Protein Expression and Purification 25, 41–49 (2002)
doi:10.1006/prep.2001.1607

Expression, Characterization, and Purification of Recombinant Porcine Lactoferrin in *Pichia pastoris*

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Received July 23, 2001

Recombinant porcine lactoferrin (rPLF) was synthesized in *Pichia pastoris* using a constitutive promoter from the glyceraldehyde-3-phosphate dehydrogenase gene. Strains expressing rPLF with its own signal sequence or with that from the yeast α-mating factor (α-MF) were able to produce and secrete rPLF, but levels were consistently higher using α-MF constructs. In contrast, *P. pastoris* strains that expressed rPLF without a signal sequence produced the protein in an insoluble intracellular form. Increasing the initial pH of shake-flask culture medium from 6.0 to 7.0 or adding ferric ions to the medium (to 100 μM) resulted in significant improvements in expression of rPLF from *P. pastoris*. Expression levels (approximately 12 mg/L) were much higher than those observed from *Saccharomyces cerevisiae* strains (1–2 mg/L). *P. pastoris*-secreted rPLF was isolated and purified via a one-step simple procedure using a heparin column. The molecular size (78 kDa), isoelectric point (8.8–9.0), N-terminal amino acid sequence, and iron-binding capability of rPLF were each similar to that of native milk PLF.

The use of prokaryotic and eukaryotic systems for the expression of recombinant proteins is well established. Yeast expression systems are often favored for their simplicity of handling and economy. *Saccharomyces cerevisiae* is well established as one yeast system, which has been utilized to express a variety of recombinant proteins (1–3). Alternatively, the methylotrophic yeast, *Pichia pastoris*, has recently been used with an increasing frequency. More than 200 of exogenous proteins have been expressed thus far (4), and certain advantages including fermentation methods and efficient promoters are well documented (5, 6). Commonly used promoters for the *P. pastoris* expression system include the methanol-inducible promoter from the alcohol oxidase I gene (*AOX1*)[2] (5, 7, 8) and the constitutive promoter from the glyceraldehyde-3-phosphate dehydrogenase gene (*GAP*) (9, 10). Methanol-inducible promoters have created potential concerns regarding the use of methanol, which is toxic and highly flammable (9). Alternatively, the *GAP* promoter is constantly active without regard to the carbon source (9). Therefore, using the *GAP* promoter, expression of recombinant proteins can be reasonably achieved unless the expressed molecule is toxic to its host.

Lactoferrin (LF), an iron-binding protein with a molecular weight of approximately 78 kDa (11), was originally found in milk. The biological functions of LF include bacteriostasis, transcriptional regulation,

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[2] Abbreviations used: AOX1, alcohol oxidase I; GAP, glyceraldehyde-3-phosphate dehydrogenase; LF, lactoferrin; rPLF, recombinant porcine lactoferrin; LB, Luria–Bertani; α-MF, α-mating factor; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PBST, PBS–Tween 20; ECL, enhanced chemiluminescence; 2D, two-dimensional; IEF, isoelectric focusing; IgG, immunoglobulin G; mPLF, milk PLF; EGF, epidermal growth factor; IGF, insulin-like growth factor.

1046-5928/02 $35.00

facilitation of intestinal iron uptake and excretion, growth promotion of intestinal epithelial cells, and the modulation of inflammation of the mucosal surface and systemic vascular compartments (12, 13). Expression levels of human LF from *S. cerevisiae* have been unsatisfactory. Liang and Richardson (14) speculated that recombinant human LF may be toxic to the yeast host (14), although LF has been successfully expressed in insect cells without apparent toxicity (15, 16). Here we describe the successful synthesis of recombinant porcine LF (rPLF) in *P. pastoris* using the constitutive *GAP* promoter. We also describe the effects of varying signal sequences and shake-flask culture conditions on rPLF secretion levels from *P. pastoris* expression strains.

MATERIALS AND METHODS

Materials

All restriction enzymes were purchased from either Boehringer Mannheim (Mannheim Germany) or New England Biolabs (Beverly, MA). T4 DNA ligase, calf alkaline phosphatase, and *pfu* DNA polymerase were purchased from Promega (Madison, WI). The heparin–Sepharose CL-6B column was obtained from Pharmacia-LKB (Piscataway, NJ). Peptone, yeast extract, yeast nitrogen base, and casamino acids were obtained from Difco Laboratory (Detroit, MI). Chemiluminescence system of ECL detection reagent was obtained from NEN Life Science (Boston, MA). PCR was performed with a Perkin–Elmer 2400 via *pfu* DNA polymerase. *Escherichia coli* DH5_ (GibcoBRL, Gaithersburg, MD) and TOP 10 (Invitrogen, Carlsbad, CA) were employed for plasmid transformation. Plasmids PGAPZ B and pGAPZα B and *Pichia pastoris* SMD1168 (pep4, His) were purchased from Invitrogen.

Growth Media and Conditions

E. coli DH5_ and TOP 10 were cultured in 1 L broth (1% tryptone, 0.5% yeast extract, and 1% NaCl, pH 7.5). As required, 25 μg/ml of zeocin was added to a low-salt LB medium containing 0.5% NaCl to select and maintain the bacterial colonies containing plasmid. The YPD plate (1% yeast extract, 2% Bacto peptone, 2% agar, and 2% dextrose) was used to support *P. pastoris* growth. Transformants of *P. pastoris* were selected from YPDS agar plate (1% yeast extract, 2% Bacto peptone, 2% dextrose, 2% agar, and 1 M sorbitol) supplemented with 100 μg/ml zeocin. For protein expression, yeast were transferred to YPD medium and grown at 30°C over time.

Construct of Recombinant Plasmid for the P. pastoris Expression System

A porcine mammary gland cDNA library was initially screened and the cDNA corresponding LF was constructed in —gt11 followed by a subcloning into *Sfi*I/*Not*I site of pGEM11Z (−) to produce pLF3-1-2. The C-terminal of LF cDNA was amplified with a RT-PCR and ligated to pBS at the *Sma*I site named pLF5. To produce PIGLTF, a *Bgl*II/*Hin*dIII fragment from pLF3-1-2 clones was inserted into a pLF5 clone at the *Bgl*II/*Hin*dIII site (17).

A full-length of PLF cDNA or mature PLF cDNA was amplified from PCR utilizing P1/P2 or P3/P2 or P4/P2 as a primer pair (Table 1) in which PIGLTF was served as a template (17). P1 primer (5′ oligonucleotide) was designed to contain a *Sfu*I restriction site and the exact coding region of PLF cDNA was situated between positions 58 and 82 (Table 1). P2 primer (3′ oligonucleotide) that allowed a *Xba*I site was designed to counter the sequence of PLF cDNA between 2095 and 2115 (Table 1). The P3 primer contained an *Sfu*I site and coding region of PLF cDNA between positions 1 and 29 (Table 1). Finally, P4 primer containing the *Xho*I site was reconstructed including *Kex2* and *Ste13* cutting sites with a coding region of PLF cDNA between positions 58 and 85 (Table 1). Following PCR amplification with a P1/P2 or P3/P2 primer pair, a fragment of PLF cDNA of roughly 2120 bp was extracted, hydrolyzed by *Sfu*I and *Xba*I, and subcloned into a pGAPZ B vector (Invitrogen). The pPSLF (with porcine signal sequence) and pMLF (without porcine signal sequence) were thus prepared. Via a P4/P2 primer pair, the PCR-amplified cDNA fragment (without signal sequence) was ligated to pGAPZα B vector following a *Xho*I/*Xba*I digestion to generate plasmid pαLF (Table 1). The major difference between expression vectors pGAPZ B and pGAPZα B was that the latter contained prepro sequence of yeast α-mating factor (α-MF). Both plasmids contained a GAP promoter, AOX1 terminator, and zeocin gene selection marker, whereas the expression cascade contained GAP-PLF-AOX1. Orientation of the inserted PLF cDNA or junction region between PLF and promoter/terminator was confirmed by restriction enzyme digestion or by DNA sequencing.

TABLE 1

Sequences of Synthesized Oligonucleotide Primers Used in PCR Amplification of PLF

Primer	Sequence
P1	5′-GCTTCGAACAAAACACAATGGCCCCTAAGAAAGG-GGTTCGAT-3′
P2	5′-GCTCTAGATTACCTCATCATGAAGGCACA-3′
P3	5′-GCTTCGAACAAAACACAATGAAGCTCTTCATCCC-CGCCCTGCTGTT-3′
P4	5′-CCGCTCGAGAAAAGAGAGGCTGAAGCTGCCCCT-AAGAAAGGGGT

Electroporation of P. pastoris with Plasmid pPSLF, pMLF, or pαLF

Linearized plasmid pPSLF, pMLF, or pαLF, prepared at 37°C for 3 h in a *Avr*II digestion mixture, was electroporated and incorporated into the chromosomal DNA of *P. pastoris* using an electroporator (MicroPulser, Bio-Rad). Briefly, YPD medium (100 ml) containing 0.1 ml of overnight culture of *P. pastoris* (SMD 1168) was cultured overnight to an OD_{600} of 1.0–1.4. The cells were washed twice and resuspended with 0.2 ml of ice-cold sterile 1 M sorbitol. The suspension of competent cells (80 μl) and of linearized DNA (1 μg/μl, 5 μl) was kept on ice, mixed, transferred to an electroporation cuvette (0.2 cm), and pulsed once (according to the manufacturer's instructions) followed by addition of 1 ml of 1 M sorbitol. The cells were then plated (YPDS with 100, 500, or 1000 μg/ml zeocin), and the plates were incubated at 30°C for 2 to 3 days.

Screening Transformant Colonies

Zeocin resistance colonies were selected and screened for both intracellular and secreted rPLF (if any). To detect protein expression of rPLF in *P. pastoris*, cells were inoculated into a 5 ml YPD medium and cultured for 2 days at 30°C on a shaker at 250 rpm. Yeast cultures were harvested and lysed using glass beads (2, 14). Following the lysis, the cell supernatant and insoluble pellets were collected, respectively. The insoluble pellets were suspended in 1 vol breaking buffer containing 7 M urea and incubated on ice for 60 min. The culture medium was concentrated prior to Western blot analyses. To study the effect of pH, 1 ml of overnight cultured cells was added into 100 ml of medium at various pH and grown for up to 4–5 days. The pH of the medium was adjusted using a 0.1 M phosphate buffer to 6.0, 6.4, 6.8, 7.0, and 8.0, respectively. $FeCl_3$ was added to a 100 ml culture medium (at initial pH 7.0) to determine the effect of ferric on cell growth. The final concentration of PLF in culture medium samples was determined by an ELISA using purified milk PLF as a standard.

Western Blot Analysis of rPLF in Media and Cell Extracts

SDS–PAGE was performed on a 0.75-mm slab gel (18). For Western blot analysis, proteins were transferred onto a Hybond C nitrocellulose membrane. Subsequently, 1% BSA–PBS-blocked membrane (2 h at 4°C) was incubated with a rabbit anti-PLF (1:2000 diluted in BSA–PBST containing 0.05% Tween 20) for 1 h at room temperature. The membrane was washed followed by an addition of a peroxidase-conjugated goat anti-rabbit IgG. Either a diaminobenzidine (19) or an ECL reagent was used as a developer. The latter was exposed to a ECL-Hyperfilm for 1 to 10 s.

Purification of Porcine Lactoferrin from Culture Media

The cells were cultured overnight and transferred (50 ml) into 3000 ml of YPD medium and incubated for 2 days. Three liters of *P. pastoris* culture medium was concentrated and partially purified using a 30 to 40% saturated ammonium sulfate. The precipitate was suspended in a 5 mM Tris buffer (pH 7.5) containing 50 mM NaCl and then dialyzed against the same buffer. The desalted fraction was then passed through a heparin–Sepharose affinity column and eluted with a step gradient of NaCl (0.2–0.6 M). The collected fractions containing immunoreactive PLF were pooled and concentrated using an Amicon Centriplus (Millipore, Bedford, MA). SDS–PAGE and two-dimensional (2D) gel electrophoresis were then used to characterize the recombinant and native milk LF. After staining with amido black, the single band corresponding to rPLF was then analyzed for its N-terminal amino acid sequence using a microsequence technique (22).

2D Gel Analysis of Purified rPLF

The method of O'Farrell was used for 2D gel analysis (20–22). Protein was loaded onto the first dimension of isoelectric focusing (IEF) gel containing Bio-Rad ampholytes and electrophoresed for 16 h at 400 V with an additional 1 h at 800 V. The IEF gels were subsequently loaded onto a 9% SDS–polyacrylamide slab gel to perform the second dimension and were processed as described (21, 22). The method previously established in our laboratory (22) proved to be reproducible as the other proteins, such as heat shock protein and haptoglobin (22), were evaluated as an IEF standard.

Iron-Binding Assay

The recombinant PLF was dissolved in 25 mM Tris buffer (pH 7.5) containing 10 mM $NaHCO_3$ followed by an addition of a final 100 μM of $FeCl_3$ and incubated at 37°C for 30 min. Unbound iron was filtered through a Bio-gel P-6 column (Bio-Rad, Hercules, CA). Absorption spectra ranged from 280 to 680 nm of samples with and without Fe^{3+} were scanned and recorded through a Beckman DU640 spectrophotometer.

ELISA

The PLF concentration in the medium was determined using a method of Yang *et al.* (23) described previously. Briefly, the antibody containing 300 ng of rabbit anti-PLF IgG fraction per well was coated overnight at 4°C. The wells were then reacted with a serial dilution of cultured medium or purified milk PLF in triplicates. After 2 h incubation, the wells were washed three times with a washing buffer (0.05% Tween-20 in

PBS) and incubated with 1:4000 mouse anti-PLF serum for 2 h at 37°C. Peroxidase-conjugated goat anti-mouse IgG were then added and developed. After the addition of 25 μl of 3 N HCl, the optical density was read at 490 nm. The concentration of rPLF in medium samples was extrapolated from a milk PLF standard curve.

RESULTS

Construction of P. pastoris Expression Vectors

To test the effect of varying signal sequence on the secretion of rPLF from *P. pastoris*, PLF cDNA without or with its own signal sequence was ligated into vector pGAPZ B to generate plasmids pMLF (without signal sequence) and pPSLF (with native PLF signal sequence) as shown in Fig. 1. A third plasmid, pαLF, was also constructed which expressed the *S. cerevisiae* prepro signal sequence of α-mating factor fused to the mature form of LF (Fig. 1). Following electroporation, linearized plasmids pSLF, pMLF, and pαLF were each integrated at the *GAP* gene promoter locus as single or multiple copies as predicted from their resistance to zeocin (0–1000 μg/ml).

Expression of Recombinant Lactoferrin in P. pastoris

As observed by others, we found that transformants resistant to high levels of zeocin (1000 μg/ml) also tended to contain high copy numbers of the integrated plasmids (24). Figure 2 shows a typical Western blot result on cell extracts and culture media samples from pSLF-, and pαLF-, and pMLF-transformed *P. pastoris* strains. An anti-PLF-immunoreactive band from each of the transformants was observed that was similar in molecular weight to purified milk PLF (mPLF). Neither Western blots nor ELISAs detected PLF protein in the soluble fractions prepared from either cell extracts or media from pMLF transformants. However, following a 7 M urea treatment on the insoluble fractions of pMLF transformants, immunoreactive rPLF was observed (data not shown). Thus, rPLF expressed without a signal sequence was synthesized, but precipitated in intracellular fractions.

Purification of Recombinant PLF from Yeast Broth

To produce PLF for purification and analysis, pSLF transformants were cultured to stationary phase and the media were collected. The media were precipitated with 30–40% saturated ammonium sulfate and precipitated proteins were redissolved and dialyzed against the starting buffer. Initial purifications were conducted according to the method of Chu *et al.* (19). Crude fractions described above were chromatographed on a heparin-affinity column using a NaCl step gradient (19). We observed the presence of one LF unrelated heparin-binding protein that coeluted with rPLF between 0.3 and 0.4 M NaCl. To remove this contaminant, the procedure was modified with an extended wash with NaCl between 0.4 and 0.6 M. rPLF was subsequently eluted with 0.6 M NaCl. The purified rPLF appeared as a single band on SDS–PAGE with a molecular weight similar to that of mPLF (Fig. 3). The ability to purify rPLF with a single heparin column step allowed us to

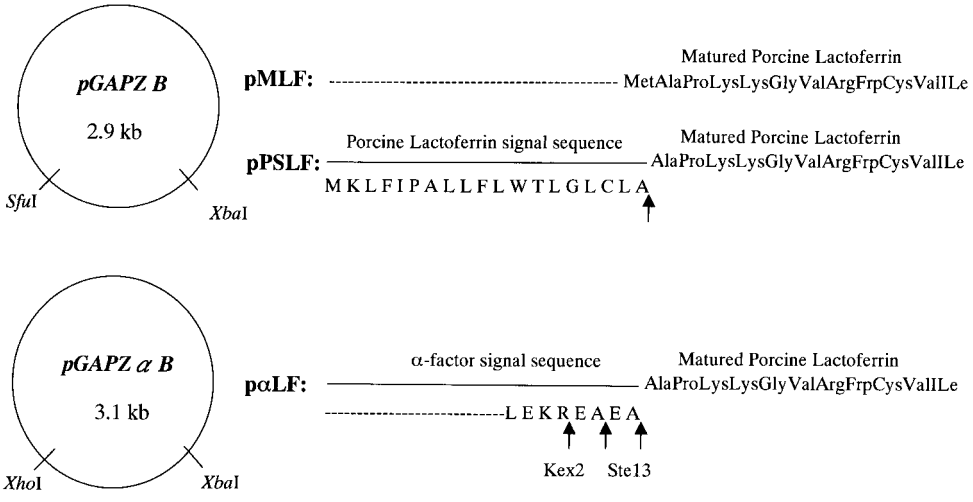

FIG. 1. Constructs designed to express PLF cDNA (with or without signal sequence) in the methylotrophic yeast *P. pastoris* (SMD 1168). LF cDNA was ligated following a PCR amplification into vector pGAPZ B to produce plasmid pMLF (without signal sequence) or pPSLF (with porcine signal sequence). Prior to ligation, both LF cDNA and pGAPZ B were digested with *Sfu*I and *Xba*I restriction enzymes, respectively. Plasmid pαLF was prepared by fusing pGAPZα B and sequences encoding the mature form of PLF. After digestion pf pGAPZαB with *Xho*I and *Xba*I, sequences encoding the yeast α-mating factor (MF) *Kex2* and *Ste 13* sites were removed. Primer P4 (Table 1) was therefore designed to reconstruct these MF sequences along with the amino-terminal encoding region of mature LF. The arrow indicates the predicted cutting site in the MF–LF fusion.

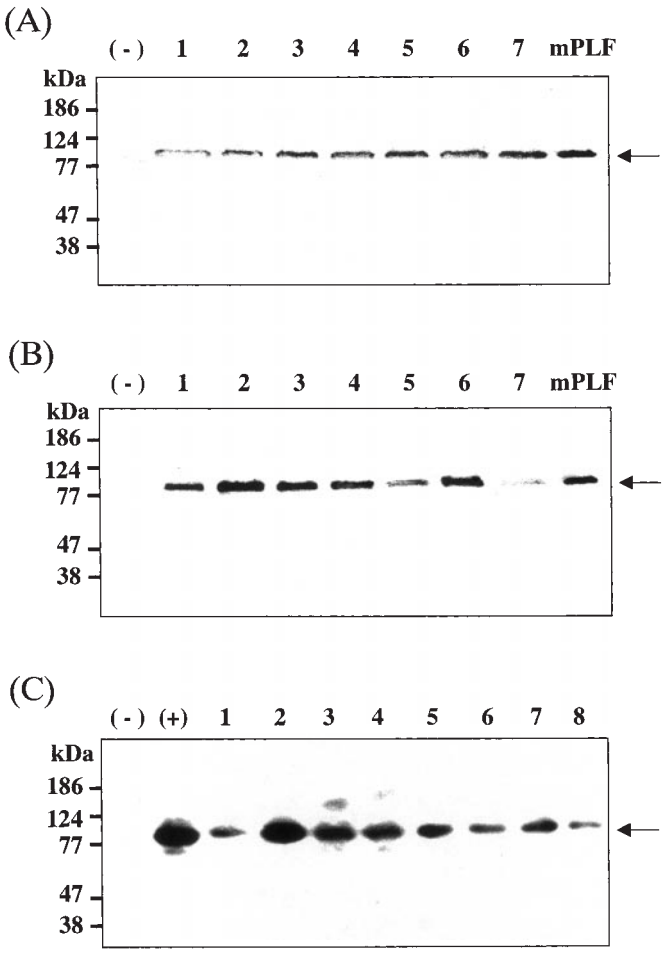

FIG. 2. Western blot analysis of rPLF from the cell extracts and cultured media samples of pPSLF and pαLF transformants. Following 2 days of growth, culture samples were harvested, ruptured with glass beads, and then centrifuged to separate soluble and insoluble fractions. The soluble fractions of cell extract (A) and medium (concentrated to 10-fold) (B) from pPSLF were analyzed by a Western blot (developed using a diaminobenzidine reagent). A Western blot of a cell extract from a pαLF-containing strain is shown in (C) (developed by an ECL reagent kit). MPLF, milk porcine LF; (−), negative control using host *P. pastoris* (SMD1168) with vector only; (+), positive control using protein extract from pPSLF.

avoid a further purification step using an immunoaffinity column described by Liang and Richardson (14) for their purification of human LF.

Characterization of Recombinant PLF

The rPLF from a pαLF-containing *P. pastoris* strain was further characterized and compared to the mPLF. 2D IEF–SDS–PAGE results illustrated that both rPLF and mPLF were basic in nature with an isoelectric point of approximately 8.8–9.0 (Fig. 4). A sample of rPLF was transferred to a polyvinylidene fluoride membrane and immobilized for solid-phase microsequencing. The N-terminal 10 amino acid residues of rPLF were found to be identical to those of mPLF confirming that the signal sequence had been processed properly and that the polypeptide was in fact PLF (Table 2). The molecular weights of both recombinant and milk PLFs decreased slightly following treatment with a neuraminidase, an enzyme that removes a portion of carbohydrate on glycoproteins (data not shown). Thus, rPLF, like mPLF, is a glycoprotein. Finally, we observed that our rPLF possessed a typical ferric ion-binding activity with an absorbance between 410 and 550 nm (Fig. 5), a property also mimicking mPLF (19).

High-Level Expression of PLF in Shake-Flask Cultures

We examined the relationship between rPLF expression and growth of our *P. pastoris* transformants. First, strains expressing PLF via its own signal sequence (pSLF) and from that of yeast (pαLF) were examined for efficiency of rPLF secretion. Figure 6 shows that there was no significant difference in maximum density achieved in cultures between strains expressing rPLF from pSLF and pαLF. The growth curves were virtually superimposable within the first 48 h of growth (data not shown) (Fig. 6). Initial rPLF production rates during the first 48 h were significantly higher with pαLF-containing strains than with pSLF-containing strains. However, after that period, production rates of rPLF

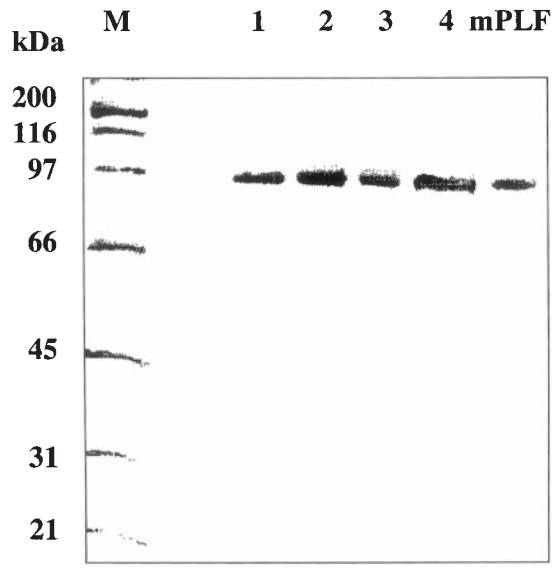

FIG. 3. Purification of rPLF from the culture medium of a pPSLF transformant using a heparin affinity column. Fractions of 1.2 ml each were collected after elution with high salt as described under Materials and Methods. Immunoreactivity for LF in each fraction was determined by an ELISA and read at 630 nm. The figure depicts SDS–PAGE analysis of purified rPLF fractions after elution with 0.6 M NaCl. MPLF, porcine LF isolated from milk; M, molecular weight markers from top to bottom are 200, 116, 97, 66, 45, 31, and 21 kDa, respectively.

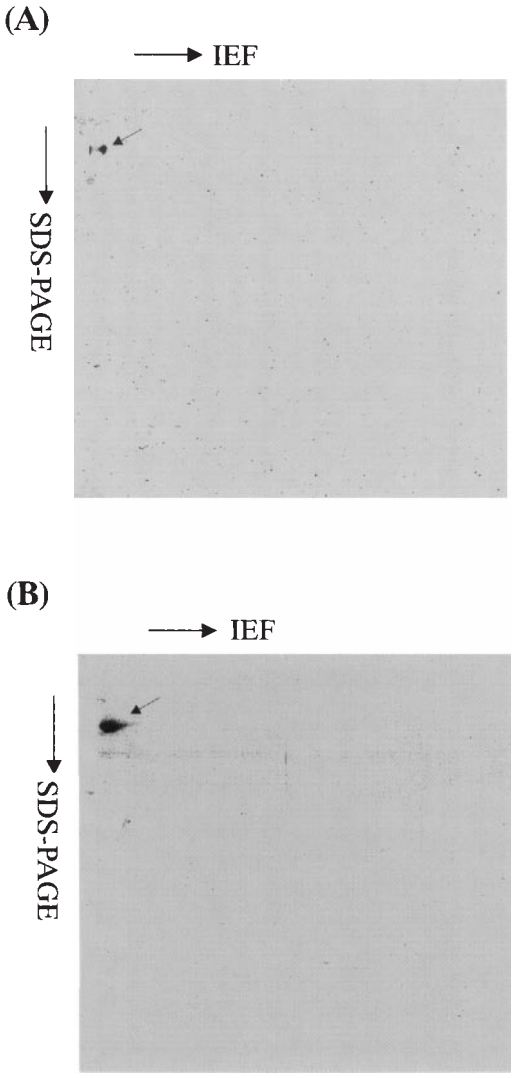

FIG. 4. Two-dimensional gel electrophoresis of purified rPLF (A) and mPLF (B). Both PLFs reveal a p*I* value of approximately 8.9–9.0. The gels were precalibrated with heat shock protein and haptoglobin (22).

TABLE 2

N-Terminal Amino Acid Sequence of PLF

Predicted sequence[a]	Ala-Pro-Lys-Lys-Gly-Val-Arg-Trp-Cys-Val
Porcine milk LF (mPLF)[b]	Ala-Pro-Lys-Lys-Gly-Val-Arg-Trp-Cys-Val
Recombinant LF (rPLF)[c]	Ala-Pro-Lys-Lys-Gly-Val-Arg--X---X---Val

[a] Predicted sequence is deduced from cDNA sequence of PLF (17).
[b] The N-terminus of rPLF and mPLF were sequenced by an automated Edman degradation procedure using a Applied Biosystems 476A sequencer.

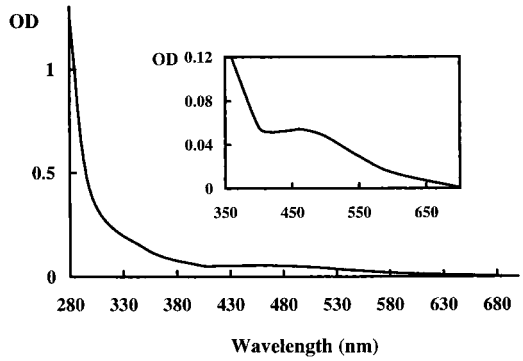

FIG. 5. Absorption spectrum of iron-saturated rPLF between 280 and 680 nm. Upon binding Fe^{3+} to rPLF, a maximal absorption of rPLF at 465 nm between visible ranges from 350 and 680 nm is observed.

from pαLF strains (0.051 μg/ml/h) were similar to those from pSLF strains (0.047 μg/ml/h). The highest rPLF levels were achieved following 168 h of culturing (~12 mg/L for pαLF-based strains vs ~7 mg/L for pSLF-based strains) (Fig. 6). Thus, the yeast signal sequence appeared to be more effective in rPLF secretion than that of the porcine signal sequence (2.0 vs 1.6 μg/ml of rPLF/h/OD_{600}).

Effect of Initial pH on PLF Production in Shake-Flask Cultures

The effect of initial pH of the culture medium on rPLF expression was investigated. Culture medium from a pαLF-based strain was adjusted over a pH range of 6.0 to 8.0. Figure 7 shows that the concentration of secreted rPLF was highest when the initial pH was 7.0. At this pH, culture medium from the pαLF expression strain

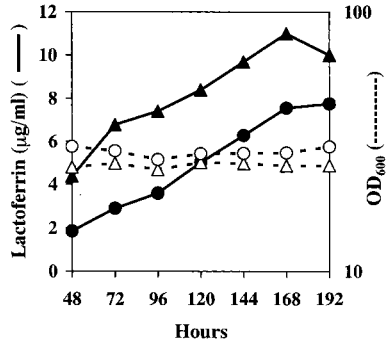

FIG. 6. Comparison of rPLF production between pPSLF (●) (with signal sequence from porcine LF) and pαLF (▲) (with signal sequence from yeast α-mating factor) transformants cultured at 30°C during 192 h. The growth of both strains reached stationary phase as determined by OD_{600} (○, pPSLF; □, pαLF) within the first 48 h (data not shown), whereas the rPLF concentrations (●, ▲) in the medium increased to their maximum level following 168 h of incubation in a shaking flask. Each value represents the mean of triplicates, typical experimental variation was less than 10%. The difference in maximal production of rPLF between the two transformants was significant ($P < 0.05$).

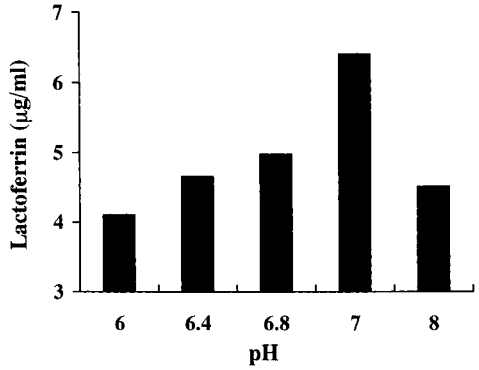

FIG. 7. Effect of pH on production of rPLF of pαLF transformant (800 μg/ml zeocin) grown in shake flasks. Cells were cultured for 4 days at the indicated pH and the medium was then assessed for PLF concentration. Each value represents the mean of triplicate determinations with a significant difference at pH 7.0 from the others ($P < 0.05$).

yielded approximately 70% more rPLF than the same strain in pH 6.0 medium. Growth of the strain at the higher pH was only marginally affected with the final OD_{600} value. The final pH value following the growth, however, was not significantly affected since there was only a slight decrease in this value (data not shown).

Effect of $FeCl_3$ on LF Production in Shake-Flask Cultures

Since LF is an iron-binding protein, we examined the effect of Fe^{3+} (100–1000 μM $FeCl_3$) addition to the culture medium on expression of rPLF from one of our pαLF-based *P. pastoris* strains. With an initial culture medium pH value of 7.0, our results revealed that addition of $FeCl_3$ to 100 μM significantly enhanced the expression of rPLF (Fig. 8) without interfering with cell growth (data not shown). Further increases in the concentration of the ion resulted in a reduction in rPLF levels.

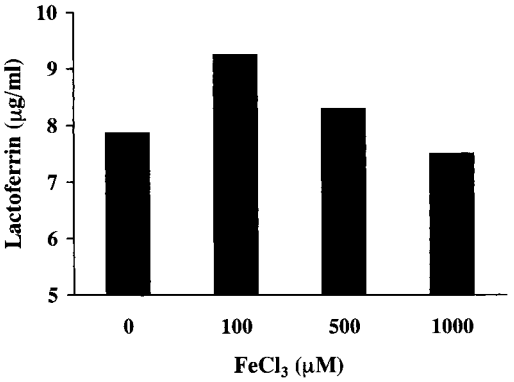

FIG. 8. Effect of $FeCl_3$ on production of rPLF in a pαLF transformant grown in shake flasks (containing 800 μg/ml zeocin). Cells were cultured for 5 days at $FeCl_3$ ranging from 0 to 1000 μM. Each value represents the mean of triplicate determinations with a significant difference at 100 μM $FeCl_3$ from the others ($P < 0.05$).

DISCUSSION

The feasibility of using *P. pastoris* for recombinant protein expression has received considerable attention. Many proteins, such as surface antigen of hepatitis B, EGF, IGF-1, and serum albumin, have been shown to express at higher levels in *P. pastoris* than that in *S. cerevisiae* (6). Similarly, we observed that the level of expression of rPLF in *P. pastoris* (~12 mg/L) was significantly higher than that in *S. cerevisiae* (1–1.5 mg/L) (14; unpublished results). The reasons for the difference in expression levels are not readily clear. However, the high plasmid copy numbers afforded by *S. cerevisiae* vectors are not always correlated with high protein expression levels (6). The present study demonstrates that there is a positive correlation between the copy number of integrated vectors in *P. pastoris* and rPLF expression levels. In addition to the low-level expression of rPLF in *S. cerevisiae*, rPLF yields were also inconsistent. Liang *et al.* suggested that LF might be toxic to its *S. cerevisiae* host (14). In contrast, our studies with *P. pastoris* suggest that rPLF synthesis does not measurably hinder the growth of this yeast. Expression levels in *P. pastoris* are consistent with or without the presence of a signal sequence. However, our highest levels of rPLF expression are achieved with constructs that synthesize the protein as a fusion with the yeast α-mating factor secretion sequence. Further increases in rPLF yield can be obtained by adjusting the initial pH of the culture medium from 6 to 7, and by the addition of ferric ions ($FeCl_3$) to 100 μM to the medium. Importantly, our characterization of rPLF indicates that it is indistinguishable from that purified directly from milk. These characteristics include the molecular size of PLF, its isoelectric point, and Fe^{3+}-binding ability.

The secretion of recombinant proteins from yeasts has been achieved using either endogenous or exogenous signal sequences, although these signals do not always lead to successful secretion (28–31). In the present study, we show that rPLF constructs, containing either the native signal peptide from porcine LF or the α-mating factor from yeast, produce and secrete rPLF from *P. pastoris*. The amino acid sequence on either side of the cleavage site for the porcine signal peptidase is identical to the yeast α-MF presequence cleavage site (Leu-Ala-Ala-Pro) (32). This suggests that the *P. pastoris* peptidase should be able to recognize the porcine LF signal sequence. Surprisingly, no secretion of rPLF was observed from *S. cerevisiae* using a porcine signal peptide (data not shown). An explanation for this discrepancy in secretion between *P. pastoris and S. cerevisiae* is not readily apparent.

Expression systems other than yeast have been utilized to produce LF. rPLF was expressed in *E. coli* as a fusion with a carrier peptide, but the protein was not glycosylated (33). rPLF was secreted from the fungus

Aspergillus oryzae at a level of about 25 mg/L (34) or higher following optimization of the system (35). The rate of production, however, was low. Expression systems using insect (15) and BHK cells (36) also produced LF at levels of roughly 10 mg/L, levels similar to those we obtained with *P. pastoris*. However, levels secreted from *P. pastoris* are likely to increase greatly when these strains are cultured at high density in fermenters. Although iron is essential for yeast growth, little is known about the distribution of iron once it penetrates the cell (38). Some studies have reported that iron storage in the vacuole affects hemoglobin and ferritin synthesis in various yeasts (39, 40). Since PLF is a high-affinity iron-binding protein, we hypothesize that rPLF in yeast may compete for and reduce iron levels in the cell below that which is optimal for its metabolic activities.

In conclusion, we have succeeded in expressing and secreting rPLF in *P. pastoris*. Levels are sufficient for the production of rPLF for structural and functional studies on the protein. Eventually, recombinant PLF may serve as a food supplement for animals and possibly humans as an aid in the modulation of certain physiologic functions of the intestinal system.

ACKNOWLEDGMENTS

The authors thank the Council of Agriculture, Taiwan, ROC (Grant 89-ST-1.3-AI-61-1), and the National Science Council, Taiwan, ROC (Grants NSC 89-2811-B-059-0003 and NSC 89-2313-B-059-017), for their research grant supports. We also thank Dr. S. R. Wang for providing the porcine lactoferrin cDNA and Dr. L. Y. Lin for his critical suggestions. We are grateful for the editorial comments and suggestions from Professor James Cregg.

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