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Commercial Production of Lactoferrin, a Multifunctional Iron-binding Glycoprotein

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Introduction

Lactoferrin is an 80 kDa member of the transferrin family of iron-binding glycoproteins (Aisen and Listowsky, 1980) which includes transferrin, the major iron-binding protein in serum (MacGillivray *et al.*, 1983; Yang *et al.*, 1984), ovatransferrin, an egg-white protein (Jeltsch and Chambon, 1982), and melanotransferrin, a membrane-bound member of the family found in melanocytes (Rose *et al.*, 1986). Although lactoferrin displays sequence and structural homology with other members of this family, the protein is unique in terms of its location and multifunctional role in the body (Metz-Boutigue *et al.*, 1984). Lactoferrin is expressed and secreted by glandular epithelial cells where it is positioned to function as a component of the primary host defense (Masson *et al.*, 1966a, b; Masson and Heremans, 1971; Pentecost and Teng, 1987). The protein is also expressed in the secondary granules of mature neutrophils and is released into the circulation upon neutrophil activation (Masson *et al.*, 1969; Wright and Gallin, 1979). The functions proposed for lactoferrin are diverse and include antimicrobial activity against a broad spectrum of bacteria (Oram and Reiter, 1968; Arnold *et al.*, 1977, 1980; Weinberg, 1978; Bullen *et al.*, 1978), immunomodulatory activity (Zagulski *et al.*, 1989; Crouch *et al.*, 1992; Machnicki *et al.*, 1993), regulation of myelopoiesis (Zucali *et al.*, 1979, 1989; Broxmeyer *et al.*, 1987; Sawatzki and Rich, 1989; Hangoc *et al.*, 1991), cellular growth promotion (Hashizume *et al.*, 1987, 1983; Nichols *et al.*, 1987, 1990) and differentiation (Zimecki *et al.*, 1991), antioxidant effects (Ambruso and Johnston, 1981; Britigan *et al.*, 1991) and regulation of iron homeostasis in the gastrointestinal tract (Cox *et al.*, 1979; Fransson and Lonnerdal, 1980; Davidson and Lonnerdal, 1989).

Abbreviations: *A.*, *Aspergillus*; ELISA, Enzyme Linked Immunosorbent Assay; GM-CSF, granulocyte/macrophage colony stimulating factor; IL-1, Interleukin-I; kDa, Kilodalton; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α.

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To exploit the potential therapeutic and nutritional applications of lactoferrin, a recombinant expression system was needed which would provide large quantities of this protein in a biologically active pure form. In the present review, we describe progress to date on the development of such a system with particular emphasis on the production of recombinant lactoferrin in the filamentous fungus, *Aspergillus awamori*. We begin by briefly summarizing the role of lactoferrin in exocrine secretions and immune cells which highlight the potential nutritional and clinical uses of this protein. For a more exhaustive summary on the biochemical properties and biology of lactoferrin, readers are referred to the recent review by Nuijens *et al* (1996) and previously by others (Baker *et al*., 1992; Sanchez *et al*., 1992; Iyer and Lonnerdal, 1993; Lonnerdal and Iyer, 1995).

Role of lactoferrin in exocrine secretions

Lactoferrin is expressed and secreted by glandular epithelial cells (Masson *et al*., 1966a). The protein is a major component of milk, where it is found at levels up to 6 g/l in colostrum (Masson and Heremans, 1971; McClelland *et al*., 1978; Brock, 1980; Hennart *et al*., 1991), and is found at lower levels in tears, nasal secretions and saliva, in addition to bronchial, pancreatic, intestinal and genital secretions (Masson *et al*., 1966b; Pentecost and Teng, 1987; Yu and Chen, 1993). A role for lactoferrin as an antimicrobial defense protein in these secretions has long been proposed (Masson *et al*., 1966b). Breast milk lactoferrin is about 6%–8% saturated with iron and is generally believed to protect the mammary gland from bacterial infection by binding to iron with high affinity and preventing the growth of iron-requiring bacteria (Nuijens *et al*., 1996; Sanchez *et al*., 1992). Furthermore, it is known that there is a lower incidence of bacterial infections associated with infants who are breast fed as compared to those fed on commercial formulae and this is likely to be due, at least in part, to the high concentrations of human lactoferrin in the former (McClelland *et al*., 1978; Bullen *et al*., 1971). However, in some cases bacteriostasis is temporary due to the ability of certain bacteria to adapt to their iron limiting environment by producing siderophores which can sequester iron from lactoferrin (Lewin, 1984) or by expressing membrane receptors which can bind iron-saturated lactoferrin (Schryvers and Morris, 1988; Otto *et al*., 1992; Tigyi *et al*., 1992; Yang *et al*., 1993; Pettersson *et al*., 1994; Staggs *et al*., 1994). A second mechanism by which lactoferrin may contribute to the non-specific immune defense of mucosal secretions is through to be direct antimicrobial action against certain Gram-negative bacteria (Arnold *et al*., 1977, 1981). Lactoferrin has been shown to bind to the bacterial outer membrane causing the release of lipopolysaccharides with concomitant increase in membrane permeability (Ellison *et al*., 1988; Ellison and Giehl, 1991). An isolated peptide corresponding to a highly cationic domain located in the N-terminus of the molecule was shown to have more potent bactericidal activity than that of native lactoferrin (Bellamy *et al*., 1992a, b; Yamauchi *et al*., 1993). However, the functioning of this peptide in the context of the full-length protein remains to be established. In addition, it has been shown that lactoferrin can bind to isolated LPS (Appelmelk *et al*., 1994; Elass-Rochard *et al*., 1995) and, in this manner, the protein may act as an antiendotoxin in local defense systems in the gastrointestinal tract by suppressing LPS induced cytokine release from the gut associated lymphatic system. Finally, at least

one report has indicated that exogenously fed lactoferrin could reduce the severity and duration of enteric infections in neutropenic patients, further supporting the hypothesis that this protein plays an important antimicrobial role in exocrine secretions (Trumpler *et al.*, 1989).

A role for lactoferrin in iron absorption by intestinal cells, although controversial, has also been proposed (Iyer and Lonnerdal, 1993). This was initially proposed on the finding that lactoferrin, but not transferrin, was capable of delivering iron to intestinal cells (Cox *et al.*, 1979). More recently, specific and saturable receptors for lactoferrin have been identified on enteric cells (Iyer and Lonnerdal, 1993; Hu *et al.*, 1988, 1990; Mikogami *et al.*, 1994; Gislason *et al.*, 1995; P.P. Ward *et al.*, 1995). However, unlike the transferrin receptor mediated iron delivery pathway, which is well established (Dautry-Varsat *et al.*, 1983; Klausner *et al.*, 1983), the downstream effects and significance of lactoferrin receptor binding remains largely not understood.

Lactoferrin may also function locally as a growth and differentiation factor in exocrine secretions. An enhanced development of the gastrointestinal tract observed in suckled animals versus formula-fed controls has been proposed to be due, at least in part, to the high concentration of lactoferrin in colostrum (Heird *et al.*, 1984; Widdowson, 1985). In addition, *in vitro* studies have shown that lactoferrin can increase thymidine incorporation into rat enteric crypt cells further supporting a growth promotion role for this protein (Nichols *et al.*, 1987, 1989, 1990).

Role of lactoferrin in the immune system

Lactoferrin is expressed during the myelocytic stage of granulocyte maturation and is stored in the secondary granules of mature neutrophils, where it can be released into the bloodstream upon neutrophil activation (Masson *et al.*, 1969; Wright and Gallin, 1979; Rado *et al.*, 1984). A human deficiency of granulocytic secondary granules highlighted the importance of lactoferrin in normal immune function (Breton-Gorius *et al.*, 1980; Boxer *et al.*, 1982). The patients had markedly reduced levels of secondary granules and altered neutrophil function, and suffered from recurrent deep-seated skin abscesses which are likely to be due, at least in part, to diminished levels of lactoferrin, a prominent component of these granules. In addition, a number of *in vitro* and *in vivo* experimental models have directly demonstrated an important immunomodulatory role for lactoferrin. First, it has been shown that lactoferrin administered systemically can provide a prophylactic protective effect to mice injected intravenously with a lethal dose of bacteria (Zagulski *et al.*, 1989). *In vitro* and *in vivo* studies indicate that this protection is due to the ability of this protein to inhibit the synthesis and/or release of TNF-α and IL-1, two cytokines which are key mediators of bacterial toxic shock (Crouch *et al.*, 1992; Machnicki *et al.*, 1993). Second, lactoferrin has been shown to be a regulator of myelopoiesis (Zucali *et al.*, 1979; Broxmeyer *et al.*, 1987; Sawatzki and Rich, 1989; Zucali *et al.*, 1989; Hangoc *et al.*, 1991). Lactoferrin can inhibit the release of IL-1 and granulocyte/macrophage colony stimulating factor (GM-CSF) from monocytes and macrophages (Crouch *et al.*, 1992; Zucali *et al.*, 1989; Broxmeyer *et al.*, 1978, 1984). The discovery of increased numbers of high affinity specific receptors for lactoferrin on myeloblasts isolated from leukemic patients raises the exciting possibility that these cells may also be potentially important target sites for lactoferrin action in normal and altered myelopoiesis (Birgens *et al.*, 1984).

Lactoferrin has also been shown to affect the function of cells derived from the lymphoid lineage. Lactoferrin has been shown to regulate natural killer cell activity (Shau *et al.*, 1992; Bezault *et al.*, 1994), suppress antibody production by B-cells (Duncan and McArthur, 1988) and selectively stimulate the differentiation of immature $CD4^-$ $CD8^-$ thymocytes to the $CD4^+$ T-helper cell lineage (Zimecki *et al.*, 1991).

The exact molecular mechanism by which lactoferrin exerts these pleiotropic effects on immune function are currently unknown but are probably due to the ability of this protein to control levels of cytokine production, which are key common mediators of these processes. Specific and saturable receptors for lactoferrin have been found on immune cell types including lymphocytes (Mazurier *et al.*, 1989; Rochard *et al.*, 1989; Legrand *et al.*, 1992; Bi *et al.*, 1994), monocytes (Birgens *et al.*, 1983; Ismail and Brock, 1993) and macrophages (van Snick and Masson, 1976; Goavec *et al.*, 1994). Whether these receptors are involved in a signaling pathway which results in the alteration of cytokine synthesis and or release remains to be established. Clearly, the molecular cloning and characterization of these receptors would provide invaluable information as to the contribution of lactoferrin/receptor interactions to the immunomodulatory role of this protein.

Heterologous expression of recombinant lactoferrin

The functions summarized above for lactoferrin highlight the potential of this protein as an important antimicrobial, anti-endotoxin and immune regulatory agent which may also have some nutritional and pharmaceutical uses with regard to development and maintenance of neonatal and adult host defence responses. However, to examine the clinical and nutritional uses of lactoferrin, it was first necessary to develop a suitable expression system which would provide large quantities of biologically active protein. To date, small quantities of lactoferrin (>50 mg/l) have been expressed in eucaryotic systems, including baby hamster cells (Stowell *et al.*, 1991) and human 293 cells (Van Berkel *et al.*, 1995). While the recombinant lactoferrin produced was biologically active, these systems are not readily amenable to scale-up to produce large quantities of lactoferrin. Recombinant lactoferrin has also been produced in the yeast, *Saccharomyces cerevisiae* (Liang and Richardson, 1993). However, the secretion of the recombinant lactoferrin in this system was inefficient, with less than 10% of the lactoferrin produced being secreted into the growth medium. High levels of recombinant lactoferrin (50 g/l) were achieved using a transgenic approach where lactoferrin was expressed in the milk of mice under the control of the bovine casein promoter (Platenburg *et al.*, 1994; Nuisens *et al.*, 1996). Using a similar approach, transgenic cows are being generated where lactoferrin is expressed in the bovine mammary gland (Nuisens *et al.*, 1996). While high levels of lactoferrin may be attained in transgenic cows, the success of this approach, including the ability to purify the recombinant protein in a viral- and toxin-free form and the cost-effectiveness of a large transgenic animal program, remains to be established. In an effort to provide a viable source of pure, biologically active lactoferrin at high levels, an alternative approach was undertaken where lactoferrin was expressed as a fusion protein in *Aspergillus* (P.P. Ward *et al.*, 1995). The most recent developments using this production system will be the focus for the remainder of this review.

Aspergillus as a host for heterologous expression of recombinant proteins

The choice of a suitable expression system for the production of heterologous proteins is dictated primarily by the size and complexity of the protein desired to be expressed, the desired production rate and the cost effectiveness of the expression system. The filamentous fungus *Aspergillus* provides several advantages for the production of relatively large (~80 kDa) secretory glycoprotein such as lactoferrin. Certain *Aspergillus* strains naturally secrete copious quantities (10–20 g/l) of glycoproteins such as α-amylase and glucoamylase (Barbesgaard, 1977; Van Brunt, 1986). Further, expression plasmids containing the heterologous cDNA of interest are homologously integrated into the *Aspergillus* genome, ensuring high mitotic stability (Jeenes *et al.*, 1991). Unlike bacteria, these organisms are capable of eukaryotic post-translational modifications, such as N-terminal processing and O- and N-linked glycosylation (Jeenes *et al.*, 1991; Saunders *et al.*, 1989). In addition, *Aspergillus* has a long history of use in the industrial production of glycoproteins with large scale fermentation technology and well established downstream processing systems (Barbesgaard, 1977). Finally, products from several strains of *Aspergillus* are generally regarded as safe and are known to produce negligible amounts of toxins, a particular concern when one needs to consider the purity of the protein for therapeutic purposes.

Over the past decade, some mammalian proteins have been produced in *Aspergillus*, with varying degrees of success (P.P. Ward *et al.*, 1995; Jeenes *et al.*, 1991, 1993; Saunders *et al.*, 1989; Upshall *et al.*, 1987; Christensen *et al.*, 1988; M. Ward *et al.*, 1990; Dunn-Coleman *et al.*, 1991; Contreras *et al.*, 1991). Some of the key factors which have been demonstrated to improve the expression of heterologous proteins in this system have been summarized in *Table 1*. In the following sections, we will summarize how a combination of three significant features has resulted in the development of an *Aspergillus* expression system which produced, for the first time, a commercially viable source of recombinant lactoferrin.

Table 1. Factors contributing to efficient heterologous gene expression in *Aspergillus*

(A)	**Use of high secretory *Aspergillus* strain**
	A.oryzae
	A.niger
	A.awamori
(B)	**Flanking heterologous cDNA with genetic control regions from highly expressed endogenous *Aspergillus* genes**
	Strong inducible promoter and signal sequence
	Efficient transcription termination and polyadenylation signal
	Stabilization of heterologous protein by fusion with endogenous gene product
(C)	**Strain Improvement**
	Fermentation condition
	Chemical and physical mutagenesis
	Rational mutagenesis

Design of an expression system for lactoferrin production in *Aspergillus awamori*

The first feature required in order to attain high expression levels of a recombinant protein in *Aspergillus* is the choice of an expression strain with high endogenous

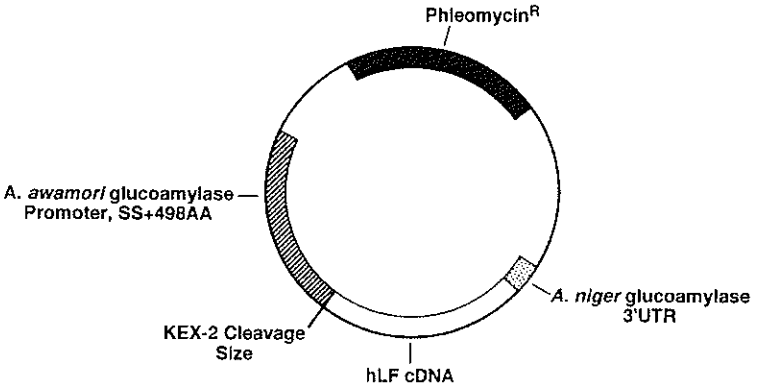


Figure 1. The *A.awamori* lactoferrin expression plasmid pPLF-19. The expression plasmid contains human lactoferrin (hLF) cDNA downstream from the *A.awamori* promoter and genomic fragment encoding the first 498 amino acids of proglucomylase. The lactoferrin and glucoamylase sequenes are separated by a KEX2 encoding hexapeptide. The *A.niger* glucoamylase 3'untranslated region (3'UTR) is positioned downstream from the lactoferrin cDNA.

protein production, for example *A.oryzae* (3 g/l), *A.niger* and *A.awamori* (20 g/l) (Barbesgaard, 1977; Van Brunt, 1986; Christensen *et al.*, 1988; Akabori *et al.*, 1954). In addition, it has been demonstrated that significant improvements in yields of recombinant proteins in *Aspergillus* can be achieved when the cDNA encoding the heterologous protein of interest is expressed as a chimera fused to a highly expressed endogenous *Aspergillus* gene. For example, production of bovine chymosin, at gram levels, was attained in *A.awamori* by fusing the precursor prochymosin cDNA with the complete gene encoding *A.awamori* glucoamylase (M. Ward *et al.*, 1990). A modification of this approach was used to achieve similar levels of production of human lysozyme (>1 gram/liter) in *A.niger* (Jeenes *et al.*, 1993). In this case, the lysozyme cDNA was expressed as a fusion with the N-terminal domain of the *A.niger* glucoamylase gene. In our case, lactoferrin was expressed in *A.awamori* as a fusion protein with the N-terminus of the *A.awamori* glucoamylase gene in an effort to improve lactoferrin expression levels. The design of the expression plasmid, pPLF-19, which was utilized for the production of lactoferrin in *A.awamori* is outlined in *Figure 1*. This plasmid contains a 2.8 kb fragment encoding the promoter, signal sequence and 5' region of the *A.awamori* glucoamylase gene (P.P. Ward, 1995). The cDNA encoding mature human lactoferrin (2.3 kb) was subcloned in frame downstream from the glucoamylase sequences. A hexapeptide containing a KEX-2 proteolytic cleavage site was inserted between the glucoamylase and lactoferrin DNA sequences to allow for *in vivo* processing of the resultant glucoamylase/lactoferrin chimeric protein. To ensure efficient transcription termination and polyadenylation, a 160 bp fragment encoding the 3' untranslated region of the *A. niger* glucoamylase gene was fused immediately downstream to the lactoferrin cDNA (Boel *et al.*, 1984). The vector also contains a 2.3 kb fragment encoding the phleomycin resistance gene from *Streptoalloteishus hindustanus* under the control of the *A. niger* β-tubulin promoter. This antibiotic-resistant marker allows selection of *A. awamori* transformants containing the integrated plasmid on growth medium containing phleomycin (Gatignol, 1987).

Expression of recombinant lactoferrin from *A.awamori* transformants

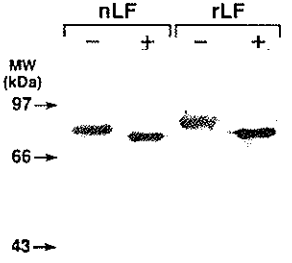
The lactoferrin expression plasmid, pPLF-19, was transformed into *A. awamori* and cultured as described previously and levels of lactoferrin expressed and secreted from these transformants was determined using an ELISA assay (Vilja *et al.*, 1985). Transformants were obtained which secreted >250 mg/l of intact lactoferrin into the growth medium. Hence, significant improvements in yields of secreted lactoferrin (~tenfold) were obtained by expression of lactoferrin as a fusion hybrid with glucoamylase relative to those obtained with the unfused cDNA (P.P. Ward *et al.*, 1992). These increases in production are likely due to enhanced translation, stability and secretion of the chimeric protein.

The third factor that contributed to high level expression of lactoferrin was the use of a chemically mutagenized strain improvement approach that has proved to be a valuable tool to improve expression levels of recombinant proteins in *Aspergillus* (Dunn-Coleman *et al.*, 1991). Increases in yield of lactoferrin were achieved using a combination of U.V. and chemical mutagenesis on *A.awamori* lactoferrin producing strains. To date, consecutive rounds of mutagenesis have resulted in the generation of transformants secreting in excess of 5 g/l of intact recombinant lactoferrin into the *A.awamori* growth medium. Since glucoamylase is secreted from improved strains of *A.awamori* at levels in excess of 20 g/l, repeated cycles of mutagenesis on optimal transformants, using a variety of physical and chemical methods, are expected to yield continual increases in levels of lactoferrin that should exceed the 20 g/l level.

Purification and biochemical analysis of recombinant lactoferrin

Lactoferrin was purified to homogeneity from the growth medium of *A.awamori* transformants using a one-step ion-exchange chromatography procedure (P.P. Ward *et al.*, 1995). N-terminal sequence of the purified protein confirmed that the recombinant

A.



B.

	Native hLF	Recombinant hLF
Molar Ratio (%):		
GalNAc	0.0	0.0
Mannose	22.9	87.7
Fucose	14.1	0.0
Glucose	N/A	N/A
Galactose	19.0	0.0
GlcNAc	32.8	12.3
NANA	11.2	0.0
NGNA	0.0	0.0

Figure 2. Glycosylation analysis of recombinant lactoferrin. **Panel A.** Western analysis of glycosylated and deglycosylated native and recombinant human lactoferrin. The first two columns contain native breast milk lactoferrin (nLF; 1 μg) undigested (–) and digested (+) with N-glycosidase F. The last two columns contain purified recombinant human lactoferrin (rLF) (1μg) undigested (–) and digested (+) with N-glycosidase F. **Panel B.** Monosaccharide analysis of native and recombinant lactoferrin. Three separate hydrolysis reactions were performed on the native and recombinant human lactoferrin to determine amine, neutral and sialic acid monosaccharides (GLYKO, Novato CA).

protein had been correctly processed from the N-terminal region of the *A.awamori* glucoamylase at the KEX2 cleavage site (P.P. Ward *et al.*, 1995). Lactoferrin is a glycoprotein containing ~5% carbohydrate which is attached through two N-glycosidic linkages to the protein (Spik *et al.*, 1982). To determine if the nature of the carbohydrate-protein linkage and the extent of glycosylation are similar in the recombinant protein, native human breast milk lactoferrin and recombinant human lactoferrin, were digested with N-glycosidase F and analyzed by Western immunoblotting with a specific IgG directed against lactoferrin. The results of this analysis are outlined in *Figure 2*. Both native and recombinant lactoferrin comigrate on the SDS-PAGE gel, suggesting that the extent of glycosylation is similar for both proteins (*Figure 2A*, lanes 1 and 3). Furthermore, both proteins comigrate following digestion with N-glycosidase F (*Figure 2A*, lanes 2 and 4) indicating that the attached carbohydrate is similarly N-linked to both the recombinant and native protein. Analysis of the released carbohydrates demonstrates that there is heterogeneity between the recombinant lactoferrin and lactoferrin isolated from human milk with the recombinant protein containing a high proportion of mannose residues (*Figure 2B*). However, it has been demonstrated that heterogeneity exists in the carbohydrate attached to lactoferrin isolated from different species (Spik *et al.*, 1988) and from different tissues within a species (Spik *et al.*, 1982; Derisbourg *et al.*, 1990). Furthermore, the lack of contribution of carbohydrate to the functional activity of lactoferrin has previously been reported and may instead contribute to the half-life of the protein in certain biological fluids (Kawakami and Lonnerdal, 1991).

Functional analysis of recombinant lactoferrin

IRON-SATURATION AND PH STABILITY OF IRON-BINDING TO RECOMBINANT LACTOFERRIN

Lactoferrin contains two highly homologous iron-binding domains, each with the capacity to bind one mole of iron with the concomitant binding of an anion such as carbonate (Aisen and Listowsky, 1980; Metz-Boutigue *et al.*, 1984; Anderson *et al.*, 1989). To determine if the binding of iron by recombinant lactoferrin was saturable and similar to that of human milk lactoferrin, an iron-saturation assay using $^{59}FeCl_3$ was performed. The results of this analysis are outlined in *Figure3A*. Both proteins bound ^{59}Fe to a similar degree at all concentrations tested and binding of iron was saturable at a 2:1 molar ratio of iron to lactoferrin as expected for the intact protein. A key feature of the iron binding activity of lactoferrin is its ability to retain bound iron over a broad pH range as is typically observed in the digestive tract. To compare the pH stability of iron-binding to native and recombinant lactoferrin, both proteins were saturated with ^{59}Fe and dialyzed against buffers from pH 7 to pH 2. ^{59}Fe bound to lactoferrin after this treatment was quantified and the results of this analysis are shown in *Figure 3B*. The pH dependent release of iron from native and recombinant lactoferrin were similar with both proteins retaining most of the iron over a pH range of 7 to 4 and were essentially iron-free at pH 2.0.

RECEPTOR BINDING ACTIVITY OF RECOMBINANT LACTOFERRIN

Specific and saturable receptors for lactoferrin have been identified on certain cell

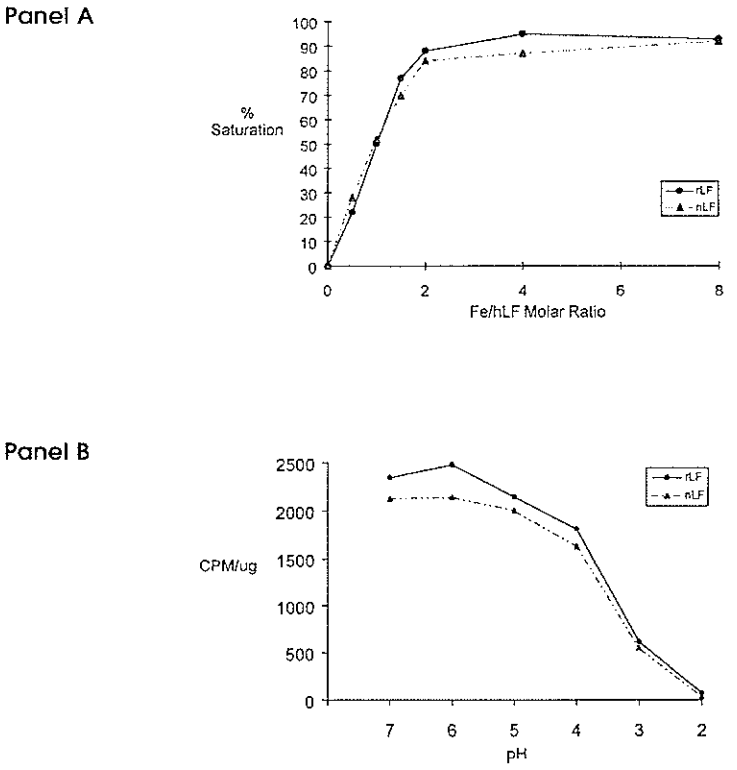


Figure 3. Iron-binding to recombinant lactoferrin. **Panel A.** Iron saturation of native and recombinant human lactoferrin. Native (nLF) and recombinant (rLF) human lactoferrin (500 μg) were incubated with increasing concentrations of ^{59}Fe for 30 min at 22°C. Free ^{59}Fe was removed by passage of the samples over a NAP-10 column. ^{59}Fe bound lactoferrin was quantified by liquid scintillation counting. **Panel B.** pH dependent release of ^{59}Fe from native and recombinant human lactoferrin. ^{59}Fe saturated native (nLF) and recombinant (rLF) human lactoferrin (200 μg) were dialyzed against buffers ranging from pH 7 to pH 2 for 48 h at 4°C. ^{59}Fe remaining bound to lactoferrin samples after dialysis was quantified by liquid scintillation counting.

types including human enterocytes (Cox *et al.*, 1979; Iyer and Lonnerdal, 1993; Hu *et al.*, 1988, 1990; Mikogami *et al.*, 1994; Gislason *et al.*, 1995; P.P. Ward *et al.*, 1995). To determine if the recombinant protein was capable of binding lactoferrin receptors on enteric cells, a receptor binding assay using biotinylated iron-saturated recombinant human lactoferrin was performed. Increasing concentrations of labeled lactoferrin (50–5000 nM) were incubated with human enteric Caco-2 membranes in the presence or absence of a 20-fold molar excess of unlabeled lactoferrin. The results of this analysis are outlined in *Figure 4A*. Binding of recombinant lactoferrin to Caco-2 cell membranes was specific and saturable. Scatchard plot analysis of the specific binding data revealed a dissociation constant (Kd) of ~1.8×10^{-7} M which is similar to that previously reported for human milk lactoferrin (Iyer and Lonnerdal, 1993).

ANTIMICROBIAL ACTIVITY OF RECOMBINANT LACTOFERRIN

Lactoferrin has previously been shown to exert a prophylactic effect in mice which

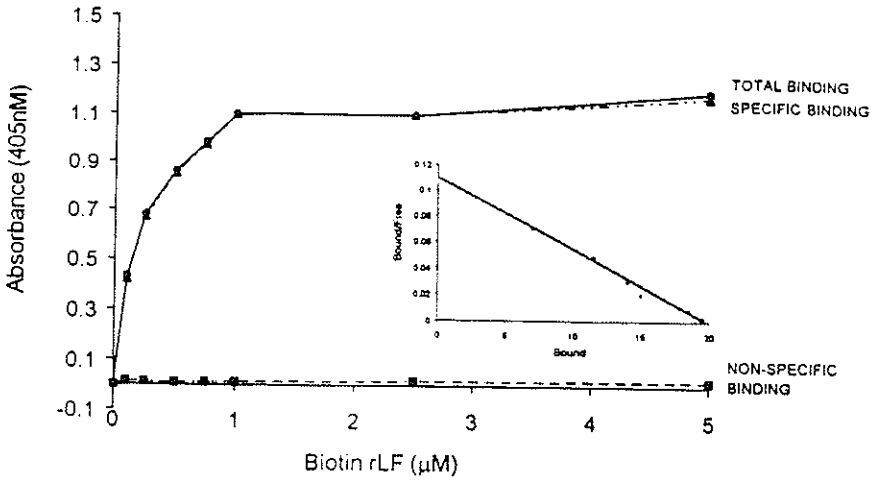


Figure 4. Binding of recombinant human lactoferrin (rLF) to human enterocyte cells. **A.** Specific and saturable binding of biotin rLF to Caco-2 cell membranes. Iron saturated biotinylated rLF (0.05–5 μM) was incubated with solubilized Caco-2 membranes (200 ng) in the presence or absence of a 20-fold molar excess of unlabeled ligand. The assay was carried out for 4 h at 4°C. Binding of biotinylated rLF to the Caco-2 membranes was determined using a Biotin/Avidin microtiter assay. The inset shows a Scatchard analysis of the specific binding data.

had been injected with a lethal dose of bacteria (Zagulski *et al.*, 1989). To determine if recombinant lactoferrin could provide the same protective function, mice were injected intraperitoneally with a lethal dose of *E.coli* 078:H11 with or without prior administration of recombinant lactoferrin. The results of this analysis are outlined in *Table 2*. Recombinant lactoferrin decreased the mortality rate from 43% to 0% in the mice tested. This analysis demonstrated that recombinant lactoferrin could display a similar *in vivo* antimicrobial prophylactic function as described for the native protein.

Table 2. Antimicrobial action of recombinant human lactoferrin *in vivo*. ICR female mice (8–10 weeks of age) were injected intraperitoneally with either recombinant apolactoferrin or BSA. After a period of 10 h, they were injected with 2×10^8 cells of *E.coli* 078:H11. The mortality rate was monitored for 48 h after the bacterial challenge.

Treatment	# Injected	Mortality
BSA (1 mg) & *E.coli* 078.H11	35	15
LF (1 mg) & *E.coli* 078.H11	36	0
LF (200 mg) & *E.coli* 078.H11	15	0

Summary and future directions

Using an *Aspergillus* expression system, we demonstrate, for the first time, the availability of a viable source of recombinant lactoferrin for commercial purposes.

The recombinant protein is functionally indistinguishable from native human breast milk lactoferrin as shown by three key criteria, iron-binding, antimicrobial and human enterocyte receptor binding activity. Although differences exist between the carbohydrate residues attached to recombinant lactoferrin and native breast milk lactoferrin, this heterogenity does not affect the functional activity of the recombinant protein. Furthermore, the oligomannose residues attached to recombinant lactoferrin are similar to the type observed on bovine milk lactoferrin, a component of human infant formula. Hence, this glycosylation pattern is unlikely to prove immunogenic, which is clearly an issue when considering the use of this protein for therapeutic applications. To date, mutagenesis and strain improvement of optimal *A.awamori* transformants have resulted in a 20-fold increase in lactoferrin expression levels in excess of 5 g/l. This has enabled the expression and purification of recombinant lactoferrin at a cost of less than $1 per gram, demonstrating the cost-effectiveness of producing this protein for therapeutic as well as nutritional applications.

The *Aspergillus* expression system which has been described here can be utilized for the production of site-directed mutants of lactoferrin which will be invaluable tools to elucidate the structure/function properties of this protein. For example, we recently reported the expression of iron-binding mutants in this system which were used to show that cooperative interactions between the two iron-binding lobes of lactoferrin contribute to the iron-binding stability that is unique to this protein (P.P. Ward *et al.*, 1996). It is anticipated that the availability of additional mutants of lactoferrin, which selectively ablate different functional domains of this protein, will provide additional important insights to the biology of this protein and may facilitate the development of a novel class of second-generation lactoferrin proteins with enhanced functional activity.

Finally, the advent of gene targeting techniques in embryonic stem cells has allowed the generation of mice deficient in lactoferrin. These lactoferrin 'knock-out' mice will provide an invaluable molecular tool to confirm the established and implicated physiological roles of this protein. Furthermore, these mice will provide a suitable genetic background to study the ability of recombinantly produced lactoferrin mutant proteins to selectively rescue altered phenotypes due to the lactoferrin ablation. Collectively, these two approaches will undoubtedly clarify further the physiological roles of this protein and may provide exciting new insights into the biology of lactoferrin.

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