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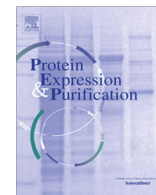

Molecular cloning, expression and purification of lactoferrin from Tibetan sheep mammary gland using a yeast expression system

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ABSTRACT

This paper reports the successful expression of a lactoferrin gene—obtained from the mammary gland tissue of Tibetan sheep—in the yeast *Pichia pastoris* GS115 using pPICZαA as the recombinant plasmid and α-factor signal sequence for secretion. The recombinant lactoferrin was purified by ammonium sulfate precipitation, ion-exchange column chromatography and gel-filtration chromatography, and it had a molecular mass of 76 kDa. We obtained an expression yield of >60 mg L^{-1} and specific activity of 2533.33 U mg^{-1}. The antimicrobial activities and iron-binding behaviors of recombinant lactoferrin indicated that it was correctly folded and functional. Additionally, recombinant lactoferrin inhibited the growth of *Escherichia coli* JM109 and *Staphylococcus aureus*. These findings indicate that recombinant lactoferrin is a potential antibiotic for use on humans. This study also demonstrates the successful expression of recombinant lactoferrin using the eukaryotic host organism *P. pastoris*, paving the way for protein engineering using this gene.

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Introduction

Antibiotics have a long history of use for fighting infection in human health treatments. However, the abuse of antibiotics is causing increasing problems such as the development of drug resistance and the accumulation of drug residues in animal food products [1]. The development of novel antibiotic substitutes will help to solve such problems.

Lactoferrin (LF), a non-heme iron-binding glycoprotein, plays an important role in host-defense responses to pathogens [2]. LF not only participates in ferric transport [3], but it also performs various biological functions related to its extensive anti-bacterial, anti-fungus, anti-virus, anti-oxidation and anti-adipogenic properties. Further, LF can regulate the immune system and improve the production performance of animals [4–6]. It is therefore considered to have tremendous potential as a new antibiotic, anti-cancer drug, and food and feed additive [7,8].

Tibetan sheep were the first artificially bred sheep in the natural ecosystem of the Qinghai-Tibetan plateau in Central Asia. They are an important species of grazing livestock with great economic value and high tolerance of the region's harsh, high-altitude conditions, including extreme cold, low oxygen concentrations and low air pressure. This represents one of the most economically efficient livestock breeds in China, generating products including feta cheese, mutton meat, and wool, so it makes economic sense to fully utilize the plateau's meadows to raise these sheep [9].

Purification of a high yield of LF from animals is generally costly and time-consuming [10]. Use of the yeast *Pichi pastoris* as an expression system has several advantages including rapid growth rate, ease of manipulation and high expression levels [11]. The many purification procedures that have been used to isolate LF include affinity chromatography [12], ion-exchange chromatography [13,14], batch extraction [15], and ultrafiltration on membranes [16]. However, a method with potential for use at an industrial level of production is not yet available.

To date, the LF of swines, horses, yaks and goats has been expressed and purified in *P. pastoris* [17–20], but little attention has been paid to sheep [21,22], with no published reports on the LF of Tibetan sheep. In this study we therefore designed an experiment to obtain recombinant Tibetan sheep LF (rLF) using the *P. pastoris* expression system. We also purified rLF using a three-step purification method, and measured its iron-binding behavior and antimicrobial activity.

Materials and methods

Animal subject and reagents

A tissue sample was collected from the mammary gland immediately after slaughter of a healthy 4-year old female Tibetan

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sheep, at Ruoergai County, Sichuan Province, China. Total RNA was extracted using Trizol reagent (Life Technologies, USA). A cDNA synthesis kit from Fermentas was used to reverse RNA. PCR reagents and pMD19-T were obtained from TaKaRa (Dalian, China), and other reagents were purchased from the Shanghai Sangon Biotechnology Corporation.

Screening and amplification of cDNA from Tibetan sheep LF

Total RNA was extracted from mammary gland tissue using a Trizol RNA extraction kit, and 1 μg of it was used as a template in the first-strand cDNA synthesis. Then, the single strain cDNA was used to clone the full length of the Tibetan sheep LF cDNA sequence. We designed oligonucleotides with sequences of 5′-TCTCTCGAGAAAAGAGAGGCTGAAGCT**GCCCCGAGGAAAAACGT**-3′ and 5′-CTGTCTAGA**TCACCTCGTCAGGAAGG**-3′ based on the open reading frame of the LF gene without its signal sequence (boldfaced). The restriction sites *Xho* I and *Xba* I (underlined above) were incorporated into the subclone of the PCR product. A boxed sequence was designed between the *Xho* I site and specific sequence of the forward primer, and coded for the amino acid sequence of Kex2 and Ste13 cutting sites to generate cleavage of the signal sequence without survival of any additional amino acids on the N-terminus of rLF. The PCR reaction was carried out for 30 amplification cycles (denaturation at 94 °C for 30 s, annealing at 54 °C for 60 s, and extension at 72 °C for 60 s).

Construction of expression plasmid and transformation of P. pastoris GS115

The PCR product was isolated and ligated into a pMD19-T vector to construct pMD19-LF, and then transformed into *Escherichia coli* DH5α for plasmid preparation. After blue-white selection and plasmid PCR, the colonies were sent to Shanghai Sangon Bio-technology Corporation for sequencing. The correct recombinant plasmids were digested with *Xba* I and *Xho* I, and then subcloned into the same restriction enzyme-digested pPICZαA vector to generate pPICZαA-LF. After linearization with *Sac* I, pPICZαA-LF was transformed into *P. pastoris* GS115 using the lithium chloride transformation method. The transformants were cultivated at 30 °C for 1–4 h, spread over YPDZ medium (1% yeast extract, 2% peptone, 2% dextrose, 2% agar and 2.5×10^{-2} mg L^{-1} Zeocin) on plates, and then cultured at 30 °C for 2–3 d. A 5-to-20-fold concentration of Zeocin ($12.5–50 \times 10^{-2}$ mg L^{-1}) was prepared to enable selection of multicopy recombinant colonies. One transformant with a high Zeocin resistance was selected for expression.

Expression of rLF

The transformant was inoculated into 10 mL of BMGY medium (1% yeast extract, 1% glycerol, 2% peptone, 4×10^{-5}% biotin and 1.34% yeast nitrogen base) at 28 °C until the optical density (OD) at 600 nm was 2–6. The yeast cells were pelleted and resuspended in 20 mL of BMMY medium (1% yeast extract, 0.5% methanol, 2% peptone, 4×10^{-5}% biotin, and 1.34% yeast nitrogen base) until the OD at 600 nm was 1. The culture was then grown at 28 °C with constant shaking at 220 rpm for 168 h, and fed every 24 h with 0.5% (v/v) methanol to maintain the induction of Tibetan sheep LF cDNA expression.

Purification of rLF from P. pastoris culture media

The supernatant was collected by centrifuging for 10 min at 5000*g* at 4 °C (ammonium sulfate was added to the crude to enable extraction to 65% saturation), stirred and then adjusted to pH 4.6. The mixture was centrifuged at 12,000*g* for 30 min at 4 °C. The protein was precipitated and dialyzed for 36 h using Tris buffer (pH 9.0, 50 mM Tris and 4.3 mM NaCl, ionic strength = 10 mM). The solution was applied to a Q-Sepharose Fast Flow column (GE Healthcare) equilibrated with Tris buffer (pH 9.0, 50 mM Tris and 4.3 mM NaCl, ionic strength = 10 mM) and eluted with a linear gradient of 0–1 M NaCl in the same buffer. The rLF was finally purified by gel-filtration on a Sephadex G-100 HR column (Pharmacia, USA). The flow rate was 0.2 mL min^{-1}, and the column was equilibrated and eluted using the same concentration Tris buffer as described above. After it was freeze-dried (Telstar*LyoQuest, Spain), rLF was precipitated and dissolved in PBS (phosphate buffer solution, pH 6.0) and finally dialyzed against 0.1 M citrate (pH 2.2) containing 0.1% EDTA in a regenerated cellulose membrane for 24 h [19]. The active fraction was desalinated and considered as the purified enzyme preparation. The rLF fraction was identified using sodium dodecyl sulfate–agarose gel electrophoresis (SDS–PAGE). The protein concentration was determined with the dye binding method, using bovine serum albumin as the standard [23].

Enzyme assay

The liquid test tube method was used to determined rLF activity [24], measured using *Bacillus cereus* as the substrate at a concentration of $10^6–10^7$ cell mL^{-1}; 0.1 mL *B. cereus* suspension and 0.1 mL rLF solution were added to 1 mL of sterile liquid culture and incubated at 37 °C for 24 h. The OD_{600} value was measured using 0.2 mL sterile water and 1 mL sterile liquid culture as a control. Antimicrobial activity was calculated using the following formulae:

$$\text{Antibacterial ratio} = \frac{OD_{600\ \text{Control}} - OD_{600\ \text{Sample}}}{OD_{600\ \text{Control}}} \times 100\%$$

$$\text{Antimicrobial activity} = \frac{\text{Antibacterial ratio} \times 100 \times 12}{0.1} \times K$$

where *K* was the dilution factor, 12 was the dilution factor of rLF by liquid culture, and 0.1 was the solution volume of the protein. All measurements were made in triplicate.

To assess the antimicrobial activity of rLF, an agarose diffusion assay was performed in standard petri dishes. *E. coli* JM109 and *Staphylococcus aureus* were cultured to the stationary phase, and each was mixed with 1% agar. Wells of 3-mm diameter were punched into the agar and filled with purified rLF protein, or with supernatant from *P. pastoris* harboring pPICZαA for the control [25]. The minimum inhibitory concentration (MIC) was also measured by monitoring bacterial growth, based on the OD at 620 nm in a double beam UV–visible light spectrophotometer [26]. *E. coli* JM109 and *S. aureus* suspended in tryptic soy broth (TSB) were diluted with 1% peptone water to achieve a density of $10^4–10^5$ cell mL^{-1}. Then, 100 μL of the diluted bacteria was added to the wells of a 96-well plate filled with 100 μL of lactoferrin solution at different concentrations (1000, 1100, 1200, 1300, 1400 and 1500 μg mL^{-1}), versus 100 μL of supernatant from *P. pastoris* harboring pPICZαA for the control. Plates were incubated at 37 °C, and absorbance was measured at 620 nm after 4, 8, 24 and 48 h, with shaking for 15 s before reading. MICs were defined as the lowest concentration at which growth was completely inhibited. Growth inhibition was defined as the concentration at which absorbance of the test wells minus absorbance of the control wells was $\leqslant 0.05$.

Iron-binding assay for rLF

Iron saturation of rLF and natural LF was measured according to the methods of Mazurier and Spik [27]: 500 mg of purified rLF or nat-

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