCGCGGAGAAAAAGGCGGATGCTGTGACCCTGGATGGTGGCATGGTGT...250
   L19982,mRNA  CATCGCGGAGAAAAAGGCGGATGCTGTGACCCTGGATGGTGGCATGGTGT......
    bLF Exon-2  CATCGCG
    rbLF-N,PCR  ATTC
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Fig. 1. The alignment of the PCR amplified rbLF-N fragment and partial bLF sequences from Genebank. The rbLF-N was cloned from bovine gDNA by PCR with the primers given in the text. The alignment showed the rbLF-N sequence correspondeing to that of bLF exon-2 (bLF exon-2, Genbank NM_180998 and L19982), with 1 base difference, except there were 3 bases different at both ends for fit into the vector.

serum albumin as the standard (Bradford, 1976). The molecular weight distribution of rbLF-N and its porcine pepsin digestion were analysed by HPLC (Waters 1515 system, Milford, MA, USA) with YMC-pack Diol-60 gel filtration column (5μm, 8 × 300 mm, Japan). The pepsin digested rbLF-N, after Sephadex G-10 column (Pharmacia Corp., USA) desalting was applied for LC-MS/MS identification. The peptide sequence of rbLF-N peptide was analysed on Finnigan LCQ™ Deca XP MA**X** LC/MS^n (Thermo Electron Corporation, Waltham, MA) with an ODS column (3 μm, 0·175 × 4 cm) and an Electro Spray Ionization source (Delahunty & Yetes, 2005). The column was washed with buffer A (0·1% formic acid in water) for 10 min, and then with linear gradient of buffer B (0·1% formic acid in acetonitrile) from 10 to 90% over a period of 160 min with total mobile phase flow rate 200 nl/min. The mass-to-charge ratio (m/e) of the peptide data were analysed by Bioworks™/Turbo SEQUEST™ (Thermo Electron, San Jose, CA).

Antimicrobial activity assay

Esch. coli C84010, *Staph. aureus* 1844, *Kleb. pneumoniae* and *Str. mutans* were used as target pathogenic microorganisms in the antimicrobial activity assays of rbLF-N. MIC and MBC methods as described by Vorland et al. (1999) and Groenink et al. (1999).

Results and Discussion

Cloning of the bLF gene target fragment and recombinant plasmids, and construction of expression vector

The sequence authenticity of the pGEM-T easy-bLF-exon 2 subclone (pGEM-T/rbLF-N) was verified at this step (bLF-N, PCR; Fig. 1). DNA sequencing of the fragment was in accordance with the deposited genes in Genebank (Accession Number NM_180998, and L19982). The 162 bp DNA fragment (bLF exon-2 gene) corresponded to a 54 amino acid residual peptide. After sequence analysis, the target DNA fragment was excised from the pGEM-T/rbLF-N with *Bam*H. and *Eco*R. and cloned to pGEX-4T1 expression vector digested with the same enzymes. The generated recombinant pGEX-4T1/rbLF-N was verified by enzyme digestion and gel electrophoresis (Fig. 2).

Recombinant plasmids are expressed and purified

Recombinant pGEX-4T1/rbLF-N was introduced into *Esch. coli* BL21(DE3) and the host strain containing the verified recombinant plasmid was induced with IPTG. A specific new protein band was observed at 33·9 kD on the Urea/SDS PAGE (Fig. 3). The fusion protein was up to 20·4% in total proteins, and the approximate yield of the fusion protein was 1·68 g/l. In this study, pGEX-4T1 was used as the expression vector and the recombinant

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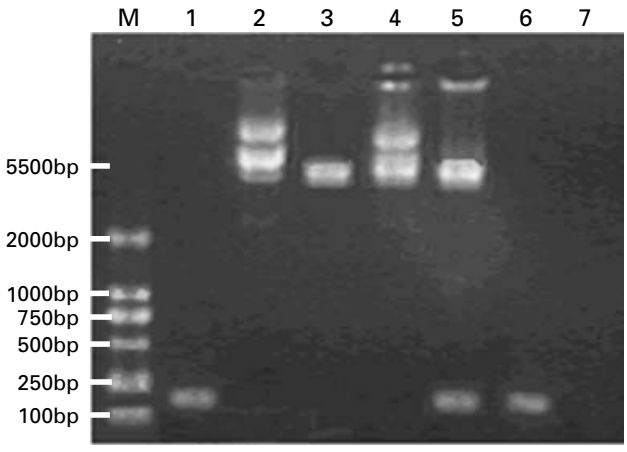

Fig. 2. The bLF-N PCR fragment and pGEX-4T1/rbLF-N recombinant expressing plasmid. lane M, DNA ladder; lane 1, bLF-N Amplified fragment from gDNA; lane 2, pGEX-4T1 vector; lane 3, linear pGEX-4T1 vector digested by *Bam*H and *Eco*R; lane 4, pGEX-4T1/rbLF-N recombinant; lane 5, linear pGEX-4T1/rbLF-N digested by *Bam*H and *Eco*R; lane 6, rbLF-N PCR product with pGEX-4T1/rbLF-N as template.lane 7, negative control.

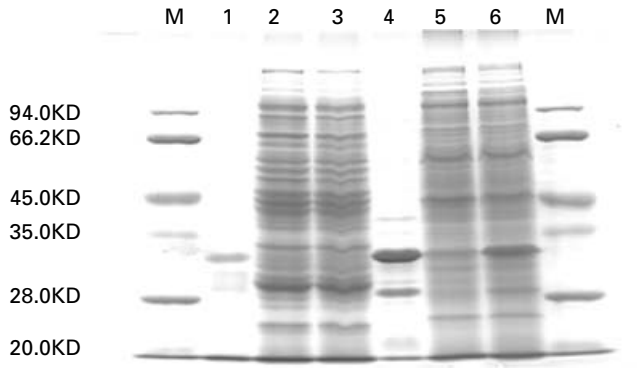

Fig. 3. Heterologous expression of the GST-rbLF-N fusion protein in *Esch. coli* BL21(DE3). Lane M, Molecular standard markers; lane 1, purified fusion protein by affinity chromatography column; lane 2, supernatant from *Esch. coli* BL21(DE3) harboring empty pGEX-4T1 after centrifugation at 15,000 ***g***; lane 3, supernatant from *Esch. coli* BL21(DE3) harboring empty pGEX-4T1 after centrifugation at 2000 ***g***; lane 4, pellet from IPTG-induced *Esch. coli* BL21(DE3) harboring pGEX-4T1/rbLF-N after centrifugation at 15,000 ***g***; lane 5, supernatant from no induced *Esch. coli* BL21(DE3) harboring pGEX-4T1/rbLF-N after centrifugation at 2000 ***g***; lane 6, supernatant from IPTG-induced *Esch. coli* BL21(DE3) harboring pGEX-4T1/rbLF-N after centrifugation at 2000 ***g***.

pGEX-4T1/rbLF-N was successfully expressed in *Esch. coli* BL21(DE3). The fusion protein was tested for proteolytic digestion. The GST-rbLF-N fusion protein was cleaved by thrombin to release the rbLF-N. The separated rbLF-N was digested by pepsin to release the native part of the bLFcin. The purity of rbLF-N was 93·23%.

Further purified fusion protein from the inclusion body was achieved by surfactant treatment solubilisation, affinity chromatography isolation, enzyme digestion and molecular cut-off membrane separation. The pepsin-digested rbLF-N peptides were successfully identified on LCQ^{TM} Deca XP MAX LC/MS^{n} mass spectrometer (Fig. 4). GST expressed by the blank vector pGEX-4T1 vector was clearly identified. The purified rbLF-N and its peptide fragments after pepsin digestion were also identified to be correct (Fig. 4). The identified peptide fragments were: (L)AAPRK, KKLGAPSI(T), and TCVRRAF(A). The results confirmed the correct ORF in the pGEX-4T1/rbLF-N expression vector and the recombinant peptide rbLF-N containing native bLFcin peptides derived from pepsin-digested bLF.

Antimicrobial activity reconstitution

The results showed that the purified rbLF-N fusion protein displayed antimicrobial activity only after peptic cleavage while no antimicrobial activity was detected with that of untreated (no pepsin hydrolysis) inclusion body and fusion protein. The MIC and MBC of rbLF-N towards different bacterial pathogens *Staph. aureus*, *Str. mutans*, *Esch. coli* and *Kleb. pneumoniae* were 11·7, 11·7, 11·7, 23·4 μg and 23·4, 11·7, 11·7, 46·4 μg, respectively.

Cloning and expression of LF have been well documented. As reported (Chong & Langridge, 2000; Liu et al. 2005; Nandi et al. 2005), hLF has been expressed in *rice*, *Saccharomyces cerevisiae*, *Aspergillus nidulans*, tobacco, insect and potato. The full length hLF was also expressed in galactophore bioreactor from mouse and bovine. However, few reports were presented with bLF and its N-terminal polypeptide which is the active region for biological function. Our data suggest that bLFcin have been separated from the N-terminal of LF generated in rbLF-N in this study. Theoretically, bLFcin and hLFcin can be cleaved from the N-terminal of LF by enzymatic digestion of their native protein. bLFcin and hLFcin share one similar peptide motif believed to be responsible for their antimicrobial activity. Activity of bLFcin with two tryptophan residues was higher than that of hLFcin with only one tryptophan residue (Schibli et al. 1999). Fusion expression of N-terminal polypeptide derived from bLF lay foundation for further study of its structure and function. Most of the LF antimicrobial and antifungal pathogen activity studies used synthetic peptides to study the structure and function of the peptides. Results confirmed that the recombinant N-terminal peptide of bLF could be expressed as fusion protein and provided a new route to produce the native antimicrobial peptide with activities. Many studies attributed the biological functions to LF and LFcin, including enhancement of iron-transfer and absorption, anti-inflammation, antioxidant, antiviral, strengthening the immune system, balance of enteric microflora, and most importantly their broad antimicrobial activity (Shimazaki,

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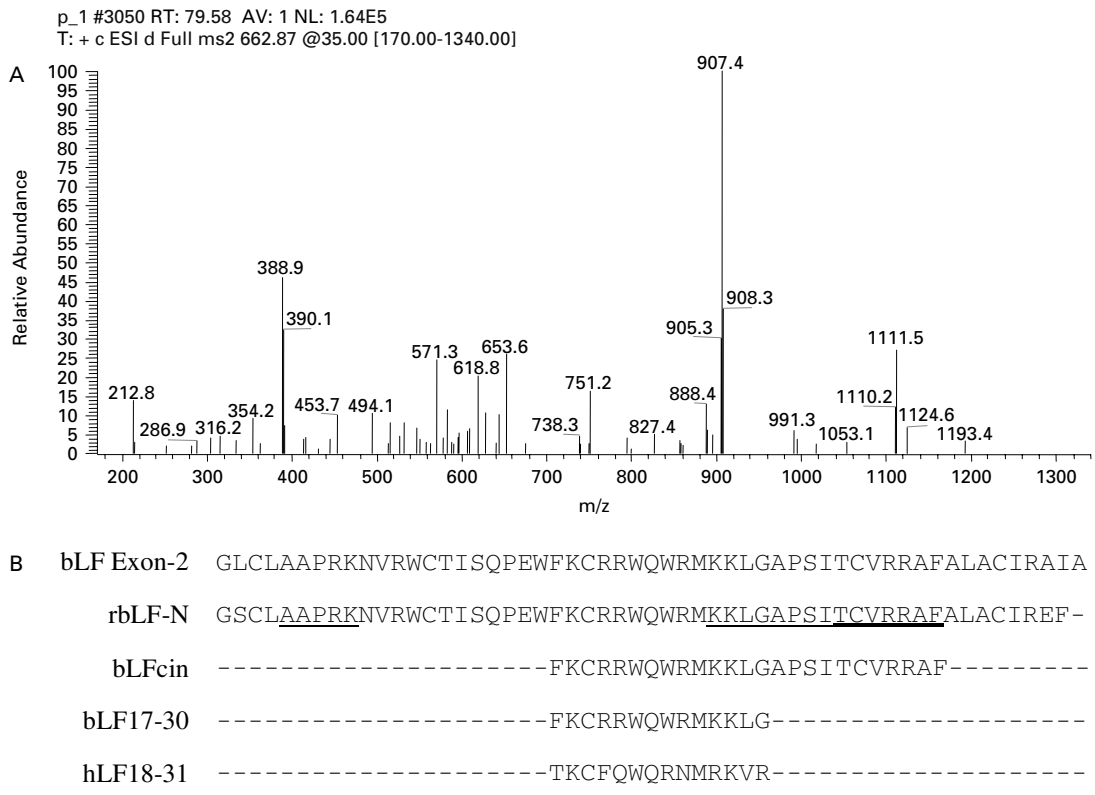

Fig. 4. Identification of the expressed products and peptide sequence analysis by LC-MS/MS. The isolation and purification of rbLF-N peptide was as described in the text. The de novo peptide sequence analysis from data of MS^2 M/+662·57 peptide fragment (r_t: 79·55 min) was performed on Bioworks™/TurboSEQUEST™ Station. A: the profile of MS^2 of M/+662·57 peptide fragment (r_t: 79·55 min); B: The alignment of different LF N-terminal peptide sequences and identified peptide fragments in the rbLF-N. The underlined sequences (L)AAPRK, KKLGAPSI(T), and TCVRRAF(A) were identified peptides.

1989). Small polypeptides from pepsin-digested bLF displayed antimicrobial activity against Gram-positive and Gram-negative bacteria and their activity was stronger than that of LF (Tomita et al. 1991, 2002). Antimicrobial activities of bLFcin and hLFcin derived from the N-terminal of bovine and human LF were higher than that of native LF. Antimicrobial activity of bLFcin was stronger than that of hLFcin (Bellamy et al. 1992b). The results of MIC and MBC showed that the purified fusion protein demonstrated antimicrobial activity after pepsin digest while no antimicrobial activity was observed without pepsin digestion. The activity may arise from a small peptide generated by the digestion. Bovine lactoferricin is proposed to exert its effect at the surface of the bacterial membrane (Bellamy et al. 1993) and positive charges within the peptide are thought to aid interaction with membrane components. The greater the number of positive changes, the greater the number of interactions with negatively charged membrane components (Nikaido & Vaara, 1985). The cationic small peptide is capable of binding anionic lipopolysaccharides (LPS) found in the cell membrane of Gram-negative bacteria, alter the permeability of the cell membrane causing the release of LPS which disrupt the physiological function of the cell membrane and lead to death of bacteria (Schröder et al. 1992). The sensitivity of Gram-positive and Gram-negative bacteria varied with Gram-negative being relatively more sensitive. This may be due to variations in composition of LPS, and structure and amount of phospholipids between the two bacteria (Rana et al. 1991). bLFcin and hLFcin share one similar region (Tomita et al. 1991). Antimicrobial activity of synthetic bLF peptide 17–30 was tested with different indicator strains, MIC and MBC towards *Staph. aureus*, *Str. mutans*, *Esch. coli* and *Kleb. pneumoniae* were 75, 37, 37, 75 μg/ml and 150, 37, 37, 150 μg/ml, respectively, and hLF peptide 18–31 with MIC and MBC of 300 μg/ml (Groenink et al. 1999). MIC of bLFcin (17–41) and hLFcin (derived from pepsin digested bLF and hLF, respectively) towards *Esch. coli* ATCC 25922 were 30 and 100 μg/ml (Samuelsen et al. 2004).

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References

Bellamy W, Takase M, Wakabayashi H, Kawase K & Tomita M 1992a Antibacterial spectrum of lactoferricin B, a potent bactericidal peptide derived from the N-terminal region of bovine lactoferrin. *The Journal of Applied Microbiology* **73** 472–479

Bellamy W, Takase M, Yamauchi K, Wakabayashi H, Kawase K & Tomita M 1992b Identification of the bactericidal domain of lactoferrin. *Biochimica et Biophysica Acta* **1121** 130–136

Bellamy W, Wakabayashi H, Takase M, Shimamura S & Tomita M 1993 Role of cell-binding in the antibacterial mechanism of lactoferricin B. *Journal Applied Microbiology* **75** 478–484

Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72** 248–254

Chong DK & Langridge WH 2000 Expression of full-length bioactive antimicrobial human lactoferrin in potato plants. *Transgenic Research* **9** 71–78

Dapsanse V, Defer MC, Follezou JY, Dugas B, Postaire E, Picard O & Damais C 2001 Differential pattern in circulating nitrogen derivatives, lactoferrin, and anti-lactoferrin antibodies in HIV type 1 and HIV type 2 infections. *AIDS Research and Human Retroviruses* **17** 1041–1045

Delahunty C & Yates JR 2005 Protein identification using 2D-LC-MS/MS. *Methods* **35** 248–255

Farnaud S & Evans RW 2003 Lactoferrin–a multifunctional protein with antimicrobial properties. *Molecular Immunology* **40** 395–405

Groenink J, Walgreen-Weterings E, van't Hof W, Veerman EC & Nieuw Amerongen AV 1999 Cationic amphipathic peptides, derived from bovine and human lactoferrins, with antimicrobial activity against oral pathogens. *FEMS Microbiology Letters* **179** 217–222

Hayes TG, Falchook GF, Varadhachary GR, Smith DP, Davis LD, Dhingra HM, Hayes BP & Varadhachary A 2006 Phase I trial of oral talactoferrin alfa in refractory solid tumors. *Investigational New Drugs* **24**(3): 233–240

Laemmli UV 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** 680–685

Lee WJ, Farmer JL, Hilty M & Kim YB 1998 The protective effects of lactoferrin feeding against endotoxin lethal shock in germfree piglets. *Infection and Immunity* **66** 1421–1426

Liu T, Zhang YZ & Wu XF 2005 High level expression of functionally active human lactoferrin in silkworm larvae. *Journal of Biotechnology* **118** 246–256

Mikogami T, Marianne T & Spik G 1995 Effect of intracellular iron depletion by picolinic acid on expression of the lactoferrin receptor in the human colon carcinoma cell subclone. HT 29-18-C1. *The Biochemical Journal* **308** (Pt 2) 391–397

Nandi S, Yalda D, Lu S, Nikolov Z, Misaki R, Fujiyama K & Huang N 2005 Process development and economic evaluation of recombinant human lactoferrin expressed in rice grain. *Transgenic Research* **14** 237–249

Naot D, Grey A, Reid IR & Cornish J 2005 Lactoferrin–a novel bone growth factor. *Clinical Medicine & Research* **3**(2): 93–101

Nikaido H & Vaara M 1985 Molecular basis of bactericidal outer mambrane permeability. *Microbiology Reviews* **49** 1–32

Oakley BR, Kirsch DR & Morris NR 1980 A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Analytical Biochemistry* **105** 361–363

Pierce A, Colavizza D, Benaissa M, Maes P, Tartar A, Montreuil J & Spik G 1991 Molecular cloning and sequence analysis of bovine lactotransferrin. *European Journal of Biochemistry* **196** 177–184

Platenburg GJ, Kootwijk EP, Kooiman PM, Woloshuk SL, Nuijens JH, Krimpenfort PJ, Pieper FR, de Boer HA & Strijker R 1994 Expression of human lactoferrin in milk of transgenic mice. *Transgenic Research* **3** 99–108

Rana FR, Macias EA, Sultany CM, Modzrakowski MC & Blazyk J 1991 Interactions between magainin 2 and Salmonella typhimurium outer membranes: effect of lipopolysaccharide structure. *Biochemistry* **130** 5858–5866

Recio I & Visser S 1999 Two ion-exchange chromatographic methods for the isolation of antibacterial peptides from lactoferrin. In situ enzymatic hydrolysis on an ion-exchange membrane. *Journal of Chromatography A* **831** 191–201

Samaranayake YH, Samaranayake LP, Wu PC & So M 1997 The antifungal effect of lactoferrin and lysozyme on Candida krusei and Candida albicans. *Acta Pathologica Microbiologica Immunologica Scandinavica* **105** 875–883

Sambrook J, Russell DW, Irwin N & Janssen KA 2001 *Molecular cloning: Alaborary mannual*. 3rd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NewYork, USA

Samuelsen O, Haukland HH, Ulvatne H & Vorland LH 2004 Anti-complement effects of lactoferrin-derived peptides. *FEMS Immunology and Medical Microbiology* **41** 141–148

Schibli DJ, Hwang PM & Vogel HJ 1999 The structure of the antimicrobial active center of lactoferricin B bound to sodium dodecyl sulfate micelles. *FEBS Letters* **446** 213–217

Schröder G, Brandenburg K & Seydel U 1992 Polymyxin B induces transient permeability fluctuations in asymmetric planar lipopolysaccharide/phospholipid bilayers. *Biochemistry* **31** 631–638

Shimazaki K 1989 Separation and Utilization of bovine lactoferrin. *Japanese Journal of Dairy and Food Science* **38** 277–283

Tomita M, Bellamy W, Takase M, Yamauchi K, Wakabayashi H & Kawase K 1991 Potent antibacterial peptides generated by pepsin digestion of bovine lactoferrin. *Journal of Dairy Science* **74** 4137–4142

Tomita M, Wakabayashi H, Yamauchi K, Teraguchi S & Hayasawa H 2002 Bovine lactoferrin and lactoferricin derived from milk: production and applications. *Biochemistry and Cell Biology – Biochimie et Biologie Cellulaire* **80** 109–112

Vorland LH, Ulvatne H, Rekdal O & Svendsen JS 1999 Initial binding sites of antimicrobial peptides in *Staphylococcus aureus* and *Escherichia coli*. *Scandinavian Journal of Infectious Diseases* **31** 467–473

Zullo A, De Francesco V, Scaccianoce G, Hassan C, Panarese A, Piglionica D, Panella C, Morini S & Ierardi E 2005 Quadruple therapy with lactoferrin for Helicobacter pylori eradication: a randomised, multicentre study. *Digistive and Liver Disease* **37** 496–500

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