https://doi.org/10.1017/S0007114500002191 Published online by Cambridge University Press

Occurrence, structure, biochemical properties and technological characteristics of lactoferrin

Jan M. Steijns* and A. C. M. van Hooijdonk

DMV International, R&D Center, PO Box 13, 5460 BA Veghel, The Netherlands

The structure of the iron-binding glycoprotein lactoferrin, present in milk and other exocrine secretions, has been elucidated in great detail, both the three-dimensional protein structure and the attached N-glycans. Structure–function relationships are being established. From these studies a function for lactoferrin in host defence and modulation of iron metabolism emerges. This paper describes in some detail how iron and other cations may be bound by lactoferrins from human or bovine sources and elucidates parts of the molecule that are critical for interactions with cells and biomolecules. Furthermore, the technological aspects, more specifically the heat-sensitivity, of bovine lactoferrin in different matrices are described.

Lactoferrin: Occurrence: Properties: Application

Background

Lactoferrin, an iron-binding glycoprotein of the transferrin family, has attracted increasing scientific interest since the early 1960s due to its high concentration in human breast milk (Lönnerdal & Iyer, 1995). The molecule has been identified in a number of mammalian species, like the cow, pig, equine, buffalo, goat and mouse. Many studies have been undertaken to pinpoint the biological role(s) of lactoferrin (Reiter, 1985; Sanchez *et al.* 1992a; Hambraeus & Lönnerdal, 1994). To date four conferences have been devoted to unravelling the features of its structure and establishing the structure–function relationships of this versatile and intriguing molecule (Hutchens *et al.* 1994; Hutchens & Lönnerdal, 1997; Spik *et al.* 1998; Shimazaki *et al.* 2000). Not surprisingly its functionality is related to the strong iron-binding properties, but non-iron related activities have also been described. Table 1 summarizes the various reported activities. Although its role in breast milk is still debated, it is evident from Table 1 that lactoferrin is a potential functional food ingredient for optimal dietary provision with iron and stimulation of the intestinal host defence.

This paper gives an overview of the occurrence, structure, biochemical properties and technological characteristics of lactoferrin. It focuses on bovine lactoferrin because of its similarity in structure and function to human lactoferrin and its commercial availability in metric ton amounts, thus allowing its application in infant formulas, foods, nutritional supplements or medical care. Where appropriate a comparison is made to native or recombinant human lactoferrin.

Occurrence

Lactoferrin is predominantly found in the products of the exocrine glands located in the gateways of the digestive, respiratory and reproductive systems, suggesting a role in the non-specific defence against invading pathogens. Thus one may not only find lactoferrin in the milk secreted by the mammary gland, providing protection to the neonate, but also in tears, synovial fluids, saliva and seminal fluid. Table 2 shows a selection of the lactoferrin amounts reported from these various biological fluids. Lactoferrin may also be found in blood. Plasma lactoferrin is derived from the neutrophils, which degranulate and synthesize lactoferrin during inflammation (Britigan *et al.* 1994; Levay & Viljoen, 1995). By means of immunohistochemistry, Inoue *et al.* (1993) demonstrated the presence of bovine lactoferrin in a variety of exocrine glands. Their observations support the idea that lactoferrin may have an antibacterial role.

Studies by Schanbacher *et al.* (1997) reveal that lactoferrin concentrations change during the mammary cycle. In the cow the concentrations of lactoferrin and its messenger RNA especially increase during development of the mammary gland, colostrum formation and involution of the gland, whereas during lactation the levels of lactoferrin decrease, as opposed to the increasing levels of casein. The latter suggests that casein has primarily a nutritional function for the calf, whereas lactoferrin may have growth factor-like activity and protective functions for the (non-) lactating gland and the critical phase for the newborn just after birth.

* **Corresponding author:** Dr. Jan Steijns, DMV International, Center of Expertise for Nutrition, PO Box 14, 6700 AA Wageningen, The Netherlands, fax +31 317 475 769; email steijnsj@dmv-international.com

Table 1. Reported *in vitro* and *in vivo* activities of lactoferrin

Activity	Mechanism	References
Iron absorption	Increasing solubility and receptor mediated uptake	Kawakami *et al.* 1988, 1993; Mikogami *et al.* 1995
Antioxidant	Iron scavenger	Matsue *et al.* 1994, 1995
Antimicrobial	Growth inhibition by iron scavenging or membrane desintegration	Reiter, 1985; Naidu & Arnold, 1997
Antiviral	Prevention of virus attachment	Harmsen *et al.* 1995; Marchetti *et al.* 1996; Yi *et al.* 1997
Anti-inflammatory, immune modulating	LPS binding, stimulation of NK cells, reduction of pro-inflammatory cytokines, T-cell maturation	Zimecki *et al.* 1991; Cohen *et al.* 1992; Mattsby-Baltzer *et al.* 1996; Shimizu *et al.* 1996
Anti-cancer	Unknown	Bezault *et al.* 1994; Sekine *et al.* 1997; Yoo *et al.* 1997

Structure

Lactoferrins are single chain polypeptides of about 80 000 Da containing 1–4 glycans, depending on the species (Spik *et al.* 1994). Bovine and human lactoferrin consist of 689 and 691 amino acids, respectively; the sequence identity is 69 % (Pierce *et al.* 1991). The 3-D conformations of both human and bovine lactoferrin are now known in great detail due to the pioneering work of Baker and colleagues (Haridas *et al.* 1995; Moore *et al.* 1997). The elucidation of the structure of the glycans attached to lactoferrins from various species has been the result of the extensive studies of Montreuil, Spik and co-workers (Coddeville *et al.* 1992; Spik *et al.* 1994).

The 3-D structures of bovine and human lactoferrin are very similar, but not identical. Each lactoferrin comprises two homologous lobes, called the N- and C-lobes, referring to the N-terminal and C-terminal part of the molecule, respectively. Each lobe further consists of two sub-lobes or domains, which form a cleft where the ferric ion (Fe^{3+}) is tightly bound in synergistic cooperation with a (bi)carbonate anion. These domains are called N1, N2, C1 and C2, respectively. In bovine lactoferrin N1 stands for the sequences 1–90 and 251–233, N2 for 91–250, C1 for 345–431 and 593–676, and C2 for 432–592; the sequence 334–344 represents the so-called hinge, which is a helix conformation with three turns and fulfils a role during opening and closing of the domains. The secondary structure is partly due to the presence of disulphide bridges between cysteine residues. The amino acids contributing to the binding of ferric ions in the cleft are Asp60, Tyr92, Tyr192 and His253 in the N-lobe and Asp395, Tyr433, Tyr526 and His595 in the C-lobe; in both lobes the (bi)carbonate ion acts synergistically in the iron binding. The reader is referred to the papers of Moore *et al.* (1997) and Haridas *et al.* (1995) for more structural details.

Table 2. Occurrence of lactoferrin in biological fluids

Biological fluid	Amounts reported*
Colostral breast milk	>7 mg/ml
Mature breast milk	>1–2 mg/ml
Tear fluid	>2·2 mg/ml
Seminal plasma	>0·4–1·9 mg/ml
Synovial fluid	>10–80 μg/ml
Saliva	>7–10 μg/ml
Cow's colostral whey	>1·5 mg/ml
Cow's milk	>20–200 μg/ml

* Data taken from: Korhonen, 1977; Arnold *et al.* 1979; Renner *et al.* 1989; Levay & Viljoen, 1995.

In bovine lactoferrin there are five potential sites for N-bound glycan structures: the asparagine (Asn) residues at positions 233, 281, 368, 476 and 545. The chemical analysis, however, only reveals four N-linked glycans; apparently Asn281 is not used. The residue Asn476 seems to be conserved among species. Spik *et al.* (1994) gives a nice overview on the glycans attached to lactoferrins of different species and further illustrates the complexity of these structures. The sugars found in bovine lactoferrin are *N*-acetyllactosamine, *N*-acetylglucosamine, galactose, fucose, mannose and neuraminic acid.

Biochemical properties

Iron binding

In the 'natural state' bovine lactoferrin is only partly saturated with iron (15–20 %) and has a salmon pink colour, the intensity of which depends on the degree of iron saturation. Iron-depleted lactoferrin with less than 5 % iron saturation is called apolactoferrin, whereas iron-saturated lactoferrin is referred to as hololactoferrin. In breast milk the lactoferrin found is essentially apolactoferrin.

The affinity of lactoferrin for iron is very high (about 260 times that of blood serum transferrin) with an affinity constant of about 10^{20} (Baker *et al.* 1994). The iron-binding capacity of lactoferrin is dependent on the presence of (small amounts) of (bi)carbonate. The binding site appears to be optimized for the binding of ferric iron and (bi)carbonate with respect to size, charge and stereochemistry, as evidenced from a number of structural studies with different anions and cations, or using mutant recombinant lactoferrins (Harrington *et al.* 1987; Baker *et al.* 1994; Brodie *et al.* 1994; Faber *et al.* 1997). Oxalate can replace (bi)carbonate with regard to iron binding, but citrate can not. However, citrate may bind to bovine lactoferrin during isolation (Brodie *et al.* 1994), which reflects the *in vivo* situation in the milk. Depending on the bicarbonate concentration, high concentrations of citrate can counteract the iron-binding efficiency of lactoferrin (Reiter, 1985).

Cations other than ferric iron may be bound in the cleft and change the wavelength absorption maximum; e.g. ferric iron-saturated lactoferrin has an absorption maximum at 466 nm, whereas copper (Cu^{2+})-saturated lactoferrin has a maximum at 434 nm (Brodie *et al.* 1994). Besides Cu^{2+}, Mn^{3+}, Co^{3+}, Zn^{2+} may also be bound.

Using site-directed mutagenesis Ward *et al.* (1996) purified mutant C- and N-lobe human lactoferrins from *Aspergillus awamori*; in these lactoferrins the two tyrosine residues involved in the iron binding were changed for

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Table 3. Number of amino acid residues in bovine and human lactoferrin (Pierce *et al.* 1991)

	Bovine milk	Human milk
Alanine	67	63
Proline	30	35
Arginine	39	43
Lysine	54	46
Asparagine	29	33
Valine	47	48
Tryptophan	13	10
Cysteine	34	32
Threonine	36	31
Isoleucine	15	16
Serine	45	50
Glutamine	29	27
Glutamic acid	40	42
Phenylalanine	27	30
Methionine	4	5
Leucine	65	58
Glycine	48	54
Tyrosine	22	21
Aspartic acid	36	38
Histidine	9	9
Total number of residues	689	691

alanine either in the C-lobe or the N-lobe or both. Their studies suggest that the C-lobe contributes more to iron stabilization than the N-lobe.

Legrand *et al.* (1990) studied the iron binding site of the N-lobe of both native human and bovine lactoferrins using the 30 kDa tryptic fragment N4–281 (including domains N1 and N2) and the 20 kDa tryptic fragment N91–251 (domain N2). From their pH-induced iron release studies they concluded that the absence of the Asp60 residue from domain N2 did not influence the iron stability. Furthermore, they also found evidence for iron stabilizing interactions between the N-lobe (30 kDa tryptic fragment) and the C-lobe (a 50 kDa tryptic fragment). Bovine lactoferrin started to release iron when the pH was below 4, whereas human lactoferrin was somewhat more resistant with release below pH 3. Finally they showed that full deglycosylation of both tryptic N-lobe fragments led to 50–100 % loss of the iron-binding capacity. Studies on intact deglycosylated recombinant human lactoferrin, however, did not show a decrease in iron binding (VanBerkel *et al.* 1995).

Physico-chemical properties

The amino acid compositions of bovine and human lactoferrin are shown in Table 3.

Lactoferrin has a very high isoelectric point. The theoretical pI values calculated for bovine and human lactoferrin are 9·4 and 9·5, respectively; yet, reported experimental values may deviate a lot (Shimazaki *et al.* 1993). Depending on the method used values around 8 have been reported for bovine lactoferrin, whereas a wide range of pI 5·5 to 10 have been reported for human lactoferrin. This variation may be due to variations in the arginine rich N-terminus of the molecule due to separation conditions (VanBerkel, 1998).

Electrophoretic comparisons between lactoferrins from bovine mammary secretions, milk neutrophils and human milk have been published by Hurley *et al.* (1993). These authors found differences in mobility in SDS-PAGE gels between neutrophil-derived lactoferrin and mammary gland-secreted lactoferrin; the latter migrated with an apparent molecular weight of about 4 kDa lower. Human milk lactoferrin also separated as two distinct bands in the gels. All tested lactoferrins were glycosylated; differences in relative lectin-binding properties were observed between human and bovine milk lactoferrin.

Magnusson *et al.* (1990) demonstrated evidence for shared antigenic determinants among human, bovine and pig lactoferrin.

Strong cationic N-terminus

The N-terminus of both human and bovine lactoferrin has strong cationic peptide regions that are responsible for a number of important binding characteristics.

The binding of bacterial LPS (lipopolysaccharide) involves a loop in the N1 domain of both human and bovine lactoferrin which expresses a high affinity binding site; the C-lobe appears to have low affinity binding sites (100–130 times lower affinity). The human loop apparently consists of the sequence 28–34; the bovine loop resides in the sequence 17–41 (Elass-Rochard *et al.* 1995).

VanBerkel *et al.* (1997) studied the binding of human lactoferrin to lysozyme, LPS, heparin and DNA with intact and N-terminally deleted lactoferrin molecules. They showed that the degree of iron saturation does not influence the binding to all these four compounds. But removal of one or more arginine residues (Arg^2, Arg^3, Arg^4, Arg^5) decreased the binding of lactoferrin to different extents and the more upon removal of extra arginine residues. No binding occurred with a mutant lactoferrin that had lost the first five amino acid residues (Gly^1-Arg^2-Arg^3-Arg^4-Arg^5). This demonstrates the decisive role that this stretch of four arginine residues in interaction with biomolecules plays in host defence.

Legrand *et al.* (1997) were able to demonstrate that the number of binding sites of human lactoferrin for human lymphoblast T-cells was highest for the intact molecule, but decreased progressively from about 100 000 per cell to 17 000 per cell upon removal of, respectively, Arg^2, Arg^3 and Arg^4. Bovine lactoferrin had about the same binding parameters as intact human lactoferrin. These authors propose that the binding occurs to the sulphated molecules on the cells and that the Arg^5 residue does not play a role in this respect.

The cationic N-terminus of bovine lactoferrin is of special interest because of the reported antibacterial activity (Bellamy *et al.* 1992; Tomita *et al.* 1994; Kang *et al.* 1996; Dionysius & Milne, 1997; Hoek *et al.* 1997). This aspect will be dealt with in greater detail by van Hooijdonk & Steijns in part B of this supplement.

Technological characteristics

Heat stability

The thermal stability of lactoferrin has predominantly been

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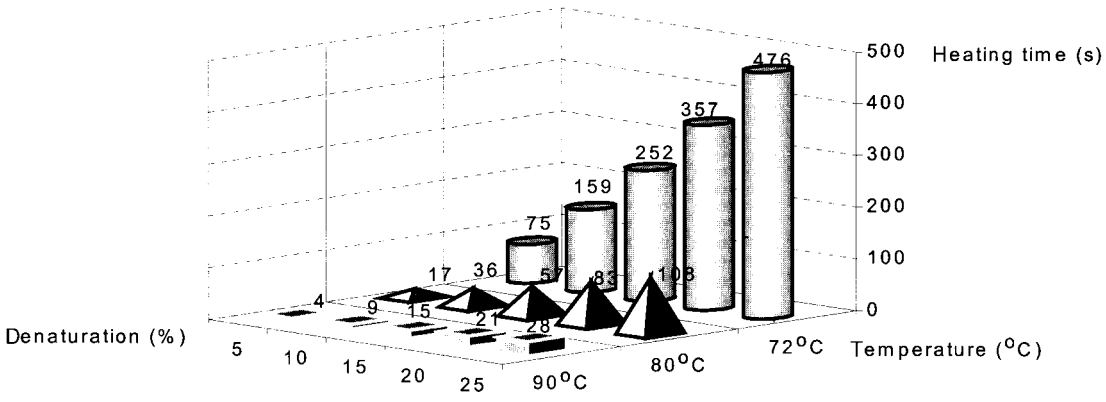

Fig. 1. Heat denaturation of bovine lactoferrin as a function of time and temperature (Kussendrager, 1994). The figure lists the heating times (s) which give either 5, 10, 15, 20 or 25 % denaturation of 0·1 % bovine lactoferrin in 10 mM sodium phosphate buffer (pH 6·5) at temperatures of 72, 80 or 90°C.

studied in model systems using buffered aqueous solutions or when added to milk.

Abe *et al.* (1991) investigated the heat stability of bovine apolactoferrin in distilled water adjusted with HCl or NaOH to different pH values. Temperatures were varied from 80°C to 120°C; a fixed heating time of 5 min was chosen. At neutral and alkaline pH (pH 6–10) turbidity and sometimes gel formation occurred, the effect being more pronounced with higher temperatures. Screening involved residual iron binding, reactivity with antiserum, antibacterial activity and HPLC homogeneity. The final conclusion was that heating apolactoferrin at pH 4 for 5 min at 90°C was a pragmatic way for batch sterilization. Preheating at 70°C for 3 min followed by UHT for 2 s at 130°C gave only 3 % loss in residual iron-binding capacity when compared with the unheated sample.

Paulsson *et al.* (1993) studied the thermal stability of (bovine) apolactoferrin and iron-saturated lactoferrin in relation to the antibacterial activity and/or bacterial interaction. Pasteurization at 72°C for 15 s had no influence when compared to the unheated control; however, UHT treatment (4 s, 135°C) abolished the ability of iron-saturated lactoferrin to bind to bacteria as well as the bacteriostatic activity of apolactoferrin.

Sanchez *et al.* (1992b) showed first-order reaction kinetics for denaturation of bovine lactoferrin between 72°C and 85°C. Apolactoferrin denatures faster than hololactoferrin; when phosphate buffer was compared to milk, it became clear that the heat-sensitivity of both lactoferrins was higher in the milk matrix. The authors concluded that the standard pasteurization regimes used in the dairy industry had practically no effect on lactoferrin structure. A similar conclusion was drawn by Luf & Rosner (1997). In a follow-up study Oria *et al.* (1993) used the interaction of lactoferrin with monocytes to assess the effects of a treatment of 8 s at 137°C on the ability to displace labelled lactoferrin or to stimulate cell proliferation. This heat treatment had only little effect.

In a kinetic approach using differential scanning calorimetry to analyse unfolding of bovine lactoferrin and immunodiffusion for aggregation, Kussendrager (1994) concluded that the thermal stability of lactoferrin is affected by environmental conditions such as pH, salts and (whey) protein, and that, as a consequence, the parameters of the heat-induced denaturation of lactoferrin have to be determined under conditions of the application of interest. Fig. 1 shows the heat denaturation of bovine lactoferrin (0·1 % in phosphate buffer, pH 6·5) in function of time at temperatures of 72, 80 and 90°C, respectively (Kussendrager, 1994).

Application examples

We used an experimental model for shelf-life improvement to investigate whether the iron-scavenging properties of lactoferrin are retained after prolonged processing at about 70°C in an industrial environment. We prepared a soy fat powder in our pilot factory with 55 % soy oil, containing unsaturated fatty acids vulnerable to oxidation, 20 % of protein (half caseinate, half whey protein), and 20 % maltodextrin; the residual 5 % consisted of moisture, minerals and other minor components. In order to protect the unsaturated fatty acids against oxidation and as a consequence the powder against development of rancidity and decrease in powder performance and dissolving characteristics, a vitamin based antioxidant mix or bovine lactoferrin were included as well. The rationale behind addition of lactoferrin was that in this way oxidation catalysts like iron and copper ions could be captured and neutralized. The estimated residence time of lactoferrin at 70°C during the liquid processing prior to spraydrying was about 30 minutes. After spraydrying the powder was stored at 5°C, 20°C and 30°C for 12 months and evaluated regularly with respect to peroxide development, an indicator of the protective effects of the antioxidant ingredients; the control contained no antioxidant additions. Fig. 2 shows the peroxide development profile at 30°C for the three different powder types. It can be concluded that lactoferrin improved the shelf life of the soy fat powder; compared to the vitamin mix the protective effect is even slightly better. At 20°C the performance of both antioxidants was comparable. The control had higher peroxide values at all three temperatures. This example shows the potential of lactoferrin as an antioxidant in food products to

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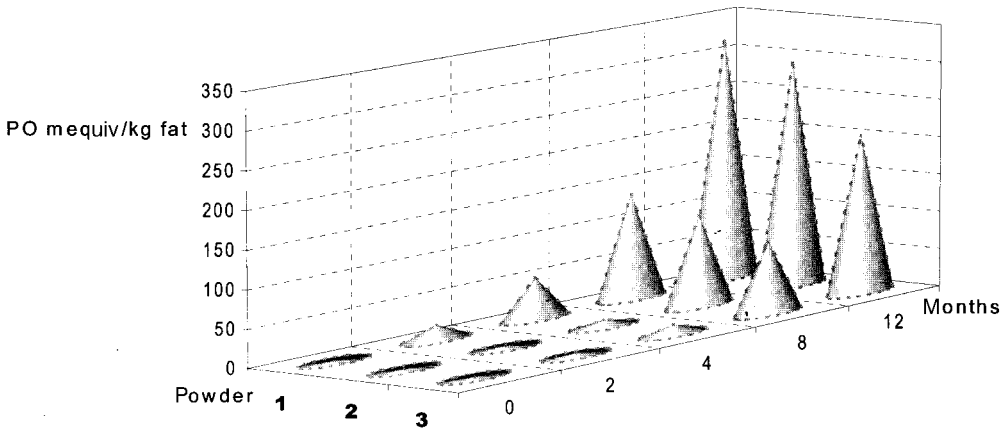

Fig. 2. Lactoferrin as an antioxidant. Powders were stored for up to 12 months at 30°C; at the indicated time points samples were analysed for peroxides (PO), expressed in mequiv/kg fat. Powder 1 is the control without added antioxidant; powder 2 contains 150 p.p.m. ascorbylpalmitate and 30 p.p.m. dl-α-tocopherol; powder 3 contains 100 p.p.m. bovine lactoferrin.

Table 4. Current commercial application of bovine lactoferrin

Market segment	Functionalities described
Milk-based infant formulas	Mimic breast milk, improved resistance against pathogens
Health supplements	Aid in iron absorption, e.g. for pregnant women, immune aid
Functional food drinks	Increases iron solubility and absorption
Cosmetics	Antioxidant
Oral care products, chewing gums	Improved oral hygiene
Feed supplements	Anti-feline virus in cats

increase shelf life. The mechanism by which lactoferrin exerts this antioxidant effect is likely the control of the iron or copper catalysed Fenton reaction, which generates the potent hydroxyl free radical from peroxides (Matsue *et al.* 1994, 1995). This mechanism is different from the radical quenching effects exerted by antioxidant vitamins like tocopherols. It is not known at present whether antioxidant vitamins and lactoferrin can act synergistically.

Finally Table 4 shows which functionalities of (bovine) lactoferrin are currently highlighted in commercial products.

Conclusion

Human and bovine lactoferrin are by now fairly well characterized biologically active proteins. Although differences in structural and biochemical properties exist, their bioactivity, as assessed *in vitro* or in animal models, is quite comparable. Bovine lactoferrin is currently used in commercial products targeting at optimal iron delivery, mimicking human breast milk or boosting the natural defence against infections. Both nutritional and functional use as a natural antioxidant are also foreseen due to lactoferrin's ability to tightly scavenge iron thereby removing an important catalyst for radical formation.

Lactoferrin provides an excellent example of how comparison of the composition of the milk from humans and cows with respect to their function for the newborn, can initiate commercial development and production of a minor milk protein component with broad application possibilities in food and feed.

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