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Production and characterization of recombinant human lactoferrin

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Chapter 10

Summary and general conclusions

1. Introduction

Human lactoferrin (hLF) is a metal-binding glycoprotein of Mr 77,000 that belongs to the transferrin family [1]. The molecule is found in milk, tears, saliva, bronchial and intestinal secretions as well as in the secondary granules of neutrophils [2]. Human LF consists of a single polypeptide of 692 amino acids [3] that is folded in two homologous lobes, designated the N- and C-lobe, each of which can bind a single ferric ion [1]. Furthermore, the molecule contains positively charged domains located in the N-terminus which can bind to negatively charged ligands such as heparin and LPS ([4-6], Chapter 4).

Based on in vitro and in vivo studies showing antimicrobial, anti-inflammatory and immunomodulatory activities, hLF is postulated to be involved in the innate host defence against infection and severe inflammation most notable at mucosal surfaces [2]. The antimicrobial activities of hLF include bacteriostasis by iron deprivation [7], bactericidal activity by destabilization of the cell-wall [8, 9] and antiviral activity by inhibition of viral infection [10]. The anti-inflammatory activities of hLF include inhibition of hydroxyl-radical formation by scavenging of iron [11], of mast cell tryptase activity by dissociation of the tryptase/heparin complex [12], of cytokine production [13] and of LPS activity [14, 15]. Besides down-regulation of immune reactions, hLF can also up-regulate immune responses by the activation of cells like monocytes/macrophages and NK cells [16]. These effects of hLF on cellular immunity likely are mediated by the binding of the molecule to cell-surface receptors and subsequent intracellular signaling pathways [17, 18]. Specific receptors for hLF have been found on a variety of cells including monocytes [19], lymphocytes [20], liver [21] and intestinal cells [22].

2. Applications of human lactoferrin in human healthcare

The diverse biological properties of hLF may allow for a wide variety of nutraceutical and pharmaceutical applications in human healthcare.

An interesting possibility would be to evaluate the use of hLF in the prevention or treatment of severe gastrointestinal disorders such as those occurring in patients with inflammatory bowel diseases or in patients receiving high-dose chemotherapy. These patients may benefit from the antimicrobial, anti-inflammatory and immunomodulatory activities of hLF, from its growth promotional effects on intestinal cells, from its probiotic effects e.g. through growth promotion of *Bifidobacterium* species, as well as from effects on iron uptake [2, 22-29]. These effects may also provide a basis for the application of hLF in clinical nutrition or infant formula.

Lactoferrin abolishing late phase airway responses (through inhibition of mast cell tryptase activity) in allergic sheep and decreasing pollen antigen-induced airway inflammation in a murine asthma model may provide a basis for evaluation the application of hLF in human allergic inflammation disorders [12, 30].

The strong antibiotic effects of hLF against *Klebsiella pneumoniae* and *Staphylococcus aureus* in mouse models ([31], Chapter 5), provide a basis for studies of the potential of hLF in treatment of patients infected with antibiotic-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) which infections have become an increasing problem in hospitals [32].

Both hLF and bLF, obtained after fractionation of bovine milk whey, can be used in applications of LF in human health care. However, the use of bLF appears restricted to oral applications because of its immunogenicity and the molecule may be inferior to hLF in applications where interactions with

specific receptors are required. Furthermore, bLF is much more susceptible than hLF to proteolysis by digestive proteases such as trypsin ([33], Chapter 7). This higher susceptibility of bLF seems relevant when considering oral applications of lactoferrin where the protein has to survive the harsh environment of the gastrointestinal tract. However, for some oral applications of hLF, it might still be necessary to develop formulations that deliver the protein intact at inflamed sites of the gastrointestinal tract.

The limited availability of human milk and purified hLF have been a major hurdle for (clinical) studies on potential nutraceutical and pharmaceutical applications of hLF. To overcome this limitation, the feasibility of large-scale production of functional recombinant hLF (rhLF) was studied in a wide variety of expression systems such as mammalian cells [34, 35], fungi [36], yeast [37] and transgenic rice [38]. The use of transgenic cows is an attractive alternative for the production of large amounts of rhLF as one cow can produce annually more than 10,000 liters of milk. This thesis emphasizes on the production of rhLF in the milk of transgenic cows.

3. Expression of rhLF in the milk of transgenic cows

The generation of transgenic cows harbouring mammary gland-specific expression vectors based on regulatory elements from the bovine α S1-casein gene and a cDNA encoding for hLF has been described previously [39]. The expression levels of rhLF obtained in milk of transgenic cows, harbouring vectors in which the hLF cDNA was replaced by genomic sequences encoding for a natural occurring hLF variant, are described in Chapter 5. The expression of rhLF in milk of transgenic cows of line 8785 was about 2.5 g/l. Furthermore, expression levels were similar between founder and offspring, among offspring as well as between hormonally- and calving-induced milk and were stable throughout the lactation period of 280 days. The expression of rhLF, even at high levels, did not affect milk-parameters such as total milk output, cell counts, total protein, fat and lactose (Chapter 5, [40]). Thus, a few hundred hLF-transgenic cows will be sufficient to supply thousands of kilograms of rhLF annually.

4. Characterization of rhLF purified from the milk of transgenic cows

Recombinant hLF purified from the milk of transgenic cows appeared saturated with iron for about 8% (Chapter 5 and 9). This low degree of iron-saturation is similar to that of natural hLF and bLF from milk [34, 41] but contrasts to the iron-saturated rhLF preparations obtained from many expression systems including mammalian cells [34, 35], transgenic mice [42] and transgenic rice [38]. Thus rhLF from transgenic cow milk does not require desaturation to obtain preparations able to bind iron.

The methods and results of comparative studies of rhLF and natural hLF from human milk are summarized in Table 1. The results indicate that rhLF and natural hLF are equivalent as to the peptide back-bone, utilization of N-linked glycosylation sites, iron-binding and -release, protein structure, susceptibility towards trypsin and effects in various murine infection models. The only apparent difference resides in the composition of attached carbohydrates. The glycans of rhLF contained N-acetylgalactosamine next to galactose, which is typical for N-linked glycoproteins produced in bovine milk such as bLF [43], and contained less fucose and N-acetylneuraminic acid than in natural hLF (Chapter 5). Based on its monosaccharide composition and susceptibility to N-glycosidases rhLF is predicted to contain oligomannose- and/or hybrid-type glycans next to complex-type whereas only complex-type glycans are found on natural hLF (Chapter 5). Similar differences in N-linked glycosylation have been described previously for antithrombin III from human plasma and the milk of transgenic goats [44]. Differences in N-linked glycosylation between recombinant and natural proteins can be expected since the structures of attached glycans is species, tissue, cell-type and protein specific [45, 46].

The predicted N-linked glycosylation pattern of rhLF was confirmed by further structural analysis of the N-linked glycans, which analysis revealed the presence of at least 17 different carbohydrates (results not shown). Importantly, none of the deduced structures contained immunogenic epitopes such as the α -

Table 1 Comparative analysis of rhLF from transgenic cow milk and natural hLF from human milk

Property	Method	Result	Chapter
Peptide back-bone	DNA sequencing N-terminal protein sequence Deglycosylation and SDS-PAGE	Equivalent	5, 7
Utilization of N-linked glycosylation sites	SDS-PAGE	Equivalent	5, 7
N-linked glycosylation	Monosaccharide analysis Deglycosylation and SDS-PAGE	Complex-, hybrid-/mannose type in rhLF; complex type in natural hLF	5
Iron-binding and -release	Spectroscopy	Equivalent	5
Protein structure of iron-saturated LF	Crystallography	Equivalent	6
Coordination of iron- and carbonate ion in iron-saturated LF	Crystallography	Equivalent	6
Susceptibility to trypsin	Tryptic proteolysis and SDS-PAGE	Equivalent	7
Biological activity	Murine infection models	Equivalent	5

galactosyl epitope [47]. The absence of immunogenic carbohydrates, i.e. structures that may elicit immune responses, in the majority of glycans is particularly important when parenteral applications of rhLF are considered.

Human LF is rapidly cleared from the circulation by binding to negatively charged chondroitin sulphate proteoglycans abundantly present on parenchymal liver cells [48]. The presence of mannose-type glycans and the low degree of sialylation of rhLF might even enhance the molecule's clearance by the liver through binding to the asialoglycoprotein- and the mannose-receptor [49]. However, pharmacological studies using intravenously injected radiolabeled rhLF and natural hLF showed similar $t_{1/2}$ values and biodistribution in multidrug resistant *S. aureus*-infected mice (Chapter 5) suggesting similar pharmacokinetics for both lactoferrins and that charge-related clearance is the predominant (fastest) clearance pathway. More importantly, intravenously administered rhLF and natural hLF were equally effective in various murine infection models despite the differences in N-linked glycosylation (Chapter 5).

5. Large scale production of rhLF from transgenic milk

5.1 Purification development

Purification development resulted in procedures which appeared useful for large scale purification of rhLF from bovine milk. The steps for large scale purification of pharmaceutical- and food-grade rhLF (Chapter 5 and 9, respectively) are summarized in Table 2. For both preparations, the transgenic milk is first defatted by centrifugation followed by extraction of the lactoferrin fraction, containing rhLF and bLF, using cation-exchange chromatography on SP Sepharose. The purity of rhLF after SP Sepharose chromatography is considered sufficient, i.e. about 95%, for use in functional foods. To obtain pharmaceutical grade rhLF, the lactoferrin fraction is processed further to remove remaining bovine milk proteins like bLF. Recombinant hLF is separated from bLF by cation-exchange chromatography on MacroPrep High S Support which results in rhLF preparations containing less than 0.1% bLF (Chapter 5). If necessary, further separation of bLF can be achieved by e.g. hydrophobic interaction chromatography [50].

Table 2 Purification of rhLF from transgenic cow milk

Step	Method
1	Defatting of transgenic milk (~2.5 g rhLF/l) by centrifugation
2	Extraction of rhLF by cation-exchange chromatography
3	Concentration and buffer exchange of rhLF by ultra-filtration
4	Bioburden removal by micro-filtration

<i>Pharmaceutical-grade</i>		<i>Food-grade</i>	
5	Viral inactivation by solvent/detergent treatment	5	Freeze-drying of rhLF
6	Purification of rhLF by cation-exchange chromatography	6	Packaging and labelling
7	Concentration of rhLF by ultra-filtration		
8	Viral removal by nano-filtration		
9	Concentration and formulation of rhLF by ultra-filtration		
10	Bioburden removal by micro-filtration		
11	Vialing and labelling		

5.2. Analytical assay development

Various analytical methods have been developed for characterization of purified rhLF. These methods can also be used for release of rhLF batches for nutraceutical or pharmaceutical use and/or to demonstrate batch-to-batch consistency.

The identity and concentration of rhLF in milk of individual cows, milk pools and purified batches can be assessed using specific antibodies against hLF. The generation of ten distinct monoclonal antibodies (mAbs) against hLF is described in Chapter 3. Experiments with proteolytic hLF fragments, rhLF lacking the N-terminal stretch Gly1-Arg2-Arg3-Arg4-Arg5 and the recombinant hLF lobes (Chapter 3, [4]) indicated that five mAbs bound to conformational epitopes residing in the N-lobe, whereas the other five bound to C-lobe conformational epitopes. The mAbs were used in hLF-specific immunoassays (Chapter 3, [4]) and structure-function relationship studies [4]. Other methods developed and/or used in the characterization of rhLF are N-terminal sequencing, monosaccharide composition analysis, iron-binding and release assays (Chapter 5), crystal structure analysis (Chapter 6) and the tryptic susceptibility assay (Chapter 7). The tryptic susceptibility assay revealed a subtle difference, i.e. slightly altered degradation kinetics, between rhLF derived from the Rey cDNA [3] and natural hLF (Chapter 7), whereas other comparative analyses including *in vitro* and *in vivo* antigenicity, iron-binding and release and binding to several ligands did not reveal any difference [42].

Characterization of a biopharmaceutical also includes qualification and quantification of impurities. For rhLF from transgenic bovine milk, these impurities can be derived from the cow (e.g. milk proteins), from the purification process (e.g. viral inactivation chemicals) or relate to rhLF (e.g. degraded molecules). A robust analytical method for determining bovine milk and rhLF-related impurities in purified rhLF is described in Chapter 2. The method, employing cation-exchange chromatography on a Mono S column, discriminates between N-terminally intact hLF and hLF molecules lacking two or three N-terminal residues, lactoferrins from other species (e.g. bLF) as well as homologous and other whey proteins. The Mono S method can also discriminate between the two glycosylation variants of bLF (bLF A and B) which differ in N-linked glycosylation at Asn²⁸¹, a site utilized in bLF A but not in bLF B (Chapter 7). Besides the use of Mono S for detecting bLF, a specific quantitative ELISA has been developed for this molecule (Chapter 5) which allows for the quantification of traces of bLF in pharmaceutical-grade rhLF batches. A specific quantitative ELISA has also been developed for a novel bovine milk protein which co-eluted with rhLF on Mono S (Chapter 8). N-terminal sequence analysis of the novel glycoprotein of Mr 25,000 revealed it to represent the bovine homologue of human neutrophil gelatinase-associated lipocalin (hNGAL) and therefore the molecule was designated bovine neutrophil gelatinase-associated lipocalin (bNGAL).

5.3 Preclinical and clinical development

Studies of possible applications of hLF in human healthcare (see section 2) have become a real option through the availability of large quantities of rhLF from transgenic cow milk. Before starting clinical studies on potential applications, preclinical studies assessing the safety of the proposed medication have to be conducted. The safety of pharmaceutical-grade rhLF has been evaluated in various preclinical studies [51] and in a Phase I clinical study in healthy volunteers which revealed that intravenously administrated rhLF at 60 mg/kg was safe and well tolerated [52]. Similarly, the safety of food-grade rhLF has been evaluated in various preclinical studies [53]. An example of a preclinical study with food-grade rhLF is shown in Chapter 9. Three doses of rhLF (200, 600 and 2000 mg/kg body weight/day) and saline as a control were daily administrated to rats via oral gavage for at least 90 days and a large variety of parameters were monitored. The results revealed no treatment-related, toxicologically significant changes on the basis of which the no observed-adverse-effect level (NOAEL) could be determined on 2000 mg/kg body weight/day.

6. Conclusions

The use of recombinant proteins in human healthcare requires a validated protein production technology, thorough comparison of the physico-chemical and biological characteristics of the recombinant protein with the natural form and extensive preclinical and/or clinical testing to assess the safety and efficacy of the proposed nutraceutical or pharmaceutical. This thesis reports on the use of transgenic cows as protein production technology for recombinant hLF and describes the characterization and safety testing of the purified molecule.

The bovine mammary gland appeared to be an attractive vehicle for producing large amounts of rhLF as constant expression levels, in the gram per liter range, have been obtained without affecting normal milk parameters. Characterization of purified rhLF revealed that the molecule closely matches the structure of natural hLF from human milk except for a difference in glycosylation. The differential glycosylation did not result in difference between rhLF and natural hLF in any of the employed in vitro and in vivo assay systems. Furthermore, rhLF appeared safe and well tolerated in various preclinical studies and in a Phase I clinical study. Taken together, transgenic cows are a valuable platform for the production of rhLF because large quantities of the molecule are expressed, the recombinant molecule displays a structure and function comparable to natural hLF and appears safe for human use. In addition, the results with rhLF illustrate the potential of transgenic cows to produce other recombinant human proteins for therapeutic use.

References

1. Anderson BF, Baker HM, Norris GE, Rice DW & Baker EN (1989) Structure of human lactoferrin: crystallographic structure analysis and refinement at 2.8 Å resolution, *J Mol Biol* **209**, 711-734.
2. Nuijens JH, van Berkel PHC & Schanbacher FL (1996) Structure and biological actions of lactoferrin, *Journal of Mammary Gland Biology and Neoplasia* **1**, 285-295.
3. Rey MW, Woloshuk SL, de Boer HA & Pieper FR (1990) Complete nucleotide sequence of human mammary gland lactoferrin, *Nucleic Acids Res* **18**, 5288.
4. van Berkel PHC, Geerts MEJ, van Veen HA, Mericskay M, de Boer HA & Nuijens JH (1997) The N-terminal stretch Arg2 Arg3, Arg4 and Arg5 of human lactoferrin is essential for binding to heparin, bacterial lipopolysaccharide, human lysozyme and DNA, *Biochem J* **328**, 145-151.
5. Mann DM, Romm E & Migliorini M (1994) Delineation of the glycosaminoglycan-binding site in the human inflammatory response protein lactoferrin, *J Biol Chem* **269**, 23661-23667.
6. Wu HF, Monroe DM & Church FC (1995) Characterization of the glycosaminoglycan-binding region of lactoferrin, *Arch Biochem Biophys* **317**, 85-92.
7. Reiter B, Brock JH & Steel ED (1975) Inhibition of *Eschericia coli* by bovine colostrum and post-colostral milk. II. The bacteriostatic effect of lactoferrin on a serum-susceptible and serum-resistant strain of *E. coli.*, *Immunology* **28**, 83-95.
8. Ellison III RT & Giehl TJ (1991) Killing of gram-negative bacteria by lactoferrin and lysozyme, *J Clin Invest* **88**, 1080-1091.
9. Ellison III RT, Giehl TJ & La FF (1988) Damage of the outer membrane of enteric gram-negative bacteria by lactoferrin and transferrin, *Infect Immun* **56**, 2774-2781.
10. van der Strate BWA, Beljaars L, Molema G, Harmsen MC & Meijer DKF (2001) Antiviral activities of lactoferrin, *Antiviral Research* **52**, 225-239.
11. Aruoma OI & Halliwell B (1987) Superoxide-dependent and ascorbate-dependent formation of hydroxyl radicals from hydrogen peroxide in the presence of iron. Are lactoferrin and transferrin promoters of hydroxyl-radical generation? *Biochem J* **241**, 273-278.
12. Elrod KC, Moore WR, Abraham WM & Tanaka RD (1997) Lactoferrin, a potent tryptase inhibitor, abolishes late-phase airway responses in allergic sheep, *Am J Respir Crit Care Med* **156**, 375-381.

13. Zucali JR, Broxmeyer HE, Levy D & Morse C (1989) Lactoferrin decreases monocyte-induced fibroblast production of myeloid colony-stimulating activity by suppressing monocyte release of interleukin-1, *Blood* **74**, 1531-1536.
14. Lee WJ, Farmer JL, Hilty M & Kim YB (1998) The protective effects of lactoferrin feeding against endotoxin lethal shock in germfree piglets, *Infect Immun* **66**, 1421-1426.
15. Kruzel ML, Harari Y, Mailman D, Actor JK & Zimecki M (2002) Differential effects of prophylactic, concurrent and therapeutic lactoferrin treatment on LPS-induced inflammatory responses in mice, *Clin Exp Immunol* **130**, 25-31.
16. Legrand D, Ellass E, Carpentier M & Mazurier J (2005) Lactoferrin: a modulator of immune and inflammatory responses, *Cell Mol Life Sci* **62**, 2549-2559.
17. Haversen L, Ohlsson BG, Hahn-Zoric M, Hanson LA & Mattsby-Baltzer I (2002) Lactoferrin down-regulates the LPS-induced cytokine production in monocytic cells via NF-kappa B, *Cell Immunol* **220**, 83-95.
18. Dhennin-Duthille I, Masson M, Damiens E, Fillebeen C, Spik G & Mazurier J (2000) Lactoferrin upregulates the expression of CD4 antigen through the stimulation of the mitogen-activated protein kinase in the human lymphoblastic T Jurkat cell line, *J Cell Biochem* **79**, 583-593.
19. Birgens HS, Hansen NE, Karle H & Kristensen LO (1983) Receptor binding of lactoferrin by human monocytes, *Br J Haematol* **54**, 383-391.
20. Mazurier J, Legrand D, Hu WL, Montreuil J & Spik G (1989) Expression of human lactotransferrin receptors in phytohemagglutinin-stimulated human peripheral blood lymphocytes. Isolation of the receptors by antiligand-affinity chromatography, *Eur J Biochem* **179**, 481-487.
21. Ziere GJ, Bijsterbosch MK & van Berkel TJ (1993) Removal of 14 N-terminal amino acids of lactoferrin enhances its affinity for parenchymal liver cells and potentiates the inhibition of beta-very low density lipoprotein binding, *J Biol Chem* **268**, 27069-27075.
22. Suzuki YA, Shin K & Lonnerdal B (2001) Molecular cloning and functional expression of a human intestinal lactoferrin receptor, *Biochemistry* **40**, 15771-15779.
23. Ward PP, Paz E & Conneely OM (2005) Multifunctional roles of lactoferrin: a critical overview, *Cell Mol Life Sci* **62**, 2540-2548.
24. Togawa J, Nagase H, Tanaka K, Inamori M, Nakajima A, Ueno N, Saito T & Sekihara H (2002) Oral administration of lactoferrin reduces colitis in rats via modulation of the immune system and correction of cytokine imbalance, *J Gastroenterol Hepatol* **17**, 1291-1298.
25. Troost FJ, Saris WH & Brummer RJ (2003) Recombinant human lactoferrin ingestion attenuates indomethacin-induced enteropathy in vivo in healthy volunteers, *Eur J Clin Nutr* **57**, 1579-1585.
26. Nichols BL, McKee KS, Henry JF & Putman M (1987) Human lactoferrin stimulates thymidine incorporation into DNA of rat crypt cells, *Pediatr Res* **21**, 563-567.
27. Zhang P, Sawicki V, Lewis A, Hanson L, Nuijens JH & Neville MC (2001) Human lactoferrin in the milk of transgenic mice increases intestinal growth in ten-day-old suckling neonates, *Adv Exp Med Biol* **501**, 107-113.
28. Petschow BW & Talbott RD (1991) Response of bifidobacterium species to growth promoters in human and cow milk, *Pediatr Res* **29**, 208-213.
29. Picard C, Fioramonti J, Francois A, Robinson T, Neant F & Matuchansky C (2005) Review article: bifidobacteria as probiotic agents -- physiological effects and clinical benefits, *Aliment Pharmacol Ther* **22**, 495-512.
30. Kruzel ML, Bacsı A, Choudhury B, Sur S & Boldogh I (2006) Lactoferrin decreases pollen antigen-induced allergic airway inflammation in a murine model of asthma, *Immunology* **119**, 159-166.
31. Nibbering PH, Ravensbergen E, Welling MM, van Berkel LA, van Berkel PHC, Pauwels EKJ & Nuijens JH (2001) Human lactoferrin and peptides derived from its N-terminus are highly effective against infections with antibiotic resistant bacteria, *Infect Immun* **69**, 1469-1476.

32. Rice LB (2006) Antimicrobial resistance in gram-positive bacteria, *Am J Med* **119**, S11-19; discussion S62-70.
33. Brines RD, and Brock, J. H. (1983) The effect of trypsin and chymotrypsin on the in vitro antimicrobial and iron-binding properties of lactoferrin in human milk and bovine colostrum. Unusual resistance of human apolactoferrin to proteolytic digestion., *Biochim Biophys Acta* **759**, 229-235.
34. van Berkel PHC, Geerts MEJ, van Veen HA, Kooiman PM, Pieper F, de Boer HA & Nuijens JH (1995) Glycosylated and unglycosylated human lactoferrins can both bind iron and have identical affinities towards human lysozyme and bacterial lipopolysaccharide, but differ in their susceptibility towards tryptic proteolysis, *Biochem J* **312**, 107-114.
35. Stowell KM, Rado TA, Funk WD & Tweedie JW (1991) Expression of cloned human lactoferrin in baby-hamster kidney cells, *Biochem J* **276**, 349-355.
36. Ward PP, Lo J, Duke M, May GS, Headon DR & Conneely OM (1992) Production of biologically active recombinant human lactoferrin in *Aspergillus oryzae*, *Bio/Technology* **10**, 784-789.
37. Liang Q & Richardson T (1993) Expression and characterization of human lactoferrin in yeast *Saccharomyces cerevisiae*, *J Agric Food Chem* **41**, 1800-1807.
38. Nandi S, Suzuki YA, Huang J, Yalda D, Pham P, Wu L, G. B, Huang N & Lönnerdal B (2002) Expression of human lactoferrin in transgenic rice grains for the application in infant formula, *Plant Science* **163**, 713-722.
39. Krimpenfort P, Rademakers A, Eyestone W, van der Schans A, van der Broek S, Kooiman P, Kootwijk E, Platenburg G, Pieper F, Strijker R & de Boer HA (1991) Generation of transgenic dairy cattle using 'in vitro' embryo production, *Bio/Technology* **9**, 844-847.
40. Hyvonen P, Suojala L, Haaranen J, von Wright A & Pyorala S (2006) Human and bovine lactoferrins in the milk of recombinant human lactoferrin-transgenic dairy cows during lactation, *Biotechnol J* **1**, 410-412.
41. Kawakami H, Shinmoto H, Dosako S & Sogo Y (1987) One-step isolation of lactoferrin using immobilized monoclonal antibodies, *J Dairy Sci* **70**, 752-9.
42. Nuijens JH, van Berkel PH, Geerts ME, Hartevelt PP, de Boer HA, van Veen HA & Pieper FR (1997) Characterization of recombinant human lactoferrin secreted in milk of transgenic mice, *J Biol Chem* **272**, 8802-8807.
43. Van den Nieuwenhof IM, Schiphorst WE, Van Die I & Van den Eijnden DH (1999) Bovine mammary gland UDP-GalNAc:GlcNAc-beta-R beta1-->4-N-acetylgalactosaminyltransferase is glycoprotein hormone nonspecific and shows interaction with alpha-lactalbumin, *Glycobiology* **9**, 115-123.
44. Edmunds T, van Patten SM, Pollock J, Hanson E, Bernasconi R, Higgins E, Manavalan P, Ziomek C, Meade H, McPherson JM & Cole ES (1998) Transgenically produced human antithrombin: structural and functional comparison to human plasma-derived antithrombin, *Blood* **91**, 4561-4571.
45. James DC, Freedman RB, Hoare M, Ogonah Ow, Rooney BC, Larionov OA, Dobrovolsky VN, Lagutin OV & Jenkins N (1995) N-glycosylation of recombinant human interferin-gamma produced in different animal expression systems, *Bio/Technology* **13**, 592-596.
46. Opdenakker G, Rudd PM, Ponting CP & Dwek RA (1993) Concepts and principles of glycobiology, *FASEB J* **7**, 1330-1337.
47. Galili U, Wang L, LaTemple DC & Radic MZ (1999) The natural anti-Gal antibody, *Subcell Biochem* **32**, 79-106.
48. Ziere GJ, Kruijt JK, Bijsterbosch MK & van Berkel TJ (1996) Recognition of lactoferrin and aminopeptidase M-modified lactoferrin by the liver: involvement of proteoglycans and the remnant receptor, *Biochem J* **313**, 289-95.
49. Drickamer K (1991) Clearing up glycoprotein hormones, *Cell* **67**, 1029-1032.

50. Nuijens JH & van Veen HA (1999) Isolation van lactoferrin from milk, *Patent*, US5861491.
51. Koopman J, van Berkel P, Van Veen H, Geerts M, Diefenbach O, Leufkens P, Olson R, Mudumbi R, Walsh G & Nuijens J (1999) Pharmaceutical development of recombinant human lactoferrin, *4th International Conference on Lactoferrin: Structure, Function and Applications*, Sapporo, Japan (May 18-22, 1999), 56.
52. Ziere B, Van Veen H, Koopman J, Van Berkel P & Nuijens J (2001) Safety, tolerability and pharmacokinetics of intravenously administrated recombinant human lactoferrin in humans, *5th International Conference on Lactoferrin: Structure, Function and Applications*, Banff, Alberta Canada (May 4-9, 2001), 43.
53. Ziere B, van Veen H, Vietsch H, Salaheddine M, Nuijens J & de Loos F (2005) Production and safety of recombinant human lactoferrin, expressed in the milk of transgenic cows for nutraceutical applications, *7th International Conference on Lactoferrin: Structure, Function and Applications*, Honolulu, Hawaii USA (October 16-19, 2005), 34.

