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# Sequence analysis of camel (*Camelus dromedarius*) lactoferrin

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## Abstract

The aim of this study was to characterise camel lactoferrin in terms of primary structure and molecular weight. The protein was eluted from a heparin-sepharose column at a sodium chloride concentration of 0.5 M, and corresponded to bovine lactoferrin in terms of N-terminal sequence and the molecular weight of 80.16–80.73 kDa. Lactoferrin cDNA was PCR amplified, using a cDNA library from lactating mammary gland of a Somali camel. The sequenced clone had a length of 2337 bp and an open reading frame of 2124 bp, which coded for a protein of 708 amino acid residues. The mature protein had a length of 689 amino acid residues, a calculated molecular weight of 75.250 kDa and a calculated isoelectric point at pH 8.14. Primary structure identity to bovine lactoferrin was 74.9%. Concentration of lactoferrin in whole, late-lactational milk was 220 mg l<sup>-1</sup>, which was higher than the lactoferrin concentration in comparable bovine milk, which was 140 mg l<sup>-1</sup>. © 1999 Elsevier Science Ltd. All rights reserved.

*Keywords:* *Camelus dromedarius*; Lactoferrin

## 1. Introduction

Lactoferrin is a protein of the innate immune system, which is also expressed in the lactating mammary gland. It is found in milk and different other body secretions, and in neutrophil leukocytes (Masson, 1970). The concentration in milk strongly depends on the species and the stage of lactation. Industrial scale purification from whey is carried out by cation exchange, and use as a preserving agent in food, drugs and cosmetics has been proposed (Saito, Takase, Tamura, Shimamura & Tomita, 1994).

Lactoferrin belongs to the family of transferrins, together with blood serotransferrin (siderophilin), egg white ovotransferrin (conalbumin), melanotransferrin of malignant melanomas, the porcine inhibitor of carbonic anhydrase, and other proteins. The common property of this protein family is the binding of two metal cations, preferably Fe<sup>3+</sup>, at structurally closely related binding sites. Most proteins of the transferrin type are needed for storage or transport of iron.

It can be assumed, that lactoferrin in colostrum milk acts as an iron scavenger, which depletes the milk from free iron and thereby slows down microbial growth.

Brock (1997) proposed, that the *in vivo* function of apolactoferrin is the prevention of iron-mediated lipid peroxidation, a property, which was already demonstrated with monocytes. This function is based on the ability of lactoferrin, to bind to cell membranes. The higher affinity for iron, as compared to other transferrins, would enable it to function at the reduced pH found in the stomach and upper intestine. The high resistance of apolactoferrin to proteolysis, compared with other apotransferrins, would enable it to maintain its iron-binding potential in the face of proteolytic activity in the gut. Since diferric lactoferrin was reported to be even more resistant to proteolysis, it was supposed, that the iron–lactoferrin complex would resist degradation, and was sequestered by hepatocytes, or was excreted from the gut. A higher lactoferrin concentration also could help to prevent lipid peroxidation by free radicals in an infected udder, which has an elevated iron content.

Iron-saturated lactoferrin, which is found in milk from the second week to the end of the lactational period, may primarily prevent microbial growth in the gut. This would help the new-born, which is easily infected, to survive the first weeks, until its own immune system becomes developed, and the gut becomes adapted to food digestion. Iron-saturated lactoferrin could also be a source of iron for the suckling, once the protein is degraded in the gut.

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Camel milk was frequently reported to have a high antimicrobial activity, and was shown to slow down the growth of pathogenic bacteria more than bovine milk (Elagamy, Ruppner, Ismail, Champagne & Assaf, 1992). Antimicrobial properties were partially attributed to well characterised proteins, such as lactoferrin, lactoperoxidase, lysozyme and immunoglobulin A. These proteins were shown to have higher concentrations or higher activity in camel milk, as compared to bovine milk. In this study we determined the basic physico-chemical and structural parameters of lactoferrin, of which the relationship between structure and function is well characterised in human and bovine counterparts.

## 2. Materials and methods

### 2.1. Isolation of lactoferrin from whey

Pooled milk of Arabian camels was stored at  $-70^{\circ}\text{C}$  until analysis. After thawing, the milk, which had a pH of about 6.6, was skimmed at 1000 g,  $4^{\circ}\text{C}$  for 15 min. Whey was obtained by acid precipitation of casein at pH 4.6 and  $37^{\circ}\text{C}$  for 20 min, using 0.1% acetic acid, followed by addition of 10 mM sodium acetate for neutralisation, and centrifugation at 4000 g for 5 min. The supernatant was dialysed twice against double distilled water for 5 h at  $4^{\circ}\text{C}$ , and once against 10 mM sodium phosphate buffer at pH 7.4 for 14 h at  $4^{\circ}\text{C}$ , using an autoclaved SPECTRA/POR membrane tubing with a molecular cutoff of 6–8 kDa (Spectrum Medical Industries, Inc., Los Angeles, CA). Prior to chromatography, samples were filtered through a hydrophilic 0.45  $\mu\text{m}$  membrane (ME25; Schleicher and Schuell, Dassel, Germany).

A Heparin-Sepharose HiTrap column (1 ml; Amersham Pharmacia, Uppsala, Sweden) was loaded with 40 ml whey. The column was washed with 10 ml PBS (10 mM sodium phosphate, 20 mM sodium chloride, pH 7.4). Elution was performed at ambient temperature by a linear gradient from 0.02 to 1 M sodium chloride over 40 min. The column effluent was monitored with an UV detector (L-7300; Merck, Darmstadt, Germany) at 280 nm. Proteins eluted were collected manually and lyophilised. Fractions were further purified, prior to micro-sequencing and molecular mass determination, by reversed-phase  $\text{C}_{18}$  HPLC (Kappeler, Farah & Puhon, 1998). Elution was performed by a linear gradient from 0.1% TFA in double distilled, nanofiltered water, to 0.1% TFA in acetonitrile, over 60 min.

### 2.2. Physico-chemical characterisation

Proteins collected from the effluent of the  $\text{C}_{18}$ -column were used directly for N-terminal sequencing (Kappeler et al., 1998).

Molecular masses of proteins were measured by matrix assisted laser desorption/ionization mass spectrometry (Kappeler et al., 1998).

### 2.3. Quantification

Lactoferrin eluted from the Heparin-Sepharose column was collected, tenfold diluted in distilled water and the absorbency measured at 280 nm. For calculation of the protein concentration, an extinction coefficient of  $84540 \text{ M}^{-1} \text{ cm}^{-1}$  was used for camel lactoferrin and of  $102890 \text{ M}^{-1} \text{ cm}^{-1}$  for bovine lactoferrin (Gill & Von Hippel, 1989). One litre whole milk was estimated to yield 0.8 l whey.

### 2.4. cDNA Sequence analysis

PolyA-mRNA isolation and construction of a cDNA library was done as described in Kappeler et al. (1998). Overlapping fragments of the lactoferrin cDNA, which together covered the complete sequence, were produced by the polymerase chain reaction (PCR). The following protocol was applied to most of the reactions: 2  $\mu\text{l}$  of the  $\lambda$ -cDNA library were taken as templates in 50  $\mu\text{l}$  PCR assays with 2.5 units Taq Polymerase (Amersham Pharmacia), which was blended with 0.05 units Pfu Polymerase (Stratagene, La Jolla, CA), and 5  $\mu\text{l}$  10 $\times$  TaqPlus Precision incubation buffer (Stratagene), 20 nmol of each dNTP (Amersham Pharmacia) and 50 pmol of specific primers. A series of 30 cycles was run with initial 2 min denaturation at  $94^{\circ}\text{C}$ , followed by 10 s denaturation at  $94^{\circ}\text{C}$ , 30 s annealing at  $55^{\circ}\text{C}$  and 2 min 30 s elongation at  $68^{\circ}\text{C}$ . Elongation prolongation was 20 s per cycle. A final 10 min incubation step at  $72^{\circ}\text{C}$  was added to increase the concentration of full-length products. Each PCR product was generated twice and ligated into a pGEM<sup>®</sup>-T Easy vector (Promega, Madison, WI) according to the manufacturer's instructions. In case of base reading ambiguities, a third PCR product was generated. Two  $\lambda$ -gt11 vector specific general primers were constructed to cover the 5'- and 3'-ends of lactoferrin cDNA:

$\lambda$ -gt11 forward:

5'-GACGACTCCTGGAGCCCGTCAGTA-3',

$\lambda$ -gt11 reverse:

5'-CACCAGACCAACTGGTAATGGTAG-3'.

The following PCR products were generated, mostly with the help of highly conserved regions in the cDNA sequences of other species (mixed base sites according to IUB code):

A 0.4 kbp PCR product was generated with 5'-CTGTCCCATAGACCTCTGCCGCTA-3', and  $\lambda$ -gt11 reverse.

A 0.8 kbp PCR product was generated with 5'-GTTTCRRTGGTGRCCRTMTCCMMA-3', and 5'-GTCTTTGAACAGCAGGTCCTTCTG-3'.

A 1 kbp PCR product was generated with 5'-TTCCAGCTCTTTGGCTCYCC-3', and 5'-TTGAACAGAAGGTTTTTGGT-3'.

A 0.4 kbp PCR product was generated with 5'-CCAGGCAAGTTTTGCTTGTTCAG-3', and  $\lambda$ -gt11 reverse.

The ligation products were dialysed and transformed into *E. coli* XL1-Blue (Stratagene) by electroporation with a Gene-Pulser® (BioRad, Hercules, CA) at 2.5 kV, 25  $\mu$ FD, and 200  $\Omega$  in 0.2 cm cuvettes. The transformed bacteria were plated overnight at 37°C on IPTG/X-Gal/Ampicillin-selective agar. White colonies were picked and grown overnight in 20 ml LB-Ampicillin 100 (Maniatis, Sambrook & Fritsch, 1989). Plasmid DNA was purified for fluorescent sequencing with the Wizard Plus SV Minipreps DNA Purification System (Promega).

Fluorescent sequencing was carried out using an ALF automated device (Amersham Pharmacia) with standard operating procedures. Sequencing samples were prepared, using the Cy5<sup>TM</sup>-dATP labelled, vector specific primers:

Cy5-SP6:

5'-TACTCAAGCTATGCATCCAACGCG-3',

and

Cy5-T7:

5'-ACTCACTATAGGGCGAATTGGGCC-3'

and the Thermo Sequenase cycle sequencing kit RPN 2438 (Amersham Pharmacia) according to the manufacturer's instructions. The following 25 cycles were applied: 95°C, 30 s, 50°C, 30 s, 72°C, 50 s. Where sequencing results differed, a third PCR product was sequenced. Overlapping sequences were detected using the FASTA module of the gcg/egcg programme package (Genetics Computer Group, Madison, WI). Consecutive sequences were joined and vector specific sequences removed. In this way, complete cDNA sequences were obtained.

### 2.5. Computational sequence analysis

Alignments of DNA and protein sequences and DNA similarity searches were performed using the gcg/egcg programme package (Genetics Computer Group).

Protein sequence similarity searches against the Swissprot database (Swiss Institute of Bioinformatics, Geneva, Switzerland) were made using a Smith and Waterman algorithm with default values (Barton, 1997).

A low-resolution model of the tertiary structure of camel milk lactoferrin was obtained by comparative modelling (Guex & Peitsch, 1997). The primary structure,

which was revealed by amino acid and cDNA sequencing, was threaded over resolved tertiary structures of different lactoferrins from other species. Multiple sequence alignments were made for improvement of modelling reliability. Energy minimisation of the model was done with force field computation by GROMOS96.

## 3. Results

### 3.1. Primary structure

PCR amplification products of a full-length cDNA clone of camel lactoferrin were sequenced (EMBL/GenBank<sup>TM</sup> accession number AJ131674). The clone was 2337 bp long, and contained a 5'-untranslated region of 22 bp and a 3'-untranslated region of 191 bp. The 5'-untranslated region contained a partial Kozak-box (Kozak, 1989) in front of the translational start site A<sup>23</sup>TG, with a purine at -3 bp, and cytosines at -1 bp, -5 bp and -8 bp. The 3'-untranslated region contained a polyadenylation signal A<sup>2303</sup>ATAAA. The open reading frame ranged from A<sup>23</sup> to G<sup>2308</sup>, and coded for a polypeptide of 707 aa residues. The start site of the mature protein was determined by similarity as Ala<sup>1</sup>. The 19 aa signal peptide was to 94.7, 84.2 and 78.9% identical to the respective signal sequences of bovine, porcine and human lactoferrin. The mature protein was 689 aa residues long, and had a molecular weight of 75.250 kDa (Table 1), without postranslational modifications. The isoelectric point of the unmodified peptide was at pH 8.14. The protein shared 74.9% sequence identity with bovine, 74.5% with porcine, and 74.0% with human lactoferrin. The pronounced homology gave indication for a nearly identical biological function of the proteins.

### 3.2. Glycosylation

N-linked glycans contribute about 4–11% (3–9 kDa) to the total mass of bovine lactoferrin, which is about

Table 1  
Physicochemical characteristics of camel and bovine lactoferrin<sup>a</sup>

	Camel	Bovine
Amino acid residues	689	689
Molecular mass (kDa) based on mass spectrometry	80.16–80.73	84.0
Molecular mass (kDa) based on amino acid sequence	75.250	76.143
Isoelectric point <sup>b</sup>	8.14	8.18
Concentration in milk (mg l <sup>-1</sup> )	220	140
Sequence identity		74.9%

<sup>a</sup>Data on bovine lactoferrin after Schanbacher, Goodman and Talhouk (1993), Yip and Hutchens (1997).

<sup>b</sup>Calculated with the gcg programme (Genetics Computer Group, Madison, WI 53711, USA).

84.0 kDa (Spik, Coddeville, Mazurier, Bourne, Cambilaut & Montreuil, 1994); Yip & Hutchens, 1997). Glycosylation enhances the solubility of the secreted protein and may help to bind at specific cell types, such as liver parenchymal cells (Ziere, Bijsterbosch & Van Berkel, 1993). Camel milk lactoferrin was found to contain 6.2% carbohydrates in colostrum milk and 5.6% in milk collected 15–30 days after parturition (Mahfouz, El-Sayed, Abd El-Gawad, El-Etriby & Abd El-Salam, 1997). The content of *N*-acetyl-glucosamine in camel milk lactoferrin was markedly higher than in ruminants' milk lactoferrins (3.35% in colostrum camel milk compared to about 1.75% in colostrum ruminants' milk, Mahfouz et al., 1997). In our study, the carbohydrate content of lactoferrin from end-lactational milk was 6.2–6.8% of total protein mass, calculated as a difference between the protein mass measured by MALDI-MS and the protein mass of the amino acid sequence (Table 1). Possible glycosylation sites, based on pattern analysis (Gavel & Von Heijne, 1990), are Asn<sup>233</sup>, Asn<sup>366</sup>, Asn<sup>518</sup> and Asn<sup>575</sup>. In bovine lactoferrin, four of five sites with *N*-glycosylation potential, Asn<sup>233</sup>, Asn<sup>368</sup>, Asn<sup>476</sup>, and Asn<sup>545</sup>, are glycosylated (Spik et al., 1994), and contribute to an overall carbohydrate content of 11.2%. Degree of glycosylation in human lactoferrin is about 6.40% (Spik et al., 1994), and thus similar to camel lactoferrin. Human lactoferrin contains 2 glycosylated sites, Asn<sup>138</sup> and Asn<sup>479</sup>, with glycans of the *N*-acetylglucosaminic type, which were also found in camel lactoferrin. By comparison with bovine and human lactoferrin, glycosylation of two of the four possible sites of camel lactoferrin is proposed (Fig. 1).

### 3.3. Concentration in camel milk

Colostrum camel milk was reported to have an extremely high lactoferrin content of 5.10 g l<sup>-1</sup> on the second day after parturition, compared to about 0.50 g l<sup>-1</sup> in bovine colostrum milk. After 30 days of milking, the lactoferrin level in camel milk went down to 0.34 g l<sup>-1</sup>, whereas in bovine milk, only about 0.06 g l<sup>-1</sup> were found (Abd El-Gawad, El-Sayed, Mahfouz & Abd El-Salam, 1996). In our studies, we used an extinction coefficient of 84540 M<sup>-1</sup> cm<sup>-1</sup> at 280 nm to calculate a lactoferrin concentration of 0.22 g l<sup>-1</sup> in a milk sample, which was taken at the end of the lactation period, 360 days after parturition. In a sample of pooled cow milk, lactoferrin concentration was 0.14 g l<sup>-1</sup>.

If it is assumed, that the main function of lactoferrin in milk is the inhibition of bacterial growth, a differently composed microflora in the gut of the new-born could be a reason for the apparently higher lactoferrin concentration in camel milk. Nevertheless, it has to be taken into account, that the concentration of protective proteins in milk also depends on the milk yield, which was about 5 l d<sup>-1</sup> for the camels, and about 15 l d<sup>-1</sup> for the cattle studied. It also has to be considered that the

immunological situation, with regard to the placenta type, the colostrum, the development and stimulation of the immune system in the calf, and the nutritional properties in general, will strongly differ between both species, as a result of the different habitats, in which the animals live, adaptation of camels to a sub-optimal food supply and quality, differences in the way, the offspring is raised, and the more distant paleontological relationship.

### 3.4. Tertiary structure and ligand binding

The polypeptide chain of transferrins consists of about 700 amino acids and is folded into two, tandemly arranged, asymmetrical metal binding sites, designated as N- and C-lobes, which probably evolved by gene duplication. The sequence of the camel lactoferrin N-lobe, which extended from Val<sup>6</sup> to Arg<sup>332</sup>, shared 39.8% sequence identity with the sequence of the C-lobe, which ranged from Val<sup>345</sup> to Arg<sup>673</sup>.

Under physiological conditions, transferrins bind one Fe<sup>3+</sup> cation in each lobe with a low dissociation constant of about 10<sup>-20</sup> (Brock, 1997). Cation binding requires synergistic binding of a bicarbonate anion, probably for charge compensation. In bovine and probably also in camel lactoferrin, the side chains of Asp<sup>60</sup>, Tyr<sup>92</sup>, Tyr<sup>192</sup> and His<sup>253</sup> are involved in binding of the cation in the N-lobe (Baker et al., 1998; Fig. 1). Two oxygens from the bidentate CO<sub>3</sub><sup>2-</sup> anion are suggested to complete a distorted octahedral geometry (Anderson, Baker, Norris, Rice & Baker, 1989). In the N-lobe, the side chains of Thr<sup>117</sup>, Arg<sup>121</sup>, and Tyr<sup>192</sup>, and two backbone hydrogens of Ala<sup>123</sup> and Gly<sup>124</sup>, are involved in binding of the bicarbonate anion. Lactoferrin retains its iron binding potential at pH values below pH 5.5, and even in the presence of citrate, in contrast to the other known transferrins (Brock, 1997). The primary structure and a modelled tertiary structure of the binding sites of bovine and camel lactoferrin were found to be nearly identical. We therefore assume that cations are bound by both lobes of the camel protein with similar affinities as in bovine lactoferrin.

### 3.5. Bacteriostatic activity of the N-terminal end

A high amount of Arg and Lys are clustered at the N-terminal end of lactoferrin, near and between a loop, which is formed by disulphide bonding of Cys<sup>19</sup> and Cys<sup>36</sup> (Fig. 1). The N-terminus of human and bovine lactoferrin was found to have strong bacteriostatic activity on gram-negative bacteria, as a result of non-specific binding to the negatively charged outer bacterial membrane, and subsequent release of lipopolysaccharides, thereby altering the permeability properties (Ellison, Giehl & LaForce, 1988). The N-terminal part of camel lactoferrin contained 13 basic residues (Fig. 1), at sites more similar to bovine lactoferrin than to human



different bacterial strains was found to have equal strength as the activity of bovine lactoferrin (Elagamy et al., 1992).

### 3.6. Food preservation

Lactoferrin was discussed to be a promising choice for preservation in food and cosmetics, since it is highly stable towards heat treatment and at low pH conditions (Saito et al., 1994). It helps to establish a favourable microflora, promotes growth of bifidobacteria, and may therefore find attraction for use in functional food products. The antimicrobial peptides formed upon gastric digestion of lactoferrin are also promising candidates as additives for food preservation. Primary structures of peptides formed from camel lactoferrin should be studied and activity of such peptides on inhibition of bacterial growth tested, to get better understanding of the action of lactoferrin in camel milk. The higher amounts of lactoferrin in camel milk are of advantage for natural preservation of the milk in arid regions, where technology for milk preservation is often not available.

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