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ORIGINAL PAPER

Molecular cloning and expression of yak (*Bos grunniens*) lactoferrin cDNA in *Pichia pastoris*

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Abstract cDNA encoding lactoferrin from yak was isolated by RT-PCR and then sequenced. The cloned cDNA (2127 bp) encodes a 709 amino acid precursor molecule of yak lactoferrin with a signal peptide of 19 amino acids. The yak lactoferrin cDNA was expressed in *Pichia pastoris*. The recombinant protein, purified by Ni-NTA affinity column, had a molecular weight of 76 kDa and reacted with an antibody raised against native bovine lactoferrin. The iron-binding behavior and antimicrobial activity of the purified protein indicated that it was correctly folded and functional.

Keywords Cloning · Expression · Lactoferrin cDNA · *Pichia pastoris* · Yak (*Bos grunniens*)

Introduction

The yak (*Bos grunniens*) is an important grazing livestock on the Qinghai-Tibetan plateau and adjacent areas. It provides draught power for transportation, milk and meat for human nutrition, and the other by-products such as hides and fiber for making tents.

Lactoferrin, an iron-binding glycoprotein with a molecular weight of approximately 80 kDa, is one of the members of the transferrin family. It consists of a single polypeptide chain of 660–690 amino acids folded into two homologous *N*- and *C*-lobes. Each lobe encloses an iron-binding site. Lactoferrin occurs naturally in numerous bodily secretions, including milk, tears, mucus, blood and saliva (Kanyshkova et al. 2001). In addition to sequestering and transporting iron, lactoferrin is involved in many other biological processes such as protection against microbes including cytomegalovirus (CMV) and anti-inflammation (Kanyshkova et al. 2001), regulation of immune function (Kanyshkova et al. 2001), myelopoiesis (Hangoc et al. 1991), intestinal iron absorption (Davidson and Lonnerdal 1989), and transcription (Kanyshkova et al. 2001). Presently, a number of cDNAs for lactoferrin have been cloned and characterized, such as those of bovine (GenBank accession No. X57084), goat (GenBank accession No. U53857), pig (GenBank accession No. AY306198), human (GenBank accession No. AY875691) etc. However, the cDNA for yak lactoferrin has not been cloned. In this study, the lactoferrin cDNA was cloned from yak and expressed in *Pichia pastoris* subsequently. Additionally, a comparison between

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lactoferrin cDNA of yak and that of other mammalian species was conducted.

Materials and methods

Materials

Cubes of tissue containing mammary cells were excised from the mammary gland of lactating yak cow (Lhasa, Tibet Autonomous Region, China). *Escherichia coli* JM109 was used as the host strain for plasmid amplification and cultured in LB medium supplemented with 100 μg ampicillin ml^{-1} or in low salt LB medium with 25 μg zeocin ml^{-1} at 37°C when necessary. All reagents were from commercial sources.

Media

Yeast extract peptone dextrose medium (YPDS) contained 10 g yeast extract l^{-1}, 20 g peptone l^{-1}, 20 g dextrose l^{-1}, 182.2 g sorbitol l^{-1} and 20 g agar l^{-1}. Buffered glycerol-complex medium (BMGY) contained 10 g yeast extract l^{-1}, 20 g peptone l^{-1}, 20 g dextrose l^{-1}, 0.4 mg biotin l^{-1}, yeast nitrogen base without amino acids at 13.4 g l^{-1} and 10 g glycerol l^{-1}, and was supplemented with 100 mmol l^{-1} potassium phosphate (pH 6.0). Buffered methanol-complex medium (BMMY) was identical to BMGY but containing 0.5% (v/v) methanol instead of 1% (v/v) glycerol.

Reverse transcription/polymerase chain reaction

Total RNA was isolated from the mammary gland of lactating yak cow using Trizol kit (Invitrogen, USA). The cDNA was synthesized at 42°C by using oligo (dT)-adaptor primer and random 9-mer, avian myeloblastosis virus (AMV) reverse transcriptase, and 0.5 μg total RNA. According to the cow lactoferrin gene sequence (GenBank X57084), the primers P1: 5′-AATCCGCGGCAAGCTCTTCGTCCCCG-CCTGCTG-3′, and P2: 5′-AGATCTA-GACCTCGTCAGGAAGGCGCAGGC-3′ (the sequences underlined are the *Sac*II and *Xba*I restriction sites, respectively) were used to amplify the cDNA of yak lactoferrin. After pre-denaturation at 94°C for 4 min, they were subjected to 30 cycles consisting of 30 s denaturation at 94°C, 30 s annealing at 57°C, and 2 min extension at 72°C, followed by a final 10 min at 72°C. The PCR product was confirmed by agarose electrophoresis and the purified PCR product was cloned into pMD18-T (TaKaRa Biotechnology Co. Dalian, PR China). The recombinant plasmid was named as pMD18-YLF and the inserted cDNA fragment was sequenced.

Transformation of *Pichia pastoris*

The cDNA fragment encoding the mature lactoferrin (MYLF) cDNA was amplified by PCR using the pMD18-YLF as template. PCR was performed with the following primers P3: 5′-TTATCGATGGCCCCGAGGAAAAACGTT-3′, and P4: 5′-AGATCTAGATCCCTCGTCAG-GAAGGCGCAGGC-3′ (the sequences underlined are the *Cla*I and *Xba*I restriction sites, respectively). The PCR product was purified and cloned into pMD18-T. The recombinant plasmid was digested with *Cla*I and *Xba*I. The small fragment was isolated and cloned into the expression vector pPICZαC of *P. pastoris* (Invitrogen). The recombinant plasmid was named as pMYLF and confirmed by sequencing. The plasmid pMYLF and pPICZαC were linearized with *Pme*I and transformed into *P. pastoris* X33 by electroporation according to the manufacture's manual (Invitrogen, Easy Select *Pichia* Expression Kit). The transformed cells were plated on YPDS plates containing 100 g zeocin ml^{-1} and incubated for 3 days 28°C.

Expression of yak lactoferrin cDNA

The transformant was cultivated at 28°C in 100 ml of BMGY medium until the optical density at 600 nm was 2–6 (16–18 h). The cells were pelleted and resuspended in BMMY medium at OD_{600} value of 1. Cultures were supplemented daily with 0.5% (v/v) methanol to maintain the induction of yak lactoferrin cDNA expression.

SDS-PAGE and Western blotting analysis

Polyclonal antibody against bovine lactoferrin was generated by immunizing rabbit with native bovine lactoferrin and the sera were partially purified by DEAE-Sepharose column chromatography.

SDS-PAGE was performed with 5% (v/v) stacking gel and 10% (v/v) separating gel, and the proteins were visualized by silver staining.

For Western blot analysis, protein samples were subjected to SDS-PAGE and then electrically transferred onto nitrocellulose membrane. The membrane was blocked with 2% fat-free milk in TBST (20 mmol l^{-1} Tris/HCl, pH 7.6, 150 mmol l^{-1} NaCl, 0.1% Tween 20) at room temperature for 2 h, and then incubated with a 1:100 dilution of rabbit polyclonal antisera against bovine lactoferrin. After washing 3×10 min with TBST, the blot was incubated with a polyclonal goat anti-rabbit IgG conjugated to alkaline phosphatase in TBST with 1% BSA (diluted 1:5000). The blot was washed 3×10 min with TBST, pre-rinsed in substrate buffer (100 mmol l^{-1} Tris/HCl, 100 mmol l^{-1} NaCl, 5 mmol l^{-1} $MgCl_2$, pH 9.5), and then covered by the substrate solution which was made freshly before each assay by mixing 50 μl NBT stock (Sigma, 75 g l^{-1} in 70% v/v dimethyl formamide) and 50 μl BCIP stock (Sigma, 50 g l^{-1} in 100% formamide) with 10 ml substrate buffer. Finally, the blot was incubated in dark with occasional agitation for 30–60 min, rinsed with dH_2O and the result was recorded.

Purification and antimicrobial activity of recombinant yak lactoferrin

The supernatant from 2 l of induced cells harboring pMYLF was used as raw material for the purification of yak lactoferrin. $(NH_4)_2SO_4$ was added to supernatant to give 65% saturation, stirred and then adjusted to pH 4.6 using 1 mol l^{-1} HCl. The mixture was kept at 4°C for 30 min, centrifuged at 15,000 g for 20 min at 4°C. The supernatant was made to 85% saturation with $(NH_4)_2SO_4$, stirred and then adjusted to pH 8.5 using by Tris base and kept at 4°C for 30 min. After centrifugation at $10{,}000 \times g$ for 20 min at 4°C, the supernatant was discarded and the precipitate was suspended in native binding buffer (50 mmol l^{-1} sodium phosphate buffer, 0.5 mol l^{-1} NaCl, pH 8.0). The supernatant was applied to a Ni-NTA column and the recombinant protein was eluted by native elution buffer (binding buffer plus 250 mmol l^{-1} imidazole).

To evaluate the biological activity of recombinant yak lactoferrin (rYLF), agarose diffusion assay was performed in standard Petri dishes. *E. coli* JM109 was grown to stationary phase and mixed with 1% agar. Wells (3-mm diam.) were punched in the agar and filled with purified rYLF protein or supernatant from *P. pastoris* X33 harboring pPICZαC, which was used as the negative control.

Iron-binding assay of recombinant protein

Iron saturation of rYLF and native bovine lactoferrin was carried out according to Mazurier and Spik (1980). Five micrograms purified protein in 1 ml dH_2O were equilibrated by dialysis with 2 mmol l^{-1} $FeCl_3$ in 0.1 mol l^{-1} $NaHCO_3$/0.1 mol l^{-1} sodium citrate buffer (pH 8.0). The excess reagent was removed by dialysis against water. The iron-saturated yak lactoferrin at pH 8.0 was equilibrated with 0.1 mol l^{-1} citrate/phosphate buffer at different pH values for 24 h at 4°C. The absorbance value at 465 nm was determined. The iron binding at different pH was recorded as percentage of iron saturation at pH 8.0.

Results and discussion

Cloning and sequence analysis of yak lactoferrin cDNA

The cDNA encoding the yak lactoferrin (GenBank accession No. DQ387455) was amplified by reverse transcription-polymerase chain reaction (RT-PCR) and inserted into vector pMD18-T. The sequence analysis showed that the yak lactoferrin cDNA (2127-bp) encodes a 709 amino acid precursor of yak lactoferrin with a signal peptide of 19 amino acids followed by a mature protein of 690 amino acids (Fig. 1). The calcu-

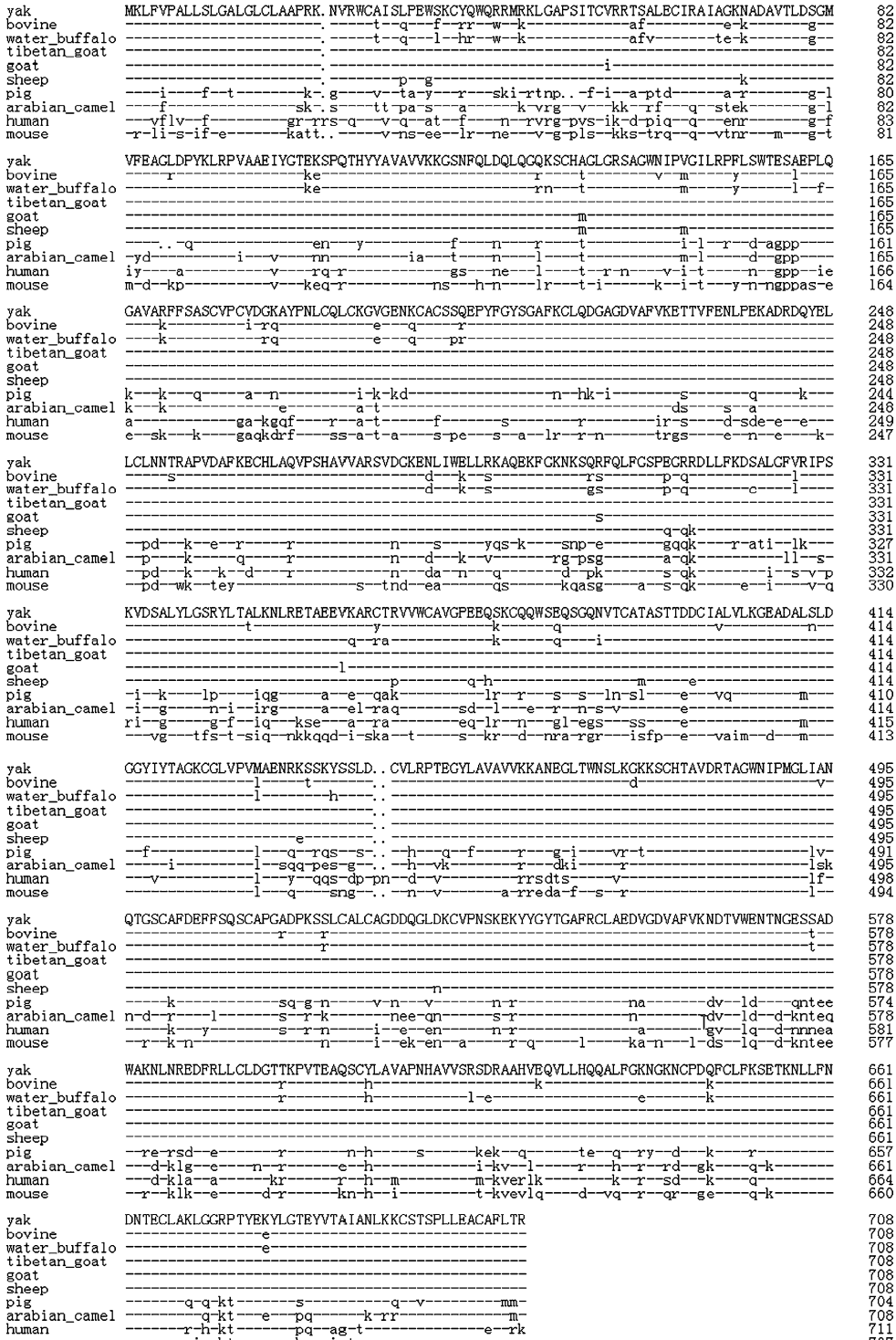

Fig. 1 Multiple alignment of the amino acid sequence of lactoferrin between yak and other mammalians. Yak lactoferrin contains the signal peptide (1–19) and mature peptide (20–708). Data were collected from Genbank with homology search at http://www.ncbi.nlm.gov/blast using the yak lactoferrin as query. Sequences were analyzed with the alignment tool DNAMAN, ''-'', the identity of amino acid residues

lated molecular mass and the estimated pI of yak lactoferrin are 76.1 kDa and 8.58, respectively. The deduced amino acid sequence of the yak lactoferrin contains four putative N-glycosylation sites at the positions: 300-303 (NKSQ), 387-340(NVTC), 495-498(NQTG), and 564-568 (NDTV).

Comparison of the amino acid sequences of lactoferrin from yak and other mammals

The comparison of amino acid sequence of the lactoferrin between yak and other mammalians are given in Table 1 and the further details of the alignment are shown in Fig.1.

Four amino acid residues of two lobes that are involved in the coordination of iron ions, His_{253}, Tyr_{92}, Tyr_{192}, Asp_{60} in the *N*-lobe and His_{253}, Tyr_{435}, Tyr_{528}, Asp_{395} in the *C*-lobe, are conserved in yak lactoferrin as in all other lactoferrins (Kanyshkova et al. 2001). Cysteine residues and disulfide bonds are highly conserved in this protein family (Le Provost et al. 1994, Goodman and Schanbacher 1991). Based on the amino acid sequence, the yak lactoferrin should possess 17 disulfides bonds, the same as with the lactoferrins of bovine, goat, and sheep, whereas human and murine lactoferrins have only 16 (Pierce et al. 1991).

The intramolecular peptide, Y_{679}-K_{695}, of human lactoferrin is an active domain inhibiting cysteine proteases (Ohashi et al. 2003), whereas the Y_{679}-K_{695} peptide of yak lactoferrin showed a different consistency with that of bovine (100%), Tibetan goat and sheep (94%), pig (88%), camel (76%), human (65%) and mouse (59%).

Lactoferrin and its peptides (lactoferricin and lactoferrampin) have broad spectrum antimicrobial activity that might attributed to removal of iron from the microbial environment and release of the membrane's lipolysaccharide molecules from the microbial cell wall (Bellamy et al. 1994; Kanyshkova et al. 2001; van der Kraan et al. 2004). Lactoferricin or lactoferrampin was generated from the pepsin or AspN digestion of lactoferrin. These peptides in all known lactoferrin are conserved, but several different amino acids are present among yak, bovine, goat, human, and mouse. The study on this difference is highly desirable.

Expression of the yak lactoferrin cDNA

As a basis for functional and structural studies, yak lactoferrin cDNA encoding mature protein was heterologously expressed. *P. pastoris* was chosen to produce recombinant protein in the native folded state. The yak lactoferrin cDNA was cloned into the *P. pastoris* vector pPICZαC in frame with the α-factor signal sequence for secretion. The lactoferrin protein was successfully secreted into the culture medium (Fig. 2).

SDS-PAGE and Western blotting analysis

SDS-PAGE and Western blotting were used to confirm the expressed product of yak lactoferrin cDNA in *P. pastoris*. As shown in Fig. 2, the

Table 1 Comparison of deduced amino acid sequences of lactoferrin between yak and other mammalians

Organism	Signal peptides[a]	Mature peptides[a]	Accession number[b]
Bos taurus (bovine)	100	96	P24627
Bubalus bubalis (domestic water buffalo)	100	96	O77698
Tibetan goat	100	90	DQ387456
Capra hircus (goat)	100	90	Q29477
Ovis aries (sheep)	100	89	Q5MJE8
Camelus dromedairus (Arabian dromedary camel)	95	73	Q9TUMO
Sus scrofa (pig)	84	71	Q6YT39
Homo sapie (human)	74	69	Q5EK51
Mus musculus (mouse)	63	63	P08071

[a]Percentage of homology

[b]Accession numbers from the Swiss-Prot or trEMBL

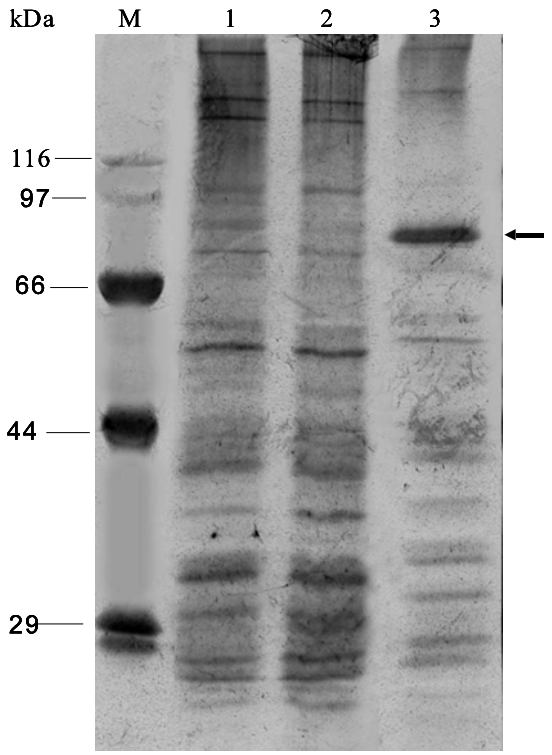

Fig. 2 SDS-PAGE analysis of the recombinant yak lactoferrin in *Pichia pastoris*. Lane M: protein molecular weight standard; lanes 1–3: supernatant from *P. pastoris* harboring pPICZαC and pMYLF after 0 and 3 days, respectively. The arrow on the right side indicates recombinant yak lactoferrin

molecular weight of yak lactoferrin secreted from *P. pastoris* is about 80 kDa, which is the same as the bovine lactoferrin. Western blotting analysis also confirmed that this protein was the lactoferrin (Fig. 3).

Yak lactoferrin was expressed at about 40 mg l^{-1} in *P. pastoris*. The rYLF could be easily purified by using Ni-NTA affinity column under native condition.

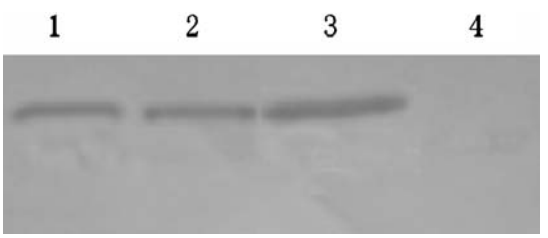

Fig. 3 Western blotting analysis of the recombinant yak lactoferrin in *Pichia pastoris*. Lane1: the native bovine lactoferrin; lanes 2–3: supernatant from *P. pastoris* harboring pMYLF after 2 and 3 days; lane 4: supernatant from *P. pastoris* harboring pPICZαC

The lactoferrin cDNAs of porcine (Wang et al. 2002), equine (Paramasivam et al. 2002) and human have been expressed in *P. pastoris.* A yield of 12 mg porcine lactoferrin l^{-1} was obtained in shake-flask cultures (Wang et al. 2002) and of 40 mg l^{-1} for equine lactoferrin (Paramasivam et al. 2002). Although the final yield of a protein is greatly influenced by its inherent properties, the yield can be significantly enhanced by manipulating the factors influencing gene expression and product stability (Sreekrishna 1993). The factors include: gene copy number, site and mode of chromosomal integration of the expression cassette, 5′- and 3′-untranslation region (UTR) of mRNA, translation start codon (AUG) context, A+T composition of cDNA, transcription and translational blocks, nature of secretion signal, endogenous protease activity, host strain physiology, media and growth conditions, and fermentation parameters. All these factors should be considered in designing an optimal production system (Sreekrishna et al. 1997). These problems encountered in yak lactoferrin cDNA expression could be overcome by improving above factors (in progressing).

Antibacterial activity of recombinant yak lactoferrin

The agarose diffusion assay showed that the rYLF could inhibit the growth of *E. coli* JM109 effectively (Fig. 4), whereas the control could not. The antibacterial activity of lactoferrin might be attributed to its iron-binding capacity and prevent iron utilization by bacteria. Many microorganisms express surface receptors for the binding of lactoferrin resulted in cell death via one of several mechanisms (e.g., initiation of lipopolysaccharide release from the cell wall) (Kanyshkova et al. 2001). As a result, the rYLF was identical to the native bovine lactoferrin in antibacterial activity.

Iron-binding analysis with recombinant protein

As the pH value of the lactoferrin solution is lowered, the iron-binding property of lactoferrin

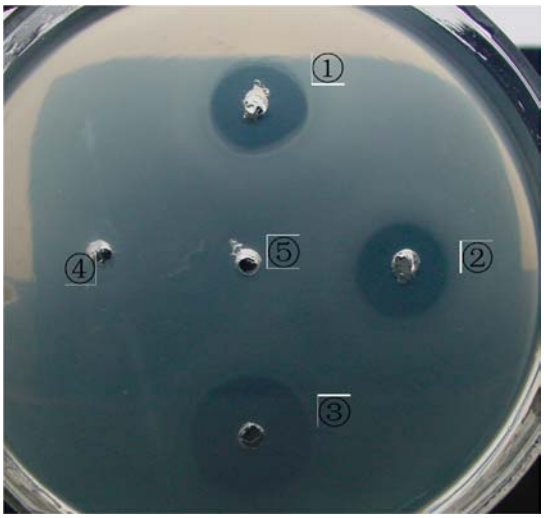

Fig. 4 Effect of inhibiting bacterial growth by recombinant yak lactoferrin (rYLF) on *E. coli* JM109. (1) and (2): 5 μl of rYLF; (3): 10 μl of rYLF; (4): control 1 (supernatant from host); (5): control 2 (supernatant from host harboring pPICZαC)

was used to assess the functional state of recombinant protein. This pH-dependent iron release was monitored by the different absorbance at 465 nm (Mazurier et al. 1980). The pattern of iron release for the native and recombinant lactoferrin was found to be identical (Fig. 5). The

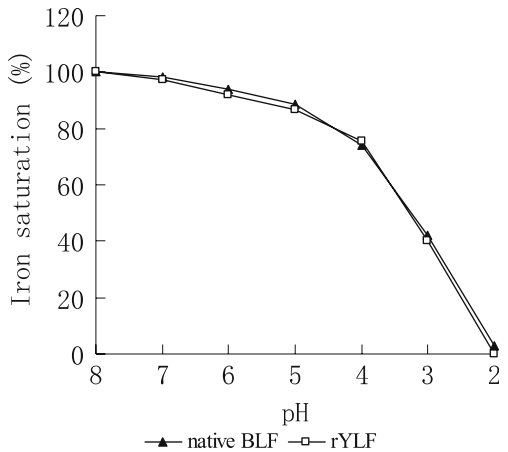

Fig. 5 pH-dependent iron release from iron-saturated yak lactoferrin. The saturation at pH 8.0 was taken as control. The figure showed absorbance of lactoferrin at 465 nm from pH 8.0 to pH 2.0: the native bovine was 0.320, 0.315, 0.301, 0.283, 0.237, 0.135 and 0.009, respectively; and the yak lactoferrin was 0.319, 0.311, 0.293, 0.276, 0.241, 0.128 and 0, respectively

results confirmed that the expressed protein was correctly folded and functional.

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