Expression and Characterization of Human Lactoferrin in Yeast *Saccharomyces cerevisiae*

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Lactoferrin (LF) has certain chemical and biological properties that are significant to the dairy food industry. To study the relationship between protein structure and functionality of LF with the aim of increasing the thermostability of LF, we established a yeast system for heterologous expression of cloned human LF cDNA. Human LF was successfully synthesized in yeast cells by placing the cloned cDNA under the regulation of yeast chelatin promoter. Both human LF and yeast invertase secretion signal sequences were used to direct the secretion of recombinant human LF synthesized in yeast cells. The construct of the expression unit containing the yeast invertase signal sequence resulted in relatively high levels of secretion of recombinant human LF (1.5–2.0 mg/L), whereas the other containing human LF secretion signal sequence produced quite low levels of secretion of the protein. The secreted recombinant human LF was purified from the yeast broth by heparin affinity and immunoaffinity chromatographies. The highly purified recombinant LF was confirmed to be bioactive, having iron- and copper-binding activities. Also, the recombinant LF synthesized in yeast was glycosylated. The levels of synthesis of human LF in the yeast system were not constant, and LF might be toxic to yeast cells.

INTRODUCTION

Lactoferrin (LF) is a nonheme, iron-binding protein found not only in milk but also in various external secretions such as saliva, tears, pancreatic juice, and semen (Groves, 1960; Johansson, 1960; Masson et al., 1966), as well as in leucocytes (Baggiolini et al., 1970). It is a monomeric glycoprotein comprised of about 700 amino acid residues, folded into two globular lobes (Legrand et al., 1984; Metz-Boutique et al., 1984). Each lobe binds reversibly a single Fe^{3+} ion together with one CO_3^{2-} anion (Anderson et al., 1989) and is conjugated to one glycan chain through an N-glycosidic linkage (Spik et al., 1982). LF is quite basic with a p*I* of 9.0 (Malamud and Drysdale, 1978) and has a very high affinity for iron (K_a = 30), 300 times that of transferrin (Aisen and Listowsky, 1980).

LF has a variety of biological activities, such as regulation of myelopoiesis (Broxmeyer et al., 1978), modulation of the inflammatory response (Oseas et al., 1981), essential growth factor for lymphocytes (Hashizume et al., 1983), DNA binding (Bennet and Davis, 1982), and RNase activities (Furmanski et al., 1989). The primary significance of LF to the dairy food industry is essentially fourfold: (1) LF possesses antimicrobial activity against a variety of bacteria; (2) LF may regulate, control, and facilitate intestinal absorption of iron; (3) LF is a dietary source of iron; (4) LF possesses antioxidant activity. Concentrations of LF in human milk generally range from 3.4 (Goldsmith et al., 1983) to 4.2 mg/mL (Evans et al., 1978). However, bovine milk contains a substantially lower concentration of LF with mean values in the range 0.02–0.2 mg/mL (Reiter, 1978), and only a small portion of LF can survive in the pasteurization process. If the cow's mammary gland can be genetically engineered to produce thermostable LF at high levels, it may help in protecting the udder from mastitis and ensuring safer, more healthful dairy products.

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The progress on characterization of LFs has provided a relatively clear picture for studies on the structure and functionality of the protein. The X-ray structure of human LF has been analyzed to 2.8-Å resolution (Anderson et al., 1989). Each iron atom is coordinated to four protein ligands, two Tyr, one Asp, one His, and the specific CO_3^{2-}. LF can also bind other metal ions, such as Cu^{2+}, Zn^{2+}, Al^{3+}, Mn^{3+}, Cr^{3+}, Co^{3+}, and others (Ainscough et al., 1979; Cochran et al., 1984). The two lobes of the molecule, representing the N-terminal and C-terminal halves, are connected by a three-turn α-helix and have very similar folding and about 40% identity of amino acid sequence. The presence of internal homology suggests that the LF gene is evolved by gene duplication. There are small differences found in the two iron-binding sites. The N lobe has 6 disulfide bridges, whereas the C lobe has 10 (Anderson et al., 1989). The N-lobe site is thermodynamically less stable and more acid-labile than the C-lobe site (Brock, 1985). It releases iron faster (Kretchmar and Raymond, 1986). A major contributor to the greater stability and slower iron release of the C lobe may be the two extra connections between domains in the iron-binding pocket made by two disulfide bridges not present in the N lobe. These extra links may reduce thermal fluctuations or restrict or alter cleft opening and so increase stability (Anderson et al., 1989; Baker et al., 1987). With an aim to increase LF thermostability in transgenic bovine milk by site-directed mutagenesis, we need to establish a suitable system for use in expression of cloned human LF cDNAs.

This paper describes synthesis of recombinant human LF in yeast cells, purification of the LF from yeast culture broth, and characterization of the recombinant human LF in terms of iron binding and glycosylation.

MATERIALS AND METHODS

Materials. All restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, T4 DNA polymerase, mung bean nuclease, and peptide-*N*-glycosidase F were purchased from either Boehringer Mannhein Biochemicals (Indianapolis, IN) or New England Biolabs (Beverly, MA). DNA linkers, heparin Sepharose

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CL-6B, and agarose adipic acid hydrazide were products of Pharmacia LKB Biotechnology (Piscataway, NJ). Oligonucleotide primers were synthesized by Operon Technologies, Inc. (Alameda, CA). Peptone, yeast extract, yeast nitrogen base, and casamino acids were obtained from Difco Laboratory (Detroit, MI). Polyclonal antibodies against human LF (rabbit) and the secondary antibody (goat anti-rabbit) were from Accurate Chemical and Scientific Corp. (Westbury, NY). The enhanced chemiluminescence (ECL) Western blotting detection system and high-performance luminescence detection film were purchased from Amersham Corp. (Arlington Heights, IL). Human LF cDNA, available under the GenBank Accession No. M83202, was cloned in our laboratory. All chemicals used in the experiments were of reagent grade.

Strains and Plasmids. *Escherichia coli* DH5α (*rec A*$^-$, *F*$^-$, *endAl, gyrA96, thi-1, hsdR17, SUP44, relAl*) was used for plasmid transformation and preparation. Plasmid vector used for DNA recombinant manipulation was pTZ19R. Yeast strain AB116 (MATα, *leu2, trp1, ura3-52, prb1-1122, pep4-3, prc1-407*, cir°), a cir° derivative of BJ2168 (Jones, 1977), was used as the host for synthesis of human LF. Strain AB116, obtained from Dr. Anthony Brake (Chiron Corp., Emeryville, CA), is deficient of major proteases including protease A, protease B, carboxypeptidase Y, and aminopeptidase I. The yeast invertase signal DNA (SUC2 secretion signal coding sequence) and plasmid pCGY1444 were kindly supplied by Dr. Hitzeman of Genentech, Inc. (South San Francisco, CA). Vector pCGY1444 contains the chelating promoter, Trp$^+$ and Amp$^+$ selective makers, and yeast 2-μm and *E. coli* origins of replication. The chelating promoter is inducible with Cu^{2+}. The recombinant plasmids using vector pCGY1444 were expected to maintain a high copy number in yeast cells because of the 2-μm origin of replication and the presence of the dominant selectable marker Trp$^+$.

General DNA and Protein Methods. DNA subcloning, restriction enzyme digestions, gel electrophoresis, and DNA fragment recoveries from low melting agarose gels were performed according to current procedures (Ausubel et al., 1987). Polymerase chain reactions were performed on the basis of standard protocols (Innis et al., 1990). The SDS/PAGE procedure of Laemmli (1970) was used for protein separation. Electrophoresis was performed on a 1.5-mm-thick slab, and gels were stained with Coomasie Blue or silver nitrate. Immunoblotting with the enhanced chemiluminescence (ECL) system of detection was done according to current protocols (Harlow and Lane, 1988) and the recommended protocols for optimizing Western blots for ECL detection supplied by the manufacturer with some modifications. The proteins in SDS gels were transferred onto a nitrocellulose membrane using an ISS semidry electroblotter (Integrated Separation System, Hyde Park, MA). After being dried in the refrigerator overnight, the membrane was blocked by incubation for 1 h in 3% Tween 20 in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4) at 37 °C on a shaker. Following the removal of the blocking solution, 10 mL of primary antibody solution [1:10000 diluted in TPBS (0.5% Tween 20 in PBS)] was added to the membrane, and the incubation was continued for 30 min. The membrane was rinsed using two changes of TPBS and then washed once for 5 min with 5% nonfat dried milk in TPBS and twice for 10 min with TPBS at room temperature on a roller. After 10 mL of secondary antibody solution (horseradish peroxidase-conjugated goat anti-rabbit Ig diluted 1:40000 in TPBS) was added, the membrane was incubated at 37 °C on the shaker for 30 min, then rinsed twice with 5% nonfat dried milk in TPBS, and washed once for 5 min and twice for 10 min with TPBS at room temperature. Finally, the membrane was incubated in ECL reagents for 1 min and exposed to Hyperfilm-ECL for 20 s–1 min. In a series of antibody titration tests, the primary antibody was specifically bound to human LF in the dilution range 1:5000–1:15000. However, the secondary antibody yielded a high background (nonspecific binding). After being preincubated against a crude protein extract from yeast (Harlow and Lane, 1988), the secondary antibody showed specificity in the dilution range 1:25000–1:50000.

Construction of Yeast Expression Vectors for Synthesis of Human LF. Since the yeast cells permitted considerable flexibility in the use of the secretion signal sequence (Kaiser et al., 1987) and were capable of recognizing and processing the secretion signal of human interferons (Hitzeman et al., 1983), two different designs for synthesis of human LF in yeast were established as.

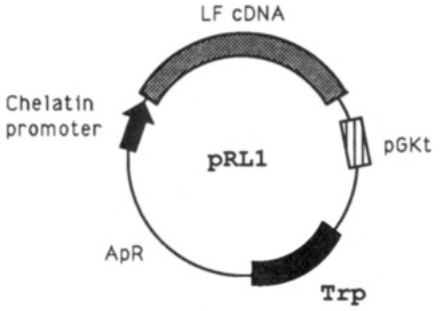
(1) Construct of Recombinant Plasmid for Expressing Human LF in Yeast with Human LF Secretion Signal Sequence. The full length of human LF cDNA in pRL100 was isolated by a double digestion of *Xho*I and *Kpn*I and cloned into pCGY1444 downstream from the chelating promoter by forced ligation. The junction region between chelating promoter and the 5′-end region of human LF cDNA was confirmed to be correct by DNA sequencing. The expression plasmid was named pRL1 (Figure 1A).

(2) Construct Recombinant Plasmid for Expressing Human LF in Yeast with Yeast Invertase Secretion Signal Sequence. To place human LF cDNA (without the leader sequence) under the influence of the invertase secretion signal sequence in the right reading frame, PCR was used to obtain the 5′ region of human LF cDNA (excluding the leader sequence). The 3′ region of human LF cDNA was isolated by *Kpn*I/*Acc*I digestions. Vector pIS9 containing the invertase leader sequence was opened with *Xho*I digestion, treated with mung bean nuclease to generate blunt ends, then digested with *Kpn*I. Finally, the two LF cDNA fragments and the linearized pIS9 were mixed and ligated with T4 DNA ligase. The recircularized plasmid was named pLFIS10 (Figure 1B,C). The junction regions (promoter/invertase signal sequence/LF cDNA) were confirmed to be in the right positions by DNA sequencing analysis.

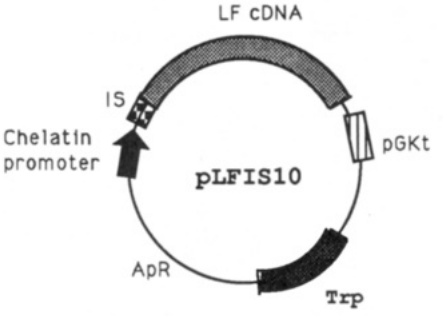
Growth Media and Conditions. *E. coli* DH5α was grown in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.5). Thirty to forty micrograms of ampicillin per milliliter was added for selective pressure on screening and maintaining the plasmid-containing bacterial colonies. YPD medium (1% yeast extract, 2% bactopeptone, 2% glucose, pH 7.0) was used for yeast AB116 growth and transformation. Yeast selective (Trp$^+$) minimal medium contained 0.67% yeast nitrogen base without amino acids and ammonium sulfate, 2% glucose, 0.02% L-leucine, and 0.005% uracil (for minimal plates, plus 2% agar). The selective minimal medium was supplemented with 2% casamino acids to form MMC medium (pH 7.0). For expression of human LF in yeast, the cells were inoculated in a 2-mL selective minimal medium and grown for 2 days at 30 °C on a shaker with a speed of 250 rpm. Then the cell culture was transferred to a 100-mL selective minimal medium in a 250-mL flask, and the same incubation conditions were continued for 2 days. After being transferred to 2.5 L of MMC medium in a 4-L flask, the cell culture was grown at 30 °C on a shaker at 150 rpm for 2 days (about 1×10^8 cells/mL). Finally, LF synthesis in yeast cells was induced by adding 0.2 mM $CuSO_4$ and 0.2% glucose. After 3–10 h of induction, the broth was saved by centrifuging the culture at 3000*g* to remove yeast cells.

Yeast Transformation and Screening Transformant Colonies. Yeast AB116 cells were transformed with the recombinant plasmid pRL1 and pLFIS10 carrying the human LF cDNA using the lithium acetate method (Ito et al., 1983). The transformed yeast cells were plated onto the selective minimal medium plates to select Trp$^+$ colonies. The plasmid DNAs of colonies growing on the selective plates were isolated and screened by PCR as described before (Liang and Richardson, 1992). The positive colonies identified by PCR were screened for the presence of human LF in yeast cells. Yeast cells from each colony were grown in 2 mL of selective minimal medium in a glass culture tube at 30 °C for 2 days. Then, 2 mL of MMC medium was added to each tube culture, and the incubation was continued for 30–36 h. Finally, the culture was induced to synthesize human LF by adding 0.3 mM $CuSO_4$. The yeast cells were harvested from 2 mL of yeast culture after 3–10 h of induction. Cell pellet of each tube was resuspended in 1 volume of glass bead disruption buffer (20 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 1 mM EDTA, 5% glycerol, 1 mM DTT, 0.3 M ammonium sulfate, 1 mM PMSF, 1× protease inhibitor mix) and 2 volumes of 2× SDS/sampling buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS 2% β-mercaptoethanol, 0.001% bromophenol blue). After addition of 1 volume of acid-washed glass bead (0.45 mm), the cell suspensions were vortexed at full speed for four intervals of 1 min each, with 1 min of cooling in ice between vortexing intervals. Following 5 min of boiling, the protein extracts were centrifuged

A.



B.



C.

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AATTCATGATGCTTTTGCAAGCTTTCCTTTTCCTTTTGGCTGGTTTTGCAGCCAAGATCTCGGCTGGCCGTAGGAGAAGGAGT----
    MetMetLeuLeuGlnAlaPheLeuPheLeuLeuAlaGlyPheAlaAlaLysIleSerAlaGlyArgArgArgArgSer----
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Yeast invertase signal sequence ↑ mature human LF

Figure 1. Constructs of recombinant plasmids. (A) Construction of recombinant plasmid pRL1 for expression of human LF in yeast with human LF secretion signal sequence. The expression unit is chelatin promoter–human LF cDNA–PGK terminator sequence. (B) Construction of recombinant plasmid pLFIS10 for synthesis of human LF in yeast with yeast invertase secretion signal sequence. The expression unit is chelatin promoter–invertase signal sequence–human LF cDNA without signal sequence–PGK terminator sequence. (C) Junction region between the DNA coding for invertase signal sequence and cDNA coding for mature human LF in pLFIS10.

for 5 min at 15000*g*. Twenty-five microliters of supernatant fluid from each sample was loaded onto SDS/polyacrylamide gels. The presence of human LF in the yeast cells was detected using Western blots with polyclonal antibodies against human LF (Figure 2).

Ultrafiltration. After removal of yeast cells, 2.6 L of yeast culture broth was passed through Whatman No. 1 filter paper and then diafiltered against 4 L of 20 mM Tris-HCl/1 mM EDTA (pH 8.0) in a tangential flow filtration system with a Pellicon mini-cassette filter (PLGC membrane with 10 000 MW cutoff) (Millipore Corp., Bedford, MA) at 4 °C to a final volume of 200 mL. The retentate was diluted to 400 mL with 50 mM Tris-HCl (pH 9.2)/1 mM PMSF/1 mM EDTA and then passed through Whatman No. 1 filter paper to remove cell debris. Finally, the retentate was passed over a 30-mL heparin Sepharose affinity column.

Chromatography. The chromatographies were performed according to standard procedures (Ausubel et al., 1987).

(*1*) *Heparin Affinity Chromatographies.* Thirty milliliters of heparin Sepharose was packed into a 35 cm × 1 cm column. After the column was equilibrated with 50 mM Tris-HCl, pH 9.2 (as running buffer), the retentate from the ultrafiltration was loaded into the column at a flow rate of 10 mL/h. Then, the column was washed with 300 mL of running buffer containing 0.1 M NaCl. Proteins were eluted from the column using a linear gradient of NaCl (0.1–1.0 M) in 250 mL of running buffer and collected into 80 tubes using a fraction collector (Figure 3A). The positive fractions identified by dot-blot immunodetection were pooled and dialyzed against 4 L of 25 mM Tris-HCl (pH 8.0)/1 mM EDTA buffer. Then, the protein solution was loaded to either a heparin affinity column with a running buffer of 50 mM Tris-HCl (pH 7.4) or an immunoaffinity column.

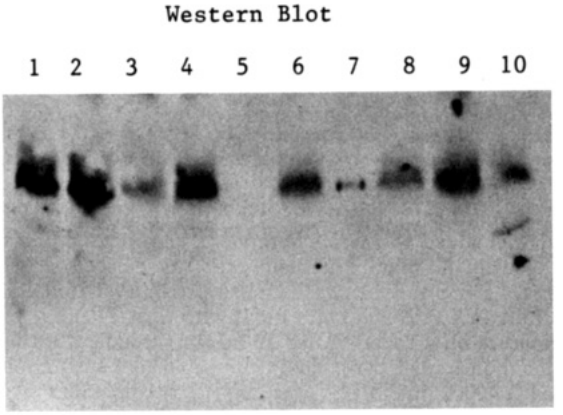

Figure 2. Immunodetection of human LF from yeast protein extracts of different transformed yeast colonies. The signals were obtained by enhanced chemiluminescence detection on Western blotting with polyclonal antibodies against human LF. In this experiment, the primary antibody was in a dilution of 1:10000; the secondary antibody was in 1:40000-fold dilution and preincubated against a crude protein extract from yeast cells. (Lane 7) Human milk LF (as positive control); (lanes 1–4) protein extracts from four indivdual colonies carrying pLFIS10; (lane 5) protein extract of yeast strain AB116 harboring vector pCGY1444 (as a negative control); (lanes 6, 8–10) protein extracts from four individual colonies carrying pRL1.

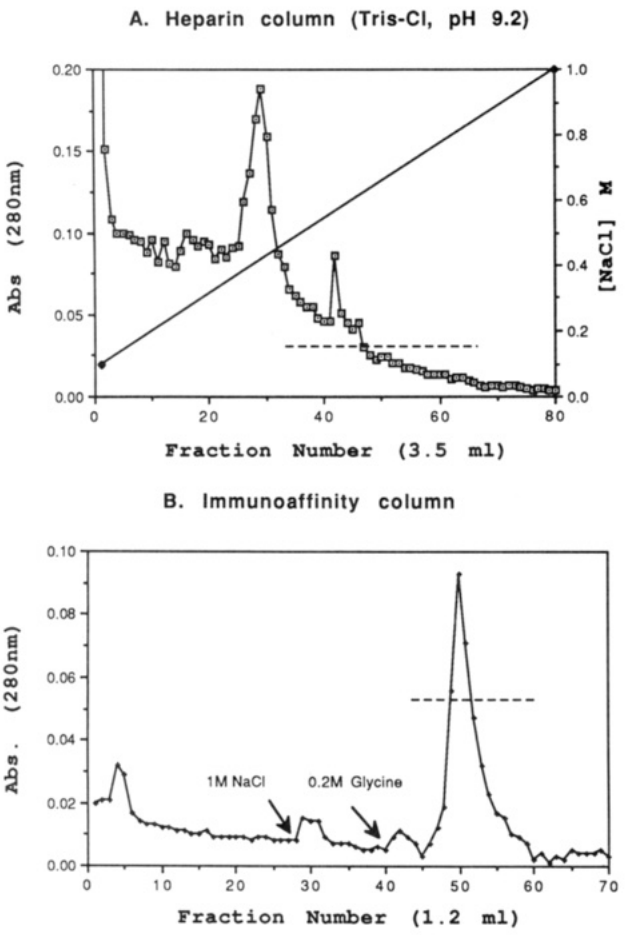

Figure 3. Elution profiles of affinity chromatographies. LF containing fractions identified by dot-blot immunodetection are indicated by dashed lines. (A) Elution profile of proteins from yeast broth through heparin chromatography with NaCl gradient in 50 mM Tris-HCl, pH 9.2. About 500 mg of proteins in 400 mL of buffer derived from 2.6 L of yeast broth was loaded onto the column, and about 5 mg of proteins (approximately 50% was human LF) was obtained from the positive fractions of the gradient elution. (B) Elution profile of recombinant human LF from immunoaffinity column. About 5 mg of proteins derived from the heparin column in 100 mL of buffer was applied onto the column. Because of the low binding capacity of the immunoaffinity column, we had to run three times the immunoaffinity chromatography, each time obtaining about 400 μg of highly pure LF.

(2) *Immunoaffinity Chromatography.* Polyclonal antibodies against human LF were coupled to agarose adipic acid hydrazide through aldehyde groups according to the protocols recommended by Pharmacia. The matrix (1.2 mL) was packed into a small column, washed with 20 volumes of high-salt buffer (1 M NaCl in 25 mM Tris-HCl, pH 7.4), and equilibrated with 25 mM Tris-HCl (pH 8.0). The column was run following the procedures described by Derisboug et al. (1990). One hundred milliliters of protein solution was passed through the column at a flow rate of 10 mL/h. The column was washed by 100 mL of 50 mM Tris-HCl (pH 8.0) containing 0.15 M NaCl, followed by 50 mL of the same buffer containing 1 M NaCl. Then LF was eluted by a 0.2 M glycine/HCl buffer (pH 2.8) containing 0.1 M NaCl. Each fraction was immediately adjusted to about pH 7.0 by adding 100 μL of 1 M Tris-HCl solution (pH 9.0) (Figure 3B). Fractions containing LF were pooled, dialyzed against distilled H_2O, and concentrated by lyophilization.

All protein samples containing the recombinant human LF obtained after each chromatographic column during the purification process were further analyzed using SDS/PAGE and Western blots (Figure 4).

Iron-Binding Assay. The chelating complex Fe^{3+}–nitrilotriacetate (Fe^{3+}–NTA) was prepared as described by Awai et al. (1979). Samples of human milk lactoferrin and the recombinant protein were dialyzed against 25 mM Tris-HCl (pH 7.6)/0.1 M $NaHCO_3$/0.1 M NaCl and saturated with iron by addition of 0.15 μM Fe^{3+}–NTA. Excessive Fe^{3+}–NTA was removed by dialysis of the iron-saturated samples against 25 mM Tris-HCl, pH 7.6/0.01 M $NaHCO_3$/0.1 M NaCl for 30 min. Then the absorption spectra of human LF and recombinant LF were measured using a Shimadzu UV-160 spectrophotometer (Figure 5).

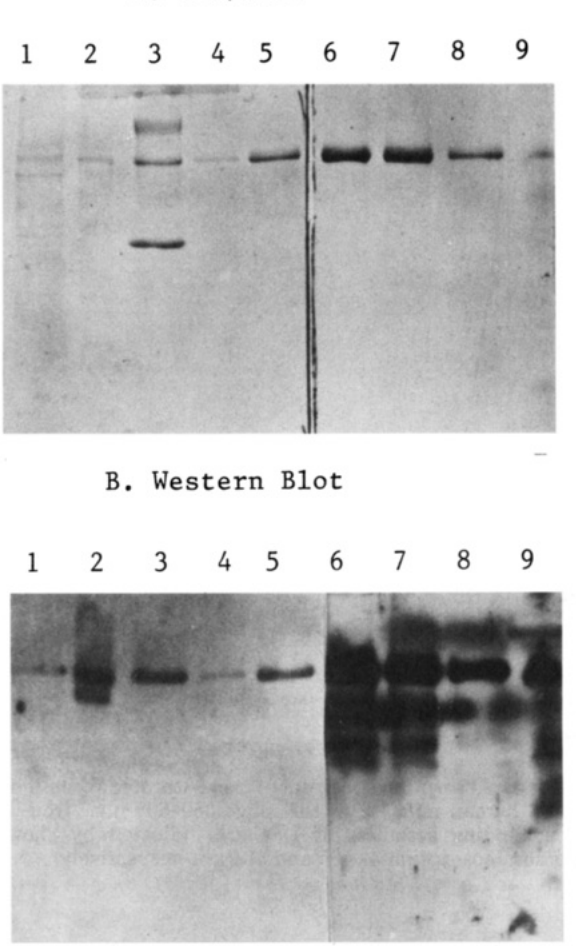

Figure 4. SDS/PAGE and parallel Western blot analyses of all protein samples containing the recombinant LF obtained in each chromatographic column during the purification process. On the SDS/PAGE, protein bands were visualized by Coomassie blue staining; on the Western blots, signals were detected by enhanced chemiluminescence. (Lane 1) Retentate of ultrafiltration; (lanes 2, 9) human milk LF; (lane 3) the pool of positive fractions from the first heparin column (pH 9.2); (lanes 4, 5) the positive peak from the immunoaffinity column; (lanes 6, 7) the pool of positive fractions from the second heparin column (pH 7.4); (lane 8) the pool of positive fractions from a small gel filtration column (Sephadex G-100).

Deglycosylation Tests. Deglycosylations of recombinant LF and human LF were performed under identical conditions using Peptide-N-Glycosidase F (PNGase F) according the protocols described by Tarentino et al. (1989). The proteins were boiled for 5 min in the presence of 0.5% SDS/0.1 M β-mercaptoethanol to expose fully all glycosylation sites, and then potassium phosphate buffer (pH 8.6) and NP-40 were added to stabilize the enzymes. After addition of enzymes, the samples were incubated at 37 °C overnight. The digestions were analyzed by SDS/PAGE (Figure 6).

RESULTS

Synthesis of Human LF in Yeast Cells. Crude protein extracts were prepared from yeast cells carrying either pRL1 or pLFIS10 and analyzed by SDS/polyacrylamide gel electrophoresis. The presence of human LF in

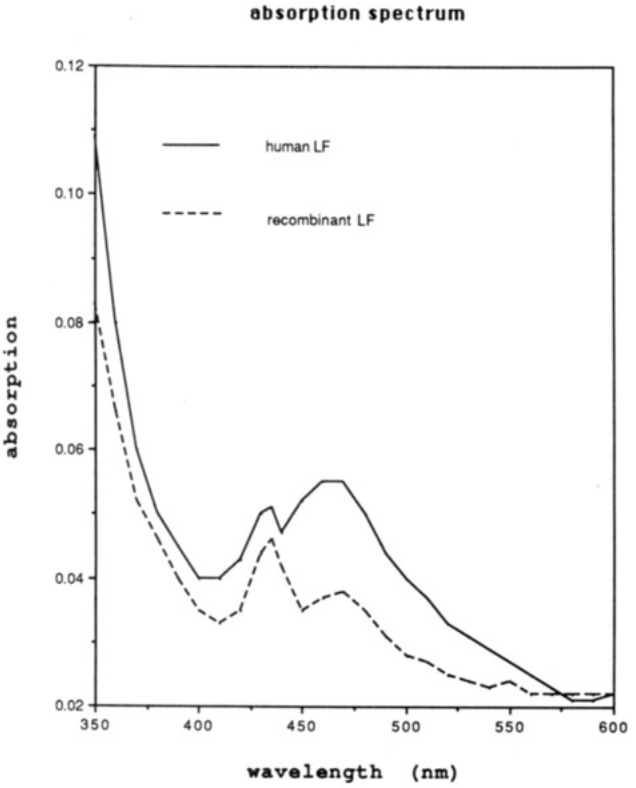

Figure 5. Absorption spectrum of iron-saturated recombinant LF and human milk LF in the range 350–600 nm. Iron- and copper-binding activities of LFs are indicated by showing maximum absorption at 465 and 434 nm, respectively.

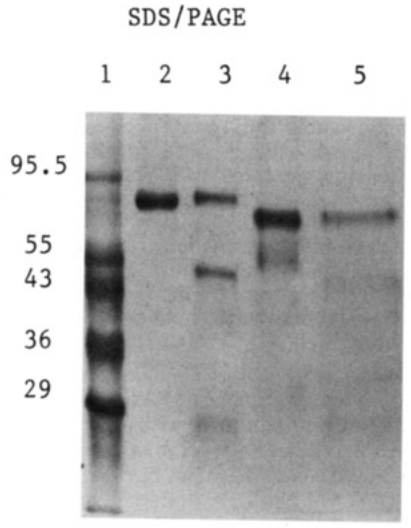

Figure 6. Deglycosylation test of recombinant LF and human milk LF. The protein bands on the SDS gel were visualized by silver staining. (Lane 1) Protein MW markers; (lane 2) human milk LF; (lane 3) recombinant LF (the low MW bands are degraded LF fragments); (lane 4) human milk LF digested with PNGase F; (lane 5) recombinant LF digested with PNGase F.

yeast cells of each colony was screened using immunoblotting detection. The results from the positive colonies are shown in Figure 2. The proteins from the transformant yeast colonies containing either pRL1 or pLFIS10 revealed a single band on the Western blot, which was not present in yeast AB116 (as negative control). This unique band had mobility similar to that of human LF on the SDS/PAGE, but it appeared as a large smeared band, not as sharp as that of human LF. This may be due to heterogeneities from posttranslational modifications. Because the protein extracts were obtained from ruptured yeast cells, the recombinant LF might have different levels of glycosylation at different stages of the secretory pathway. From Figure 2, one can see that the levels of LF are different in different colonies. The colonies carrying pLFIS10 had relatively higher levels of LF than those containing pRL1 in an overall comparison. The LF contents in colonies pRL1-9 and pLFIS10-2 were estimated to be 20 and 30 mg/L of culture, respectively, by scanning the Western blotting film with an enhanced laser densitometer (ULTROSCAN XL, LKB, Bronma). However, no recombinant LF could be detected from the media of yeast cultures on small scales (4–20 mL). Colonies of pRL1-9, pLFIS10-2, and pLFIS10-4 which produced the highest levels of LF as indicated in the immunodetection were selected for large-scale preparations of yeast cultures (2.5 L) to isolate recombinant LF from the yeast medium. The results indicated that secreted LF levels in colonies carrying pRL1 were quite low (detectable), and we were unable to isolate the LF protein from the yeast broth. The secreted LF levels in pLFIS10-2 and pLFIS10-4 were relatively high. We obtained about 500 μg of highly purified recombinant human LF per liter of yeast broth after a series of chromatographies. So, the concentrations of lactoferrin in the yeast culture medium were estimated to be 1.5–2.0 mg/L.

Purification of Recombinant Human LF from Yeast Broth. After ultrafiltration, the viscosity of the retentate was decreased. When passing through the heparin affinity column, most proteins were washed out except a few, including LF, which have some affinity for heparin. The elution profile of the heparin column is shown in Figure 3A. In the heparin column with the running buffer (50 mM Tris-HCl) at pH 9.2, the recombinant LF was mainly eluted between 0.5 and 0.7 M NaCl in a linear salt gradient as detected by dot-blot immunodetection. The proteins from the positive fractions were pooled and were found to contain two or three major bands upon SDS/PAGE (Figure 4A, lane 3). After dialyzing against 25 mM Tris-HCl (pH 8.0)/1 mM EDTA to remove the salt, the pooled protein solutions were applied to another heparin affinity column with a running buffer (50 mM Tris-HCl) at pH 7.4 or to an immunoaffinity column.

The elution profile of the immunoaffinity column is shown in Figure 3B. The peak containing recombinant LF was sharp, but the LF binding capacity of the column was low. Only about 400 μg of the recombinant LF could be obtained during each running of the column.

The protein isolated from yeast culture broth had mobility identical to that of human LF on SDS/PAGE (see Figure 4A). After heparin columns, the protein obtained from either filtration or immunoaffinity columns was highly pure, appearing as a single clear band on SDS/PAGE. In Western blots (Figure 4B), the protein was specifically recognized by the polyclonal antibodies against LF. The protein was further characterized in comparison with human milk LF.

Iron-Binding Activity. Both human LF and the recombinant protein (1 mg/mL) were saturated with Fe^{3+}–NTA. The iron-binding activity for the proteins was detected from the increase of absorbance at 465 nm and by the absorption spectrum shown in Figure 5. After iron saturation, the 465-nm absorbance of human LF increased from 0.020 to 0.056, and that of the recombinant LF was from 0.019 to 0.038. The absorption spectrum of the recombinant protein was very similar to that of human milk LF between 250 and 700 nm (data not shown). In the spectrum between 350 and 600 nm, two peaks were observed for each protein (Figure 5). The first peak was

at 465 nm, corresponding to the maximum absorption of iron-bound LF; the second peak was at 435 nm, which was the maximum absorption of copper-bound LF. The absorption ratio 280 nm/465 nm was 24 for the recombinant LF and 19 for human LF. The higher absorbance ratio 280 nm/465 nm or lower 465-nm absorbance for the recombinant LF in the comparison with those of human LF may be because the recombinant LF synthesized in yeast was partially saturated with copper, since the yeast broth contained 0.2 mM $CuSO_4$ for activating the chelating promoter. The recombinant LF was thus partially saturated with Cu^{2+} in the yeast medium previous to the iron saturation. This was further confirmed by the appearance of a strong absorption peak at 435 nm for the recombinant LF. To further evaluate the iron-binding activity of the recombinant LF, both human milk LF and the recombinant LF were dialyzed against acetic acid/sodium acetate buffer ($I = 0.2$) containing 40 mM EDTA and 0.2 M sodium phosphate (pH 4.0) overnight followed by dialysis against distilled H_2O to remove metal atoms from the proteins (Mazurier and Spik, 1980) and then resaturated with iron. The absorption spectra were essentially identical for the two proteins after the above treatments, but the maximum absorption peaks were shifted 10–15 nm. The maximum absorption of copper-bound LF appeared at 420 nm, and that of iron-bound LF was at 455 nm (data not shown). The reason for such absorption shifts is not clear. However, there is a report that LF binding copper together with oxalate shifted the absorption maximum from 434 to 424 nm (Smith et al., 1991).

Deglycosylation of LFs. To evaluate the glycosylation levels of the recombinant LF, deglycosylation of both recombinant LF and human LF was performed under identical conditions with PNGase F. The results of deglycosylation are shown in Figure 6. Before the digestion, the recombinant LF and human milk LF were estimated to be 82 kDa in the SDS/PAGE. After the deglycosylation with PNGase F, the molecular masses of both proteins were decreased to 76.5 kDa, which corresponds to the molecular mass of human LF polypeptide (692 aa). So, PNGase F appears to remove the glycan moieties of the LFs completely. The glycan moiety of the recombinant LF has essentially the same molecular mass as that of human milk LF.

DISCUSSION

Yeast *Saccharomyces cerevisiae* has been successfully used in producing foreign glycoproteins including hepatitis B virus surface antigen (Valenzuela et al., 1982), interferon (Hitzeman et al., 1983), and monoclonal antibody (Wood et al., 1985). Since LFs are glycoproteins, we used yeast as a host for expression of cloned human LF cDNA with the aim of establishing a suitable expression system for studies of the relationship between the structure and functionality of human LF. Two recombinant plasmids for producing human LF in yeast were constructed, pRL1 and pLFIS10. Although cells harboring either plasmid synthesized relatively high levels of human LF, which were estimated to be 3–5% of the total cellular protein, only colonies carrying pLFIS10 secreted considerable amounts of recombinant human LF into the yeast medium. The secreted LF was estimated to be 5–7% of the total LF synthesized by the yeast cells, with the majority of LF remaining intracellular or being associated with membranes and cell wall materials. This implies that yeast cells may not be able to secrete mammalian proteins as efficiently as their own secretory proteins in spite of the presence of the yeast invertase signal sequence in the precursor recombinant human LF. Yeast cells carrying pRL1, which does not harbor the yeast invertase signal DNA but does include the human LF signal sequence, basically failed to mediate secretion of human LF into the medium. This suggests that the secretion signal peptide of human LF is not recognized properly by yeast cells in the secretory pathway. However, the recombinant LFs in the protein extracts from yeast colonies carrying either pRL1 and pLFIS10 revealed large unique bands comigrating with that of human milk LF on SDS/polyacrylamide gels. It may be true that either form of recombinant LF was directed in going through the secretory pathway but accumulated in certain rate-limited steps, from where the proteins were glycosylated at different levels. In yeast colonies harboring pRL1, some human LF might even go through the secretory pathway and be associated with the cell membrane in the periplasm or attached to cell wall components.

Proteins secreted into the medium should be easily purified. However, purification of the recombinant human LF from the yeast medium was complicated by the fact that the protein seemed to form relatively stable associations with other yeast proteins, especially with a 40-kDa yeast protein (see Figure 4A, lane 3). After the first heparin column, the proteins pooled from the positive fractions mainly consisted of the recombinant LF and a 40-kDa protein. This 40-kDa protein could not be separated from the LF but eluted with the LF in gel filtration columns (Sephadex G-100). Thus, the second heparin column and the immunoaffinity column were needed for further purification of the recombinant LF.

The highly purified recombinant human LF had iron- and copper-binding activities, having a visible absorption spectrum similar to that of human milk LF. This suggests that the recombinant human LF is bioactive and has correct folding and proper iron-binding environments. Surprisely, after desaturation of LF with chelating agents (EDTA and sodium phosphate), the copper was not effectively removed. It suggests that human LF has very high affinity in binding copper, and such affinity for binding copper may be even higher than that for iron. Since copper is a major peroxidative factor in dairy products, human LF with high affinity for binding copper may have great potential significance to the dairy industry.

The results of deglycosylation with PNGase F indicate that the recombinant LF is glycosylated. The recombinant protein band is always sharper than the milk LF band in all SDS/polyacrylamide gels. This implies that the glycosylation of secreted recombinant LF is highly uniform, since human milk LF is known to be heterogeneous in glycosylation (Spik et al., 1982).

Trp^+ selective pressure was found to be critical for synthesis of human LF in yeast. At the beginning, we used YPD-rich medium for preparing large-scale yeast cultures, but the LF levels in the medium were very low. Later, we used MMC medium, because casamino acid does not contain tryptophan, resulting in relatively high levels of LF in the yeast medium. However, after a few months, the large-scale yeast culture preparation resulted in much lower LF levels than before. About 100 μg of purified LF was obtained from 1 L of yeast broth. The reason for this is not fully clear. One major reason may be the antimicrobial effect of LF. LF may be toxic to yeast cells, especially when LF is synthesized and accumulated inside the yeast cells. Although the chelating promoter is inducible, the basal level of transcription regulated by the chelating promoter is relatively high (the induction level of transcription is 5-fold the basal level) (Etcheverry et

al., 1986). Stress of toxic LF on yeast cells may favor mutations, especially the reverse mutation for pep4-3 and prb-1122 which are nonsense mutations (Jones, 1991), and progeny cells which have lost the plasmid.

Because of the significance of human LF, one of our aims in the expression of cloned human LF cDNA in yeast cells is to explore the possibility of producing large quantities of human LF using yeast fermentations. At the synthetic level as well as the secreted level of human LF, this yeast system has not produced sufficient levels for industrial production. Further investigation is necessary to increase the expression levels of human LF. A good yeast strain and proper promoters will be necessary before this goal is obtainable. One feasible approach is to screen yeast colonies for supersecreting mutants after the cells are treated with mutagens, because many mammalian secretory proteins are not secreted efficiently from wild-type yeast strains (Hitzeman et al., 1983; Lemontt et al., 1985). Secretion of recombinant human LF at high levels may reduce the level of LF toxicity to the yeast cells.

Although the present yeast system has not been optimized for synthesis of human LF, the successful expression of human lactoferrin cDNA here provides a suitable tool for systematic study of the relationship between structure and function of LF by means of site-directed mutagenesis. Since the two lobes of LF can function independently and the iron-binding site in the N-terminal lobe of LF is thermodynamically less stable and more acid-labile than that in the C-terminal lobe of LF (Brock, 1985), LF thermostability may be increased by introducing some extra disulfide bridges in the N-terminal lobe or by replacing the N-terminal lobe with the C-terminal lobe by DNA manipulation.

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