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Expression of Bovine Lactoferrin C-lobe in *Rhodococcus erythropolis* and Its Purification and Characterization

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A *Rhodococcus erythropolis* expression system for the bovine lactoferrin C-lobe was constructed. The DNA fragments encoding the BLF C-lobe were amplified and cloned into vector pTip LCH1.2. *R. erythropolis* carrying the pTip-C-lobe was cultured at 30 °C with shaking, and expression of the rBLF C-lobe was induced by adding 1 μg/ml (final concentration) thiostrepton. The rBLF C-lobe was isolated in native and denatured (8 M urea) form by Ni-NTA affinity chromatography. To obtain a bioactive rBLF C-lobe, the protein isolated in the denatured form was refolded by stepwise dialysis against refolding buffers. The antibacterial activity of the rBLF C-lobe was tested by the filter-disc plate assay method. The refolded rBLF C-lobe demonstrated antibacterial activity against selected strains of *Escherichia coli*.

Key words: lactoferrin; *Rhodococcus*; gene expression; antibacterial activity; milk

Lactoferrin is an iron-binding glycoprotein present in milk and many other mammalian biological fluids, including saliva, tears, and mucous secretions.[1,2] Lactoferrin is widely considered to be an important component of the host defense mechanism against microbial infection.[3] Its antibacterial effects are explained by several mechanisms; one such mechanism is that lactoferrin, being an iron-binding protein, limits the amount of free iron (an essential growth factor for microorganisms) available.[4] Another mechanism involves destabilizing the outer membrane of gram-negative bacteria, resulting in the liberation of lipopolysaccharides.[5] The consequent changes to the permeability of the membrane alter the complete mechanism of antimicrobial action and allow the passage of otherwise impermeable antibiotics through the membrane. Furthermore, several peptides having antimicrobial activity have been identified from lactoferrin. Tomita *et al.*[6] discovered an antimicrobial peptide, lactoferricin, which is generated upon gastric pepsin cleavage of lactoferrin. The antimicrobial activity of lactoferricin has been shown to be 100-fold stronger than that of undigested bovine lactoferrin. Lactoferricin is active against a wide range of gram-negative[7,8] and gram-positive bacteria, fungi, and protozoa.[9–11]

Lactoferrin is folded into two globular lobes designated the N- and C-lobes, and each lobe consists of two domains. Structure and sequence analyses indicate that these molecules might have arisen by gene duplication. Moreover, a difference was observed with regard to the participation of the N- and C-lobes in binding to cell surface receptors.[12–14] Separation of the N- and C-lobes of lactoferrin is a useful approach for studying the structure-function relationship of lactoferrin, but separation of these two lobes by proteolytic enzyme digestion is very difficult. Further, studies on the characterization of the C-lobe are inherently limited due to the nonavailability of a viable source of lactoferrin for functional testing and comparative analysis.

Recently, Nakashima and Tamura developed a new expression system using the nocardioform actinomycete *Rhodococcus erythropolis* as a host cell.[15,16] Using this expression system, they found that proteins that are difficult to express in *Escherichia coli* can be expressed and isolated in soluble forms. Hence we attempted, for the first time, to investigate the expression of the recombinant bovine lactoferrin C-lobe (rBLF C-lobe) in *R. erythropolis*. We also observed antibacterial ability against selected strains of *E. coli*.

Materials and Methods

Strain, media, and growth conditions. E. coli XL-1-blue served as the host strain for plasmid maintenance

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and amplification. *R. erythropolis* L-88 and *E. coli* strains were routinely cultured in Luria-Bertani (LB) broth (1% Bacto tryptone, 0.5% Bacto yeast extract, and 1% NaCl) at 30 °C. Appropriate antibiotics, ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml), were used to select the transformants in the culture media.

Construction of bovine lactoferrin C-lobe expression vector. cDNA encoding bovine lactoferrin was prepared according to the method described by Nakamura *et al.*[17] PCR amplification of the gene encoding the bovine lactoferrin C-lobe (364 Val–708 Arg) was performed using two primers (5′-GGGTCATGAGCGTCGTGTG-GTGTGCCG-3′ and 5′-ACGTCGACCCCTCGTCAG-GAAGGCGCAG-3′) with the cDNA of bovine lactoferrin as the template. The amplified DNA fragment was digested with BspH I and Sal I and introduced into an expression vector, pTip-LCH1.2,[15] which was digested with NcoI and XhoI. The resulting plasmid (pTip-C-lobe) encoded the bovine lactoferrin C-lobe fused with a His6-Tag at the C-terminal. The pTip-C-lobe was transformed into a lysozyme-sensitive mutant of the *R. erythropolis* L-88 strain.[18]

Expression and purification of recombinant bovine lactoferrin C-lobe. The *R. erythropolis* L-88 strain carrying the pTip-C-lobe was cultured at 30 °C at 150 rpm, and expression of the rBLF C-lobe was induced by adding 1 μg/ml (final concentration) thiostrepton (Sigma, St. Louis, MO). The rBLF C-lobe was isolated in either its native or denatured form 20 h after induction. For small-scale preparation of the rBLF C-lobe (10 ml culture), harvested cells were disrupted using a MultiBeads shocker (Yasui kikai, Osaka), and the rBLF C-lobe was isolated by Ni-NTA affinity chromatography according to the manufacture's instructions.

For large-scale preparation of the rBLF C-lobe, a single colony was inoculated with 10 ml LB in a 50 ml flask and incubated at 30 °C with shaking for 2 d. Cells were then transferred to 1 l fresh LB medium in 3-l flasks and cultured at 30 °C. Protein expression was initiated by adding thiostrepton when the optical density (OD, 600 nm) reached 1.0. After a 20 h induction period, the cells were harvested and resuspended with a buffer (500 mM sodium phosphate, 300 mM NaCl, pH 8.0). Lysozyme (final concentration, 2 mg/ml) and benzonase (final concentration, 1 U/ml) were added to the cell suspension, and the cells were incubated on ice for 30 min. The cells were disrupted by sonication, followed by centrifugation (4 °C, 20,000 × *g*, 15 min). The supernatant was subjected to Ni-NTA affinity chromatography, and the recombinant protein was isolated according to the manufacture's instructions. Briefly, the supernatant was incubated with Ni-NTA superflow agarose for 1 h at 4 °C, and the mixture was transferred into a column. The proteins bound to Ni-NTA Superflow (Qiagen, Hilden, Germany) were eluted with a buffer (500 mM sodium phosphate, 300 mM NaCl, 10% glycerol, pH 6.3) containing 400 mM imidazole.

The recombinant proteins were also isolated in their denatured form in the presence of 8 M urea. Protein isolation was performed at room temperature, and the experimental procedure was the same as that described above, except for the lysozyme and benzonase treatment.

Western blot analysis. The protein samples were subjected to 12% SDS–PAGE. Proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell BioScience, Dassel, Germany) using a BioRad semi-dry transfer cell. The rBLF C-lobe was detected according to the standard protocol using an anti-6xHis monoclonal antibody (Covance Research Products, California) as the primary antibody and an alkaline phosphate-conjugated antimouse IgG antibody (Promega, Madison, WI) as the secondary antibody. The bound secondary antibodies were detected using the BCIP/NBT liquid substrate system for color development (Promega).

Refolding experiments. To obtain a bioactive rBLF C-lobe, the protein isolated in its denatured form was refolded by stepwise dialysis against refolding buffers (100 mM sodium phosphate, 10 mM Tris–HCl, and 10% glycerol, pH 8.0). The urea concentration of the dialysis buffer was changed every 12 h, and it was gradually decreased to 0 mM in steps of 2 M at 4 °C. After dialysis against a buffer that did not contain urea, the proteins were further dialyzed against 100 mM sodium phosphate (pH 8.0) containing 10 mM Tris–HCl for 12 h at 4 °C. The protein concentration was measured by the Lowry method[19] and used for antibacterial activity tests.

Measurement of the antibacterial activity of the rBLF C-lobe. The rBLF C-lobe was tested for antibacterial activity by the filter-disc plate assay method using three bacterial strains (*E. coli* XL1-blue, Top10, and BL21) as the target cells. Five milliliters of LB agar was cooled to 45 °C, mixed with 1 ml of the precultured target cells, and then overlaid on an LB plate (10 cm diameter). Sterile filter discs were placed on the plate. The rBLF C-lobe was prepared in different concentrations of 4, 2, and 1 mg/ml, and then 30 μl of these solutions were spotted on the filter disc. All plates were kept upright to dry them at room temperature before they were inverted and incubated at an optimum temperature for 18 h. Antibacterial activity was evaluated by the size of the zones of growth inhibition.

Results and Discussion

Expression and purification of the rBLF C-lobe

Preparation of a substantial amount of the rBLF C-lobe has not been successful thus far; this suggests that the host cells tested so far are not suitable for rBLF C-lobe production. Hence we attempted to apply a newly

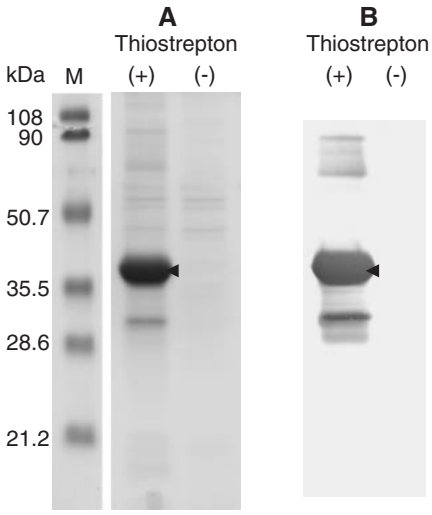

Fig. 1. SDS–PAGE (A) and Western Blot (B) Analysis of the Expression of Recombinant Bovine Lactoferrin C-lobe.

Protein samples were separated on 12% SDS–PAGE gel. Induction was performed with 1 μg/ml thiostrepton (+) and without thiostrepton (−). Harvested cells from 10 ml culture medium were disrupted using a MultiBeads shocker, and the rBLF C-lobe was isolated by Ni-NTA affinity chromatography. M, Molecular weight marker.

developed recombinant protein expression system using the nocardioform actinomycete *R. erythropolis* as the host cell. The expression vector pTip-C-lobe was transformed into the *R. erythropolis* L-88 strain, a lysozyme-sensitive mutant.[18] rBLF C-lobe expression was initiated by adding thiostrepton, and the expressed protein was isolated in its denatured form, as described in "Materials and Methods." The expressed protein appeared as a single major band in SDS–PAGE and had a molecular mass of 38 kDa (Fig. 1); this was in good agreement with the predicted molecular mass. A faint minor protein band with a molecular mass of 30 kDa was also isolated along with the 38 kDa protein, and this protein band is perhaps truncated at the N-terminus of the rBLF C-lobe, since the anti-6xHis antibody reacted with it (Fig. 1). We also attempted to isolate the rBLF C-lobe in its native form. It was possible to isolate the soluble forms of the rBLF C-lobe, but most of the expressed protein was insoluble, and the protein yield was very low (data not shown). Hence we isolated the rBLF C-lobe in the insoluble form and then attempted to refold the protein for use in the antibacterial activity tests.

Large-scale production and purification of recombinants

The rBLF C-lobe was expressed and isolated in a denatured form from 22 g of cells, as described in "Materials and Methods." The protein that was bound to Ni-NTA Superflow agarose was eluted using a 0–400 mM imidazole linear gradient. Each fraction was loaded onto SDS–PAGE (data not shown), and the fractions were pooled and subjected to the refolding experiment. The recombinant protein can be purified easily by one-step Ni-NTA affinity chromatography. The purification level of rBLF C-lobe in *R. erythropolis* cell for 1 ml was determined according to the method of Lowry *et al.*[19] with bovine serum albumin as a standard. The yield of the rBLF C-lobe is estimated to be 3.6 mg/ml culture. The pooled proteins were refolded by gradually decreasing the urea concentration of the dialysis buffer, as described in "Materials and Methods." In order to compare the yield of the soluble forms of proteins, a variety of components, such as arginine, proline, cysteine, and sucrose, were added to the refolding buffer. However, with regard to solubility, each of these components produced results similar to those produced by the refolding buffer having none of the abovementioned components (data not shown).

Recently, Tanaka *et al.*[20] and Nakamura *et al.*[17] reported the expression of rBLF and the lactoferrin N-lobe using a *Spodoptera frugiperda* (Sf9) cell as the host cell. In these studies, the expression of the rBLF C-lobe did not succeed, but we successfully expressed and isolated substantial amounts of the BLF C-lobe using the expression system with an inducible expression vector. In this experiment, *R. erythropolis* was an adequate host cell for rBLF C-lobe expression. The reason for high expression of rBLF C-lobe in *R. erythropolis* is not clear, but Nakashima and Tamura[15] reported that it might be due to differences in cellular environments such as turnover rates of proteins, folding machineries, osmotic pressures, or redox states in *E. coli* and *R. erythropolis*. They also reported that soluble protein is obtained when incubated at lower temperatures. It is well known that a lower temperature is more suitable for the production of recombinant proteins.

Antibacterial activity

Antimicrobial activity was estimated by the filter-disc plate assay method using three bacterial strains (*E. coli* XL1-blue, Top10, and BL21) (Fig. 2). rBLF C-lobe exhibited significant antibacterial activity against the three bacterial strains. The rBLF C-lobe at a concentration of 1 mg/ml exhibited weak antibacterial activity (Fig. 2C), and showed a large inhibition halo at concentrations of 2 and 4 mg/ml against all the strains (Fig. 2A and B). In a control experiment without the rBLF C-lobe, no zones of antibacterial activity against any of the strains were observed (Fig. 2D).

The lactoferrin molecule is proposed to consist of two lobes (N-lobe and C-lobe); the N-lobe contains the active domains for bactericidal action and heparin binding, whereas the C-lobe contains a functional domain for hepatocyte binding and internalization. Lactoferricin B, a 25-amino acid peptide derived from the N-lobe of bovine lactoferrin, has bactericidal activity.[21] The antibacterial properties of lactoferricin B are attributed to disruption of target cell membranes by the basic residues arrayed along the outside of the

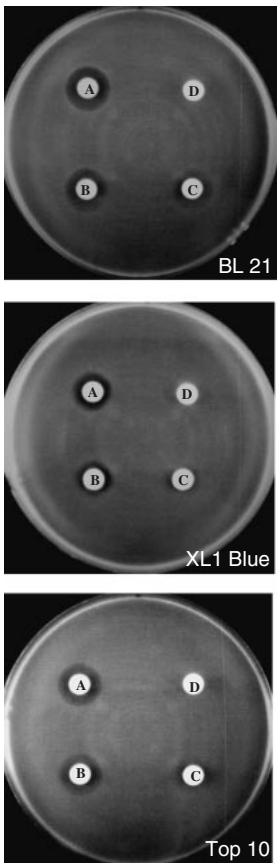

Fig. 2. Filter-Disc Plate Assay for Detection of Antibacterial Activity against *E. coli* Strains.

LB agar was cooled to 45 °C and mixed with 1 ml of the precultured target cells, and then overlaid on an LB plate. Sterile filter discs were placed on the plate and the rBLF C-lobe was prepared in different concentrations, and then 30 µl of these solutions were spotted on the filter disc. All plates were incubated at an optimum temperature for 18 h. Antibacterial activity was evaluated by the size of the zones of growth inhibition. D, only buffer (10 mM Tris–HCl, 100 mM Sodium phosphate, 10% glycerol, pH 8.0); A–C, concentration of recombinant bovine lactoferrin C-lobe (A, 4 mg/ml; B, 2 mg/ml; C, 1 mg/ml).

lactoferricin B molecule.[22] Generally, the antibacterial region of lactoferricin is known to be in the N-lobe, but in this experiment, we confirmed that the rBLF C-lobe exerts antibacterial activity against *E. coli*.

Another mechanism of antibacterial activity is the binding of iron, an essential element for the growth of almost all microorganisms. Apo lactoferrin has been found to inhibit the growth of many pathogenic bacteria.[23,24] The binding of the iron in the medium is the most well-known mechanism by which lactoferrin induces growth inhibition in bacteria. Thus the antibacterial activity of the rBLF C-lobe is perhaps related to its iron-binding activity or due to the existence of an antibacterial peptide in the C-lobe. We intend to conduct further studies on antibacterial activity with respect to the rBLF C-lobe.

In conclusion, the *R. erythropolis* expression system allowed high-level expression of the BLF C-lobe, and it demonstrated significant antibacterial activity against selected strains of *E. coli*.

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