

Expression, Characterization, and Biologic Activity of Recombinant Human Lactoferrin in Rice

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ABSTRACT

Background: Lactoferrin has been suggested to have many biologic activities, such as facilitating iron absorption and having antimicrobial and antiinflammatory effects. In humans, several of these activities are likely to only be facilitated by human lactoferrin because they depend on the binding of human lactoferrin to specific receptors. Rice may be a useful vehicle to introduce recombinant human lactoferrin to infant foods because it has low allergenicity and is likely to be safer than using microorganisms or transgenic animals.

Methods: Recombinant human lactoferrin was expressed in the rice cell culture system, and its biologic activity was assessed by iron-binding and -releasing properties, antimicrobial activity, and binding and uptake to Caco-2 cells. The authors also compared the stability of recombinant and native human lactoferrins against heat, low pH, and in vitro digestion.

Results: Biologic activity of rice-expressed recombinant hu-

man lactoferrin was similar to that of native human lactoferrin. Heat-treated proteins retained their functional activities except with severe treatment at 100°C for 8 seconds, which disturbed the iron-binding capacity of recombinant human lactoferrin. Both types of proteins retained their functional activities between pH 2 and 7.4. After in vitro digestion, 50% of both proteins were detectable by enzyme linked immunosorbent assay. The remaining native and recombinant lactoferrins retained antimicrobial and Caco-2 binding and uptake activities.

Conclusions: The results indicate recombinant human lactoferrin has stability similar to native human lactoferrin when exposed to thermal treatment, pH treatment, and in vitro digestion, suggesting it may be active when added to infant formula.

JPGN 36:190–199, 2003. Key Words: Recombinant human lactoferrin—Transgenic rice—Iron—Antimicrobial activity—Caco-2 cells—Infant formula. © 2003 Lippincott Williams & Wilkins, Inc.

Lactoferrin (Lf), an 80 kDa, single polypeptide protein, is abundant in human milk (1) and is believed to play a role in the breast-fed infant's ability to resist infectious disease. Lf has two high affinity iron-binding sites and has been suggested to be biologically active in the infant intestine based on its capacity to retain iron at low gastric pH (pH ~3) (2) and its resistance against digestion, as supported by the detection of intact human Lf (HLf) in the feces of breast-fed infants (3). Proposed biologic activities of Lf include antimicrobial properties (4), facilitating iron absorption (5), immunomodulating effects (6), and antiviral and anticancer activities (7). We have previously reported the presence of a receptor specific for HLf in infant intestine (8). Binding studies using brush border membrane vesicles from human infant small intestine revealed that bovine Lf does not bind to

the receptor to the same extent as HLf. Therefore, some of these biologic activities may be exerted only by HLf.

Recombinant HLf (rHLf) has been produced in transgenic cows (9) and *Aspergillus* (10). An attractive application for rHLf is as a supplement to infant formula, which would help formula-fed infants attain resistance to infectious disease that breast-fed infants currently possess. We developed a rice expression system for rHLf that yields high levels of expression. An advantage of using the rice expression system is that contaminating proteins are rice proteins, which are a normal part of the diet of infants and children and, therefore, impose little risk of allergenicity or toxicity. We evaluated the biologic activity of rice rHLf: iron-binding capacity, iron-releasing property, antimicrobial activity, and binding and uptake by human intestinal Caco-2 cells in culture. We recently cloned an Lf receptor gene from human fetal small intestine and found that Caco-2 cells expressed the Lf receptor, suggesting that this cell line is a useful model to assess receptor-mediated binding and uptake of Lf (11). We further assessed the stability of rHLf expressed in rice. Stability of rHLf expressed in *Aspergillus*

Received January 14, 2002; accepted September 24, 2002.

Supported in part by BioSTAR grant #S98-13.

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against heat and low pH treatments was previously evaluated with regard to iron-binding capacity (12). The iron-binding capacity was retained after heat treatment <70°C for 20 seconds (12). Low pH treatment of rHLf had no significant effect on iron-binding properties (13). Stability against digestion was not previously assessed for rHLf. Native HLf (nHLf) is known to be resistant against proteolytic digestion compared with other proteins of the transferrin family (14). Thus, rHLf may also possess resistance against digestion similar to nHLf. We evaluated the biologic activity of rHLf from rice after thermal treatment, low pH treatment, and in vitro digestion.

MATERIALS AND METHODS

Synthetic Lactoferrin Gene and Plasmid Construction

The *HLf* gene (GenBank accession #U07643) was synthesized by Operon Technologies (Alameda, CA, U.S.A.). Four hundred thirteen of the 693 codons in the *HLf* gene were modified, but the amino acid sequence remained the same. The plasmid containing the codon-optimized gene named *Lac-ger* was digested with *Sma* I and *Xho* I. The fragment containing the synthetic lactoferrin gene was cloned into pAPI137 (15), digested with *Sac* I, blunted with T4 polymerase, and digested with *Xho* I. The resulting plasmid was designated pAPI160 (Fig. 1).

Rice Transformation

The procedure of microprojectile-mediated rice transformation (16) was followed with minor modifications. Calli were raised from mature TP309 rice seeds, selected (2–4 mm in diameter), and placed on N6 medium supplemented with 0.3 mol/L mannitol and 0.3 mol/L sorbitol for 20 hours before bombardment. Gold particles were coated with plasmids, pAPI160, and pAPI76 (6:1) and bombarded into rice calli with the biolistic PDC-1000/He system (Bio-Rad, Richmond, CA, U.S.A.). Plasmid API76 contained hygromycin B phosphotransferase as selectable marker. After 2 days, bombarded

calli were transferred to N6 selection medium supplemented with 30 mg/L hygromycin B. After 45 days, transgenic calli that could be distinguished from nontransgenic calli were selected. The selected calli were dissected into 5 to 10 parts and placed back in the selection medium. The selection process was repeated twice to ensure that all transgenic cell lines were homogeneous.

Cell Culture and Induction of rHLf Expression

Transgenic cell lines were propagated on N6 medium by biweekly subculture. Induction of gene expression followed the principle of Huang et al. (15). To induce expression of rHLf, calli were incubated in a sugar-free liquid medium for 3.5 days. After incubation, calli were collected by centrifugation. Induced calli were ground with mortar and pestle in phosphate buffered saline (PBS) to obtain a rice homogenate. The homogenate was centrifuged at 220 x g at 4°C for 10 minutes to obtain a cleared crude extract. Total protein was quantified with the Bradford assay. The expression level of rHLf in each cell line was determined by enzyme linked immunosorbent assay (ELISA) and Western blot analysis.

Purification of rHLf

Transgenic calli were induced in sugar-free medium (1 g/2 mL of induction solution) for 2.5 days and kept in the dark at room temperature with shaking at 270 x g. Clumps of rice calli were dissected and incubated in PBS with 1 mol/L NaCl (1 g/2 mL) for 2 days at room temperature with shaking at 270 x g, and then were filtered through two layers of cheesecloth. The filtrate was collected and centrifuged at 33,000 x g at 4°C for 40 minutes. The delipidated supernatant was adjusted to 0.5 mol/L NaCl with 20 mmol/L Tris, pH 7.4, and centrifuged at 33,000 x g for 20 minutes. The supernatant was loaded onto an equilibrated (0.5 mol/L NaCl in 20 mmol/L Tris, pH 7.4, binding buffer A) Con A Sepharose column (4 mL/min; Amersham Pharmacia Biotech, XK26, Piscataway, NJ, U.S.A.). The column was washed with binding buffer A and eluted with elution buffer (binding buffer A + 0.1 mol/L mannoside). Fractions containing rHLf were pooled and dialyzed against 50 mmol/L NaHPO₄, pH 8 (Amicon concentrator, Millipore, Bradford, MA, U.S.A.), and loaded onto an equilibrated (0.4 mol/L NaCl in 50 mmol/L sodium phosphate, pH 8.0, binding buffer B) SP-Sepharose column (4 mL/min; Amersham Pharmacia Biotech). The column was washed with the binding buffer B and eluted with 1 mol/L NaCl in 50 mmol/L sodium phosphate, pH 8.0, and dialyzed against PBS.

N-terminal Amino Acid Sequencing

Purified rHLf from rice culture cells was separated by 10% SDS-PAGE followed by electroblotting to a PVDF membrane (Bio-Rad). The membrane was stained with 0.1% Coomassie brilliant blue R-250 in 40% methanol and 1% glacial acetic acid for 1 minute and destained in 50% methanol. The blot was thoroughly washed with H₂O, and rHLf was sequenced using an ABI 477 Sequencer by Edman chemistry at the Molecular Structure Facility (University of California at Davis).

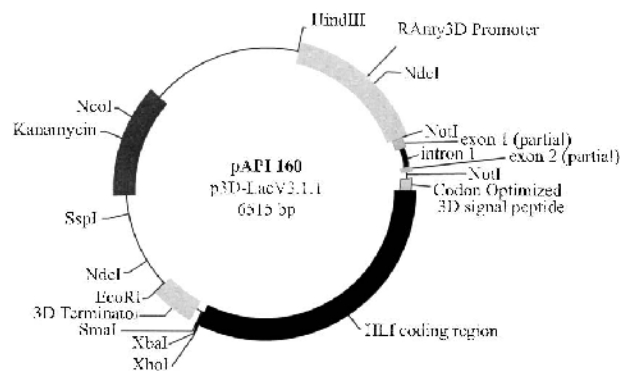


FIG. 1. Schematic representation of the expression plasmid, pAPI160. This plasmid contains a rice α -amylase 3D promoter, the synthetic *HLf* gene, and a 3D gene terminator.

Detection of Glycosylation and Determination of Sugar Content

Glycosylation of purified nHLf and rHLf was analyzed using an Immuno-Blot kit for glycoprotein detection (Bio-Rad). The molecular weight of rHLf and nHLf was analyzed by matrix-assisted laser desorption ionization–mass spectrometry (MALDI-MS; PE Applied Biosystems, Voyager System, Shelton, CT, U.S.A.).

Determination of Isoelectric Point of Lactoferrin

Reverse isoelectric focusing (IEF) gel electrophoresis was performed with a precast Novex IEF gel, pH 3 to 10. Purified rHLf (30 µg) was loaded, and electrophoresis was done at 100 V for 50 minutes and at 200 V for 20 minutes. The gel was fixed in 136 mmol/L sulfosalicylic acid and 11.5% trichloroacetic acid (TCA) for 30 minutes and stained in 0.1% Coomassie brilliant blue R-250.

Native Human Lactoferrin

Native HLF was isolated from human milk by a heparin–Sephacrose affinity column equilibrated in 50 mmol/L Tris–hydrochloride buffer (pH 8.0) with a NaCl gradient as described previously (17).

Iron Saturation

Native HLF and rHLf were incubated with 2 mol/L excess ferric iron ($\text{FeCl}_3\text{:NTA} = 1\text{:}4$) and sodium bicarbonate ($\text{Fe:HCO}_3^- = 1\text{:}1$) for 1 hour at room temperature. Excess free iron was removed with a PD-10 desalting column (Amersham Pharmacia Biotech), and iron saturation was determined by the $A_{280}\text{-to-}A_{460}$ ratio (18).

Radiolabeling

Holo-HLf was labeled with ^{125}I by the Iodogen method (19), and apo-HLf was labeled with ^{59}Fe (Amersham Pharmacia Biotech) following the iron saturation method described previously.

Heat Treatment

Holo-HLf in PBS (1.0 mg/mL) was treated in the following conditions: 1) 62°C for 15 minutes, 2) 72°C for 20 seconds, 3) 85°C for 3 minutes, or 4) 100°C for 8 seconds.

Low pH Treatment

Holo-HLf in PBS (1.0 mg/mL) was adjusted to pH 2, 4, 6, or 7.4 by the addition of 1 mol/L HCl and incubated for 1 hour at room temperature. The pH was then adjusted to 7.4 with 1 mol/L NaHCO_3 . A desalting column removed free iron that was released from HLF.

In Vitro Digestion

In vitro digestion was performed as previously described (20) with minor modifications. Holo-HLf (1 mg/mL) and human serum albumin (HSA; 1 mg/mL as a positive control) in PBS were adjusted to pH 3.8 with 1 mol/L HCl, and porcine pepsin (Sigma, St. Louis, MO, U.S.A.) was added to a pepsin-to-protein ratio of 0.08. The mixtures were incubated for 30 minutes at 37°C with shaking at 450 x g. After incubation, the pH was adjusted to 7.0 with 1 mol/L sodium bicarbonate. Porcine pancreatin (Sigma, 8× USP) was then added to make a pancreatin-to-initial protein ratio of 0.016. After 1-hour incubation at 37°C with shaking at 450 x g, enzymes were inactivated with heat (85°C for 3 minutes).

Enzyme Linked Immunosorbent Assay

Human Lf and HSA were quantified either before or after treatment by sandwich ELISA and competitive ELISA, respectively, and the survival ratio after each treatment was calculated. For quantitation of HLF, an 80,000-fold dilution of rabbit anti-HLf IgG (primary antibody, Dako, Carpinteria, CA, U.S.A.) in 50 mmol/L NaHCO_3 buffer, pH 9.6 (coating buffer), was coated onto microtiter plates (Nalge Nunc International, Rochester, NY, U.S.A.). Samples were diluted in PBS containing 0.1% Tween 20 (PBS/Tween) and added to the plate; HLF bound to the plate was detected with a 20,000-fold dilution of horseradish peroxidase (HRP)-conjugated rabbit anti-HLf IgG (Bioscience Resource Project, Kennebunk, ME, U.S.A.) in PBS/Tween. For quantitation of HSA, the plates were coated with 50 ng of HSA in the coating buffer. Samples were preincubated with a 20,000-fold dilution of rabbit anti-HSA IgG (Dako) in PBS/Tween for 2 hours and then added to the microtiter plate. The antibody bound to the coated HSA was detected with a 20,000-fold dilution of HRP-conjugated donkey anti-rabbit IgG (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) in PBS/Tween. TMB substrate (Pharmingen, San Diego, CA, U.S.A.) was used to develop the color for quantification; the reaction was stopped with 1 mol/L sulfuric acid; and the absorbance at 450 nm was measured using a Multiskan Ascent plate reader (Labsystems Inc, Franklin, MA, U.S.A.). The data were analyzed with ASCENT (version 2.3; Labsystems Oy, Helsinki, Finland) and a four-variable logistic model.

Iron-Releasing Properties

Holo-nHLf and holo-rHLf were dialyzed against buffers at pH 7.4 (0.05 mol/L Tris/HCl, 0.2 mol/L NaCl), 6.0 (0.05 mol/L MES, 0.2 mol/L NaCl), 3.0 to 5.5 (0.05 mol/L sodium acetate, 0.2 mol/L NaCl), and 2.0 to 2.5 (0.05 mol/L glycine/HCl, 0.2 mol/L NaCl) for 12 hours at room temperature, and the pH was slowly increased to 7.4. Iron saturation was determined by the $A_{280}\text{-to-}A_{465}$ ratio.

Iron-Binding and Iron-Holding Capacity

Apo-HLf was incubated with varying amounts of iron (iron-to-HLf molar ratio = 0, 0.2, 0.4, 0.8, 1.6, 2, 3, and 4) for 1 hour at room temperature. After free iron was removed using a PD-10 desalting column, iron saturation was determined by the

A₂₈₀-to-A₄₆₅ ratio. For in vitro digestion, ⁵⁹Fe-HLf was used to determine iron-holding capacity.

Antimicrobial Activity

Holo-HLf in PBS (5 µg/mL), sterilized by passing through a membrane filter with a pore size of 0.2 µm, was incubated with 10⁴ colony forming units (cfu) enteropathogenic *Escherichia coli* (EPEC)/µL in 100 µL sterile synthetic broth (Difco, Detroit, MI, U.S.A.) containing 0.1% dextrose at 37°C. Bacterial growth was determined by monitoring optical density at 630 nm from 1 to 24 hours.

Binding and Uptake to Caco-2 Cells

Caco-2 cells (50,000/well, American Type Culture Collection, Rockville, MD, U.S.A.) were seeded and grown in minimum essential medium (GIBCO, Rockville, MD, U.S.A.) containing 10% fetal bovine serum in 24- or 48-well tissue culture plates for 3 weeks. For binding studies, Caco-2 cells were incubated with varying concentrations (0–2 µmol/L) of ¹²⁵I-HLf with or without a 100-fold excess of unlabeled nHLf for 2 hours at 4°C, and cells were washed 5 times with ice-cold PBS. Cells were solubilized with 0.5 mL of 0.1% SDS, and radioactivity was quantified in a gamma counter. For uptake studies, 0.4 µmol/L of ¹²⁵I-HLf was incubated with Caco-2 cells for 0 to 24 hours at 37°C, and cells were washed and dissociated in the same way as in the binding study. TCA solution (24%) was added to the dissociated cells, and free iodine was removed by centrifugation. Free and protein-bound ¹²⁵I were quantified separately to evaluate how much HLf was degraded in the cells.

SDS-PAGE, Western Blot Assays

Samples (1–2 µg protein/well) underwent electrophoresis during reducing conditions, and gels were stained with Coomassie blue. For Western blot analysis, proteins were transferred to a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech). The membrane was blocked with 5% BSA, 0.05% Tween-20 in PBS for 1 hour at room temperature, washed 3 times with gentle shaking (15 minutes each), and incubated with HRP-conjugated anti-HLf antibody (1:20,000 dilution, Biorad) for 1 hour at room temperature. After another wash, immunologically active HLf was detected by ECL Western blot detection reagent (Amersham Pharmacia Biotech).

Statistical Analysis

Results are presented as means ± SD. Statistical comparisons were made using Student *t* test.

RESULTS

Expression of rHLf in Rice Culture Cells

One hundred one transgenic rice cell lines were screened for rHLf expression by Western blot analysis and ELISA. Expression levels from four representative

lines are shown in Table 1. Expression of rHLf represented 2% to 4% of the total soluble protein in transgenic calli. All transgenic cell lines were ranked according to expression level, and the top 17 cell lines were selected and propagated for purification of rHLf.

Purification of rHLf From Transgenic Rice Culture Cells

During the rHLf purification, we found that if the induced calli were cultured in PBS with 1 mol/L NaCl for 2 more days, most of the rHLf was secreted into the culture solution, thus eliminating the need to disrupt cell walls. This procedure also enriched rHLf to about 10% of secreted proteins (Table 2). Because Lf is a glycoprotein, the secreted proteins from cell culture were passed through a Con A column to remove proteins that do not contain branched mannose and carbohydrate with terminal mannose or glucose (Fig. 2). Sixty-seven percent of the rHLf applied to the Con A column was recovered, which corresponds to 66% of total protein in this fraction (Table 2). To remove the remaining non-rHLf proteins, an SP-Sepharose column was used, and the purified rHLf formed a single band in gel electrophoresis (Fig. 2).

Biochemical Properties of rHLf and nHLf

Sequencing analysis determined that the N-terminal sequence rHLf was identical to that of nHLf, i.e., Gly-Arg-Arg-Arg-Ser-Val-Gln-Trp()Ala. The blank bracket represents an undefined cycle, most likely the result of an unmodified cysteine residue that could not form a stable derivative during Edman degradation. Isoelectric focusing determined that both forms of Lf migrated to the same location, indicating similar surface charges. MALDI-MS analysis indicated that the mass of rHLf was less than that of nHLf (78.9 vs. 80.6 kDa). This slightly lower molecular weight of rHLf is likely the result of a lower degree of glycosylation as confirmed by glycoprotein staining analysis, which showed that rHLf did not stain as well as nHLf (data not shown). Iron-binding capacity and pH dependency for iron release was identical for rHLf and nHLf (Fig. 3A and B). The antimicrobial activity of apo-HLf against EPEC was also identical for rHLf and nHLf (Fig. 3C); both types of HLf inhibited the growth of EPEC to 20% of control. Recep-

TABLE 1. Expression of recombinant human lactoferrin (rHLf) in transgenic cell lines

Cell lines	Calli (g)	Protein (mg)	Total rHLf (µg)	rHLf/protein %
160-1	6	21.3	597	2.8
160-11	4	21.0	453	2.2
160-12	3	10.5	453	4.3
160-20	4	14.3	313	2.2

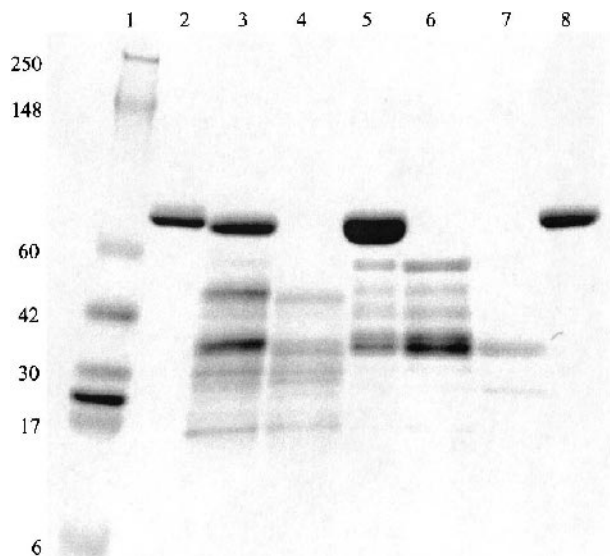


FIG. 2. SDS-PAGE analysis of fractions obtained during purification of rHLf from transgenic rice. Lane 1, Molecular weight marker; Lane 2, Native HLF; Lane 3, Crude extract loaded to Con A Sepharose; Lane 4, Unbound fraction from Con A Sepharose; Lane 5, Elution from Con A column (loaded to SP Sepharose); Lane 6, Unbound fraction from SP-Sepharose; Lane 7, Wash fraction of SP-Sepharose; Lane 8, Elution from SP-Sepharose (rHLf).

tor binding of rHLf to the human intestinal Caco-2 cell line was saturable and specific, indicating that rHLf bound to the Lf receptor (Fig. 3D). The binding constant (K_d) was similar for rHLf and nHLf, but the number of binding sites was lower for nHLf. The amount getting into the cells, however, was identical for rHLf and nHLf (Fig. 3E). This finding may be caused by the difference in glycosylation, i.e., rHLf may bind selectively to a pseudoreceptor because of the different glycans, but the pseudoreceptor cannot take up rHLf into the cell.

Stability Against Various Treatments

SDS-PAGE, Western Blot Assay

SDS-PAGE and Western blot assays revealed no difference between nHLf and rHLf at 62°C for 15 minutes, 72°C for 20 seconds, and 85°C for 3 minutes, but at 100°C for 8 seconds, rHLf almost completely lost its immunologic activity, whereas nHLf still maintained detectable immunoreactivity (Fig. 4A). A slightly smaller immunoreactive molecule (~70 kDa) was detected after exposure of nHLf to pH 2 and 4 and of rHLf to pH 2 (Fig. 4B). Native HLF and rHLf resist digestion by pepsin (at pH 3.8) and pancreatin, whereas HSA is completely digested after *in vitro* digestion (Fig. 4C). Western blot analysis revealed that immunoreactivity was also substantially maintained after digestion. Although some smaller molecules were generated during digestion of

HLf, most of the immunologically detectable HLF retained its intact size.

Survival and Iron-Binding Properties

The survival ratio of HLF was >90% for almost all treatments, except at 100°C for 8 seconds and for *in vitro* digestion (Table 3). The high temperature precipitated both types of HLF, and only about 10% of HLF was detectable. Approximately 50% of rHLf and nHLf was detectable after *in vitro* digestion, which correlates well with what was observed by SDS-PAGE and Western blot analysis. There were no significant differences in survival ratio between nHLf and rHLf for any of the treatments. More than 80% of iron was still bound to rHLf and nHLf after thermal treatments with the exception of that at 100°C for 8 seconds. In the 10% of HLF that survived after 100°C for 8 seconds, iron saturation of nHLf was >90%, whereas that of rHLf was only approximately 40%. Low pH did not have any significant effect on iron-holding capacity for either type of HLF (Table 3). After *in vitro* digestion, iron-holding capacity was retained at approximately 40% to 60%. Although this is significantly lower than control samples, both types of HLF are still able to hold substantial amounts of iron to exert their function.

Antimicrobial Activity Assay

Antimicrobial activity is presented as percent inhibition of EPEC growth by 5 µg/mL of HLF after 24 hours of incubation (Table 3). Both nHLf and rHLf significantly inhibited EPEC growth after all treatments compared with no protein, with the exception of rHLf treated at pH 6.0, where variance was high. There was no significant difference in antimicrobial activity between nHLf and rHLf for any of the treatments. Thus, heat, low pH, and *in vitro* digestion did not affect the antimicrobial activity of HLF. It is interesting to note that although the amount of HLF decreased by 50% after digestion, the antimicrobial effect was similar to that of undigested HLF.

Binding and Uptake by Caco-2 Cells

Although there was a trend that nHLf had somewhat fewer binding sites than rHLf, these differences were not statistically significant for any of the treatments except for 100°C (Table 4). When treated at 100°C, a significantly higher amount of rHLf bound to Caco-2 cells (compared with either control rHLf or with nHLf treated at 100°C), most likely by nonspecific binding resulting from more rHLf being denatured than nHLf, which was indicated by the decrease in iron-holding capacity for rHLf treated at 100°C (Table 3). Digestion did not appear to affect the K_d , but it decreased the number of binding

TABLE 2. Purification of recombinant human lactoferrin (rHLf) from transgenic rice calli

Steps of purification	Total protein (mg)	Total rHLf (mg)	Milligrams rHLf/milligrams protein	% Recovery	Fold purification
Cell extract and centrifugation	1,239	120	0.096	100	0.0
ConA column	120	80	0.660	67	7.1
SP column	69	65	0.930	54	9.7

sites (Table 4). Although the decrease in the number of binding sites after digestion was statistically significant, receptor-bound HLf still may be biologically active. Uptake properties were similar for ^{125}I -nHLf and ^{125}I -rHLf for all treatments. The milder heat treatments (i.e., 62°C, 72°C) and digestion decreased uptake compared with the control sample, but the highest heat treatment (100°C) increased uptake. The free iodine level in the cells, which reflects degradation of HLf, was approximately 30% in

the untreated sample. There was no significant difference between nHLf and rHLf. HLf treated at 100°C or with low pH was degraded more than untreated HLf, which might indicate that denaturation of HLf caused by heat treatment or by low pH treatment will make the protein more susceptible to intracellular proteases. Total iron uptake from nHLf was twice as high as that from rHLf, although this finding was seen only after in vitro digestion (Table 4).

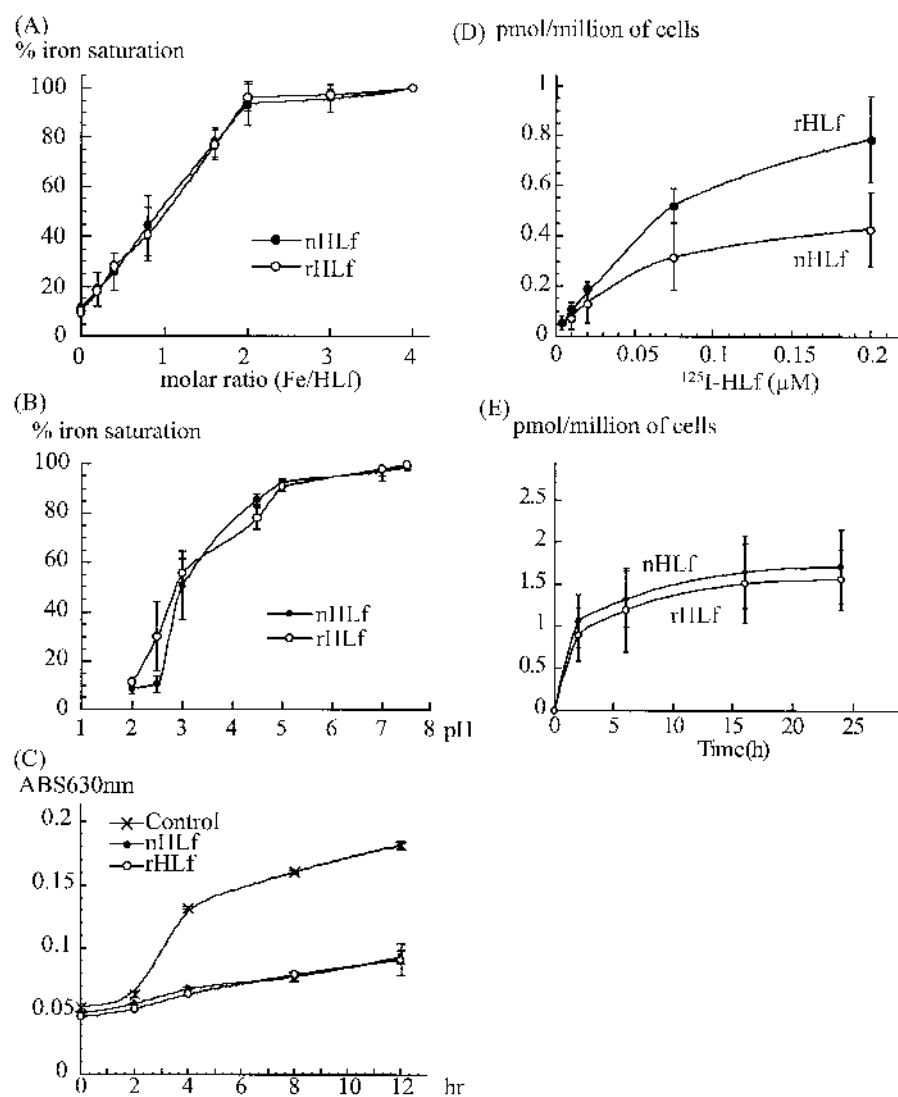


FIG. 3. Comparison of functional and biologic activities between nHLf and rHLf. (A) Iron-binding capacity. (B) pH-dependent iron-release properties. (C) Antimicrobial activity against enteropathogenic *E. coli* (EPEC). Either nHLf or rHLf, 1 $\mu\text{g}/\text{mL}$, was incubated with 10^6 cfu/mL of EPEC in the synthetic broth, and bacterial growth was measured at various times by absorbance at 630 nm. Control sample does not contain any protein except for 10^6 cfu/mL EPEC. (D) Binding of nHLf and rHLf to Caco-2 cells. (E) Uptake of nHLf and rHLf to Caco-2 cells.

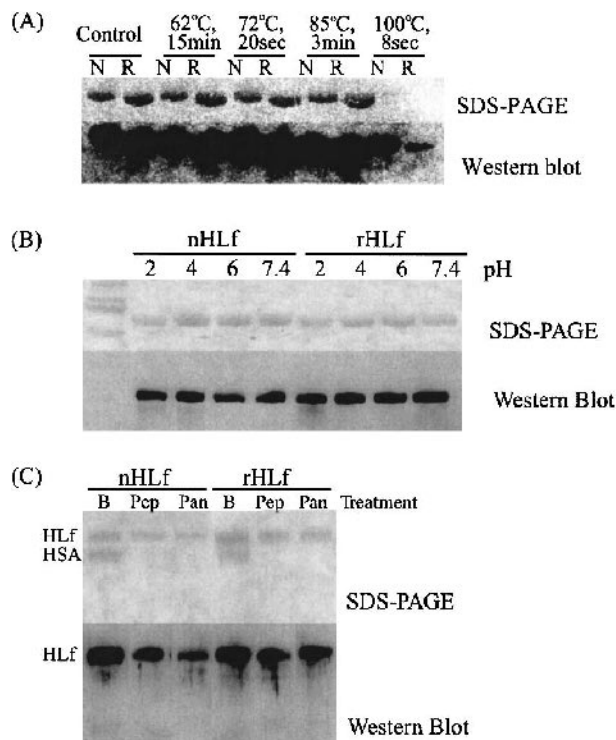


FIG. 4. SDS-PAGE and Western blot analysis after various treatments. **(A)** Thermal treatments. N indicates nHLf, and R indicates rHLf. **(B)** pH treatments. **(C)** In vitro digestion. B indicates before the digestion; Pcp, pepsin treatment at pH 3.8 for 30 minutes; Pan, pepsin treatment at pH 3.8 for 30 minutes; and then followed with pancreatin treatment at pH 7 for 60 minutes.

DISCUSSION

Our studies demonstrate that rHLf expressed in rice is biologically active and behaves similarly to nHLf as shown by iron binding, or pH-dependent iron-releasing properties, antimicrobial activity, and binding to and uptake by human intestinal cells. Furthermore, these functional activities are substantially retained after heat exposure or exposure to low pH and are still detectable after in vitro digestion.

To enhance production efficiency of rHLf, we modified 60% of the codons in the *HLf* gene. It has been shown that production of recombinant proteins in transgenic barley grain can be enhanced by using a synthetic gene with the codon preference of the barley gene (21). The codon optimization changed either an A or T at the third position of the codons to G or C. However, the amino acid sequence of the codon-optimized Lf remained unchanged by these modifications. We observed that the degree of glycosylation of rHLf was somewhat lower than that of nHLf. The lower degree of glycosylation could result in the differences in properties beyond those reviewed in this study.

Mata et al. (12) studied the iron-binding capacity of rHLf expressed by *Aspergillus* after heat treatment; it

remained >75% after most treatments applied, except for rHLf heated at 85°C for 20 minutes, when it decreased to 50%. They suggested that one of the most suitable treatments would be 72°C for 20 seconds, which corresponds to mild pasteurization. In our studies, 72°C for 20 seconds seems to be one of the most suitable heat treatments for iron binding and release, antimicrobial activity, and receptor binding and uptake. Exposure to 100°C for 8 seconds was the most severe treatment and it caused partial precipitation, but this result may not properly reflect industrial conditions, which include closed and continuous-flow systems for heat treatment. In our open system near the boiling point, water is bubbling, and the water–air interface area significantly increases. HLF present in the interface between solution and air may be more susceptible to denaturation because of physical shock by bubble disruptions. HLF, which remained in the soluble fraction after 100°C treatment, even though it was a small amount, can still bind to iron, particularly nHLf. Therefore, it is possible that the conformation of the protein may remain in intact form and that the protein has antimicrobial activity, receptor binding activity, and so on, in vivo.

Iron binding has been the only property investigated for pH stability of rHLf, and it remains normal as it does for nHLf (18,22,23). The key features for the pH-dependent iron-binding properties of HLF have been investigated with site-directed mutagenesis (24–26). The decreased iron binding is attributed solely to the loss of a histidine ligand, whereas protein conformation and interdomain interactions are unchanged (24). Using mutants that selectively ablate the iron-binding function in either lobe, Ward et al. (25) demonstrated that the unique iron-binding stability of the protein was imparted primarily by the carboxyl-terminal domain that functioned cooperatively to stabilize iron binding to the amino-terminal domain of HLF. Because rHLf expressed in rice maintains all of these structures of nHLf, it is logical that the pH-dependent iron-binding property is retained. We verified that the iron-binding and -releasing properties of rHLf are maintained, as they are for nHLf, after various pH treatments. The effect of low pH treatment on the antimicrobial activity of HLF has not been studied previously. The antimicrobial effect depends on the iron-binding capacity of Lf for some strains (27), but there also is a domain in HLF with bactericidal activity that is distinct from the iron-binding region (28,29). Therefore, it is important to evaluate the antimicrobial activity even though the iron-binding capacity is properly maintained. In this study, we demonstrated that the antimicrobial activity was maintained after all treatments we tested.

Stability of rHLf against digestion has not been investigated previously. Lf has been shown to be comparatively resistant against proteolytic degradation in vitro (14,30). Trypsin and chymotrypsin were shown to be ineffective in digesting Lf; some large fragments were formed, and proteolysis was clearly limited. Studies of

TABLE 3. Effect of various treatments on survival ratio, iron saturation, and antimicrobial activity of human lactoferrin (HLf)

Treatment	Type of HLf	Survival ratio (%)	Iron saturation (%)	Antimicrobial activity (% EPEC growth)
Control	No protein			100.0 ± 8.5
	Native	100.6 ± 5.2	97.8 ± 7.1	59.3 ± 1.5‡
62°C, 15 min	Recombinant	97.3 ± 4.1	93.3 ± 7.0	64.7 ± 2.5‡
	Native	96.5 ± 8.6	93.0 ± 6.5	59.0 ± 8.0‡
72°C, 20 s	Recombinant	91.7 ± 9.7	84.6 ± 5.9	56.0 ± 6.0‡
	Native	95.0 ± 7.2	94.3 ± 3.7	70.0 ± 6.0‡
85°C, 3 min	Recombinant	91.3 ± 7.0	90.1 ± 6.3	60.0 ± 13.0‡
	Native	91.2 ± 8.2	85.9 ± 6.1	62.0 ± 7.0‡
100°C, 8 s	Recombinant	90.9 ± 9.0	89.4 ± 6.1	60.0 ± 15.0‡
	Native	10.7 ± 4.7†	91.1 ± 7.6*	ND
pH 7.4	Recombinant	9.1 ± 4.9†	37.3 ± 8.9†	ND
	Native	97.1 ± 6.1	94.2 ± 6.7	69.3 ± 9.0‡
pH 6.0	Recombinant	93.2 ± 5.6	96.0 ± 3.5	85.3 ± 5.0‡
	Native	92.7 ± 5.7	93.6 ± 4.4	70.3 ± 9.0‡
pH 4.0	Recombinant	92.0 ± 4.7	93.8 ± 4.7	84.3 ± 23.4
	Native	96.4 ± 4.4	95.2 ± 5.1	70.7 ± 12.1‡
pH 2.0	Recombinant	95.7 ± 7.2	95.0 ± 9.2	75.0 ± 13.0‡
	Native	97.5 ± 8.1	96.3 ± 5.5	75.7 ± 12.1‡
In vitro digestion	Recombinant	94.1 ± 8.2	93.7 ± 8.7	66.7 ± 10.5‡
	Native	48.0 ± 8.2†	48.7 ± 7.7†	65.6 ± 2.3‡
	Recombinant	47.5 ± 12.0†	60.1 ± 6.0†	76.4 ± 17.2‡

* $P < 0.05$: comparison between native and recombinant in the same treatment.† $P < 0.05$: comparison between control and each treatment for the same type of HLf.‡ $P < 0.05$: comparison against control (no protein) for antimicrobial activity.EPEC, *Escherichia coli*; ND, not determined.

breast-fed and formula-fed infants have subsequently shown that significant quantities of intact HLf can be found in the stool (3,31). Thus, nHLf can survive digestion by pepsin and pancreatic enzymes in the infant gut

and possibly perform biologic functions in the gastrointestinal tract. In this study, we demonstrate that rHLf is able to survive a sequential in vitro digestion with pepsin and pancreatin. The antimicrobial activity of rHLf was

TABLE 4. Effect of various treatments on binding and uptake of human lactoferrin (HLf) by Caco-2 cells

Treatment	Type of HLf	Binding		Uptake		
		Binding sites (pmol/10 ⁶ cells)	K _d (nmol/L)	¹²⁵ I-HLf (pmol/10 ⁶ cells)	⁵⁹ Fe-HLf (pmol/10 ⁶ cells)	Degradation (%)
Control	Native	0.94 ± 0.30	192.5 ± 74.3	1.74 ± 0.46	6.81 ± 2.53	34.7 ± 11.4
	Recombinant	1.75 ± 0.59	410.7 ± 191.9	1.71 ± 0.46	6.03 ± 2.94	32.3 ± 14.8
62°C, 15 min	Native	1.90 ± 0.59	114.0 ± 98.6	1.27 ± 0.33	ND	25.3 ± 14.5
	Recombinant	2.09 ± 1.06	335.0 ± 133.9	0.90 ± 0.44†	ND	24.3 ± 16.5
72°C, 20 s	Native	1.84 ± 0.63	163.6 ± 53.5	0.90 ± 0.33†	ND	19.7 ± 5.5
	Recombinant	1.66 ± 0.82	324.0 ± 209.2	0.83 ± 0.46†	ND	21.3 ± 16.2
85°C, 3 min	Native	1.21 ± 0.61	109.8 ± 98.8	1.58 ± 0.51	ND	33.7 ± 22.9
	Recombinant	3.91 ± 2.21	352.0 ± 332.6	1.40 ± 0.63	ND	11.3 ± 7.0
100°C, 8 s	Native	1.44 ± 0.64*	107.4 ± 85.3	2.10 ± 0.98	ND	52.0 ± 6.6†
	Recombinant	8.70 ± 1.21†	890.4 ± 432.7	3.22 ± 1.08†	ND	40.3 ± 8.7
pH 7.4	Native	1.02 ± 0.38	255.5 ± 142.1	2.04 ± 0.41	ND	39.7 ± 6.4
	Recombinant	1.38 ± 0.29	684.8 ± 467.1	2.04 ± 0.24	ND	37.3 ± 2.6
pH 6.0	Native	0.92 ± 0.14	140.6 ± 114.4	2.09 ± 0.20	ND	45.2 ± 6.1
	Recombinant	1.04 ± 0.34	208.0 ± 192.1	2.14 ± 0.35	ND	52.8 ± 1.3†
pH 4.0	Native	0.90 ± 0.13	46.8 ± 8.4	1.91 ± 0.21	ND	47.5 ± 3.4
	Recombinant	1.38 ± 0.37	613.2 ± 573.5	2.21 ± 0.15	ND	52.3 ± 13.2
pH 2.0	Native	0.93 ± 0.09	168.2 ± 100.8	2.23 ± 0.68	ND	52.0 ± 16.4
	Recombinant	1.15 ± 0.05	444.7 ± 163.4	1.73 ± 0.25	ND	52.3 ± 1.5†
In vitro digestion	Native	0.03 ± 0.01†	94.0 ± 64.8	0.82 ± 0.47	3.10 ± 0.45*†	27.3 ± 15.0
	Recombinant	0.03 ± 0.01†	230.0 ± 156.0	0.66 ± 0.29†	1.42 ± 0.27†	52.3 ± 17.0

* $P < 0.05$: comparison between native and recombinant in the same treatment.† $P < 0.05$: comparison between control and each treatment for the same type of HLf.

ND, not determined.

retained after in vitro digestion, indicating that rHLf may have antimicrobial activity similar to nHLf in the infant gut. We observed that holo-HLf was partially digested, which caused iron to be released from HLf. Bacteria may use this iron as a nutrient and thus grow better. However, no difference in antimicrobial activity was observed before and after digestion. One possibility for this finding would be the generation of antimicrobial peptides. HLf partially digested by pepsin generates small peptide fragments, such as lactoferricin, a peptide that has potent bactericidal effects, possibly restraining the growth-enhancing effect by released iron. In the Caco-2 cell uptake study with or without in vitro digestion, we used ^{125}I -HLf and ^{59}Fe -HLf (Table 4). The digested samples were applied to a desalting column to remove free ^{125}I and ^{59}Fe that had been released during in vitro digestion. Considering that approximately 50% of ^{59}Fe still bound to both types of HLf that survived digestion (Table 3), if all ^{59}Fe in the cells is transported as HLf-bound ^{59}Fe , uptake of ^{59}Fe should be the same as the uptake of ^{125}I -HLf. However, uptake of ^{59}Fe from rHLf and that from nHLf was two and four times as high as uptake of ^{125}I -rHLf and that of ^{125}I -nHLf, respectively (Table 4). Uptake of either type of HLf, thus, is not enough to carry all Fe to cells. This was also the case for undigested HLf (^{59}Fe -HLf uptake was 3.5–4 times as high as ^{125}I -HLf). There are several possible mechanisms to explain how this could happen. First, HLf may be recycled back to the apical side after releasing iron into cells, and, therefore, uptake of ^{125}I -HLf was less than uptake of ^{59}Fe . If this is the case, nHLf may be recycled more readily than rHLf because more ^{125}I -rHLf was in the cells than ^{125}I -nHLf. Second, HLf may release Fe when it binds to the cell membrane receptor, and Fe uptake may mainly depend on the free iron transport pathway via divalent metal transporter-1 (DMT-1). If this is the case, binding of HLf to the receptor would not be primarily for taking up iron, and it would make sense that the intracellular ^{125}I level is much less than the intracellular ^{59}Fe level. In this case, our results suggest that nHLf releases Fe more easily than rHLf when it binds to the receptor. These possibilities still remain to be explored. However, it is evident that both forms of HLf effectively deliver iron to the intestinal cells.

In summary, we have demonstrated that rHLf expressed in rice possesses biologic activities similar to nHLf, with the exception of binding to Caco-2 cells, to which rHLf binds more than nHLf, and that it retains those activities after common thermal processing conditions that occur during the manufacture of food products. Further, we have demonstrated rHLf retained biologic activity after low pH treatment (simulating gastric pH conditions) and also largely after in vitro digestion, although there was a tendency toward less degradation and more uptake by nHLf relative to rHLf. Taken together, these observations indicate that rHLf may retain most of its biologic activity in vivo throughout the infant's gas-

trointestinal tract and in a manner similar to nHLf. Therefore, the expression of biologically active recombinant proteins in rice offers a feasible solution for the large-scale and safe production of recombinant proteins and may be a valuable source of HLf for infant formula supplementation.

Acknowledgment: The authors thank Frank E. Hagie, Jr. and Raymond L. Rodriguez for their support of this study.

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