Lactoferrin : A Multifunctional Protein in Milk

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Introduction

Major antimicrobial proteins found in milk are immunoglobulins, lactoperoxidase, lactoferrin and lysozyme and these antimicrobial factors act in a cooperative manner in living systems. These proteins are secreted not only by the mammary gland but also by other exocrine glands, such as intestinal and salivary glands, and neutrophils. Immunoglobulins act via specific antigen-antibody reactions. Lysozyme is an enzyme with the ability to hydrolyze polysaccharides in the cell wall of Gram-positive bacteria, such as *M. luteus* and *B.subtilis*, thereby inducing bacteriolysis. Lactoperoxidase, a heme protein, catalyses an oxidation reaction with thiocyanate ion(SCN^-) and hydrogen peroxide(SCN^-/H_2O_2/lactoperoxidase system) which produces hypothiocyanite($OSCN^-$), a potent bacteriostatic or bactericidal substance effective against *S.mutans*(suppression of growth and acid production), *E.Coli*(growth inhibition), some lactic acid bacteria(suppression of acid production) and other microorganisms.

Lactoferrin is a metal-binding glycoprotein and is a member of the transferrin family of proteins, which share a high degree of amino acid sequence homology and conformational similarities. As lactoferrin shows not only antimicrobial action but also a very broad range of other biological functions relating to the host defense system, it is called a multifunctional protein. This report outlines the biochemical properties of lactoferrin, its antimicrobial activity, ligand interactions of lactoferrin, and inhibition of tumor metastasis by lactoferrin.

General description of lactoferrin

Lactoferrin is present at a concentration >2 ㎎/㎖ in human milk and at 20-200㎍/㎖ in bovine milk(Masson and Heremans, 1971). Colostral milk contains more lactoferrin than mature milk and the lactoferrin content rises at the end of lactation and in mastitic milk. Human lactoferrin has a molecular weight of 82.4kDa(703amino acid residues, Metz-Boutigue et al., 1984) and bovine lactoferrin has a molecular weight of 83.1 kDa (689 amino acid residues, Pierce et al., 1991). The carbohydrate content of microheterogeneity of sialic acid content(Shimazaki et al., 1993, Spik et al., 1988). The amino acid sequence of lactoferrin has been determined from analysis of its peptides, and by cDNA or mRNA analysis. The molecular structure of lactoferrin as been reported from X-ray crystallographic analysis performed on human lactoferrin(Anderson et al., 1989).

Lactoferrin works as a component of the host defense system, displaying activities such as direct and indirect anti-bacterial action, anti-virus effects, regulation of cell growth and myelopoietic regulation, effects on phagocytic cell function and on immuno-regulatory activities, regulation of inflammatory reactions, stimulation or suppression of iron-absorption in the gut, inhibition of bacterial translocation in the gut and other related biological functions(Brock, 1995, Nuijens et al., 1996)

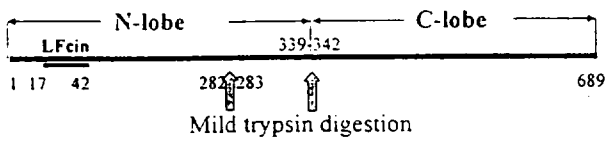
Antimicrobial activity of lactoferrin and its peptic fragment

Lactoferrin inhibits the growth of many Gram-positive and Gram-negative bacteria, some molds and yeasts(Arnold et al., 1980, ellison, 1994). Bacteriostatic effects of lactoferrin are due to its ability to bind environmental iron ions. Adding to its effect as an iron ion chelator, its direct binding to the cell membrane and destruction of microorganisms have been demonstrated. Growth inhibitory effects on parasites, such as *Toxoplasma gondii*, also have been reported(Tanaka et al., 1996, 1997) but the mechanism of action is still unresolved. Some kinds of bacteria, e.g. *E. coli* secrete chelators to enhance iron uptake, and the bacteriostatic effects of lactoferrin are thought to be due to its ability to bind environmental iron(Arnold et al., 1977). Moreover apo-lactoferrin has been shown to bind to the outer membrane of Gram-negative bacteria and causes the release of lipopolysaccharide(Ellison, 1994)

Recently, hydroysates produced by gastric pepsin cleavage of human or bovine lactoferrin were found to contain a potent

bactericidal peptide, named lactoferrincin® H and B, respectively(Bellamy et al., 1992). The microbial killing effect of these peptides was ten to one-hundred times stronger than that of undigested lactoferrin. These active peptides display broad-spectrum antimicrobial properties, having effectiveness against Gram-positive and Gram-negative bacteria, yeast and filamentous fungi and, in the case of some species, with varying degrees of effectiveness due to strain-to-strain variation in susceptibility(Bellamy et al., 1992, 1993, Jones et al., 1994, Wakabayashi et al., 1992). It is interesting that lactoferricin B shows stronger antimicrobial activity in vitro than lactoferricin H. parasiticidal effects on *Toxoplasma gondii* have been demonstrated (Tanaka et al., 1995), too. Lactoferricin B consists of a single peptide chain of 26 amino acid residues having the sequence FKCRRWQWRMKKLGAPSITCVRRAFA, derived from the N-terminal region(17-42) of bovine lactoferrin.

Lactoferricin has been shown to have an affinity for cell membranes and may exert its lethal effect by disruption of essential membrane functions. It binds directly to lipopolysaccharide and disrupts the permeability barrier of the outer membrane of Gram-negative bacteria(Yamauchi et al., 1993). Also, lactoferricin B acts to disrupt the permeability properties of the cytoplasmic membrane(Bellamy et al., 1993). On the other hand, *Bifidobacterium bifidum* shows resistance to lactoferricin(Tomita et al., 1994)



Function

* Metal binding
* Bacteriostatic & bactericidal effects(both dependent on & independent of Fe-binding)
* Receptor binding
* LPS binding
* Heparin binding
* Binding to microorganisms and many others !

* Metal binding
* Fe-chelation dependent bacteriostatic effect and others !

Figure 1. Bovine lactoferrin biological function map

Structural features of lactoferrin

The lactorerrin molecule is composed of two structural lobes, the N-lobe and the C-lobe, each of which consists of 3 domains(Baker et al., 1991). Isolation of the C-lobe has been accomplished by gel-filtration and ion-exchange chromatography after mild-tryptic digestion of bovine lactoferrin(Shimazaki et al., 1993). However, there are some difficulties involved in obtaining the N-lobe because some of the peptide bonds (282K-283S) in the N-lobe are very sensitive to trypsin digestion.

The functional difference between the N-lobe and C-lobe is another interesting aspect. It is reported that one of the N-terminal domains of human lactoferrin is responsible for binding to receptors expressed on phytohemagglutinin-stimulated peripheral blood human lymphocytes and receptors on T-lymphocytes(Rochard et al., 1989), as shown in Figure 1. Also, we have obtained experimental results showing that the C-lobe shows no ability to bind to *Trypanosoma cruzi*, in contrast to intact lactoferrin(Shimazaki et al., 1993)

Antimicrobial activity of the C-lobe

In our laboratory, we are investigating the function of the C-lobe. We thought that the C-lobe may have an antimicrobial function depending on iron-binding. Therefore, the antibacterial activity of the C-lobe was compared with that of bovine apo-and holo-lactoferrin, as shown in Figure 1. In this case, the C-lobe was isolated by reverse-phase HPLC using acetonitrile gradient elution. Cultures of *E. coli* O111 treated with the C-lobe at 5 ㎎/㎖ showed a turbidity about 80% of the control level and those treated with intact apo-or holo-lactoferrin at the same concentration showed a turbidity about 20-30% of the control level. As C-lobe purified by reverse-phase HPLC was used for the assay, there remains the possibility that the C-lobe was not in its native conformation even after the removal of acetonitrile followed by dialysis against phosphate buffer for 2 days in the refrigerator.

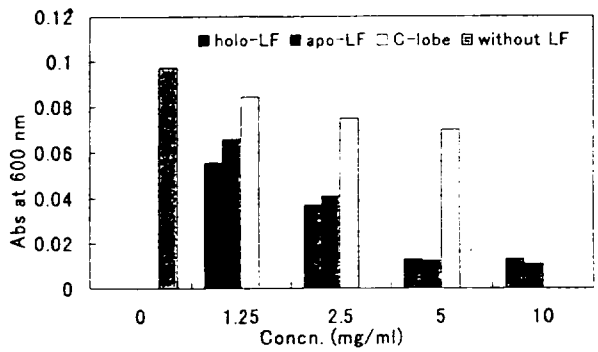


Figure 2. Comparison of the antimicrobial activity of bovine apo－and holo－lactoferrin and C－lobe. Each value is the average of 8 measurements.(*E. coli* O111 cultured in 1% Bactopeptone, pH 6.8, at 37℃for 5h)

Ligand interactions and the structure of lactoferrin

Lactoferrin can bind metal ions such as Fe^{3+}, Cu^{2+}, Zn^{2+}, Al^{3+}, Ga^{3+} and Ca^{2+}. In the natural state in milk, lactoferrin is 25－35% saturated with iron ions. The holo－lactoferrin molecule contains two Fe^{3+} ions, one in each lobe, and HCO_3－ions are necessary for iron binding. Other substances that have been found to bind to lactoferrin include many kinds of small molecules and biopolymers, such as dyes, drugs, ferritin, immunoglobulin, albumin, β－lactoglobulin, phospholipid, DNA, lipopolysaccharide, agarose and heparin. Lipopolysaccharide and phospholipid are cell－surface components and heparin is produced by lung, liver, skin and mast cells. Therefore, it is important to study the mode of interaction between lactoferrin and these substances to understand the functions of lactoferrin mediated through its interaction with somatic cells and microorganisms.

Determination of the heparin－binding region of lactoferrin

It is well known that lactoferrin displays affinity for heparin and this interaction has been used for the isolation of lactoferrin. From a hydrolysate of bovine lactoferrin produced by pepsin digestion, we have isolated a heparin-binding peptide by chromatography using an immobilized heparin column and an ODS column. N-terminal amino acid sequence analysis showed that this heparin-binding peptide starts from the 17th amino acid residue of lactoferrin and the molecular mass of this peptide was determined to be 3195.5 by MALDI-TOF mass spectrometry. Therefore, the sequence of the heparin－binding peptide was taken to be FKCRRWQWRMKKLGAPSITCVRRAFA, within which the two Cys residues form a disulfied bridge, and this sequence is the same as that of the bactericidal peptide lactoferricin B. By ELISA, we confirmed that this peptide showed reactivity with monoclonal antibody against lactoferricin B(Shimazaki et al., 1996).

We consider that the heparin－binding sites of bovine lactoferrin are KCRR(18－21), RMKK(25－28) and RR(38－39), as shown in Figure 3. In bovine lactoferrin, the sequence BXBB is found at two locations in the N－lobe and one in C－lobe (KDKK, 452－455). Here, B means basic amino acid residue. The heparin－binding site of human lactoferrin is reported to be at 1G－2－5R(Mann et al., 1994) or 5R, 25R－XX－28R－29 K－X－31R(BXXBBXB)(Wu et al., 1995). The consensus sequences BBXB or BBBXXB have been reported for heparin －binding sites of vitronectin, etc.(Cardin and Weinstraub, 1989).

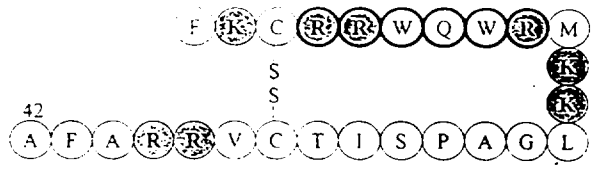


Figure 3. The Heparin binding sites of bovine lactoferrin. The sites for binding of heparin(thick circle), and the antimicrobial subregion(gray dotted circle) are shown. The sequence "RRWQWR" is essential for the antimicrobial activity of lactoferricin B(Tomita et al., 1994)

We have synthesized peptides having the heparin－binding sequence of bovine lactoferrin to confirm the above described results. Peptides were synthesized from Fmoc amino acid active esters on a pre－activated cellulose membrane using the Simple Precise Original Test System for epitope analysis(SPOT$_s^{TM}$, a product of Genosys Biotechnologies, Inc).

The membrane was treated with blocking solution containing casein, washed out and incubated with heparin solution. To detect hearin binding to the synthesized peptides, the membrane was incubated with a solution of human vitronectin, a heparin－binding protein, after excess heparin was washed out. Next, the membrane was incubated with monoclonal antibody against

human vitronectin. Finally, alkaline phosphatase-labeled anti-mouse antibody(goat) was used to detect the binding of anti-vitronectin antibody. This experiment clearly showed that the sequence BXBB exhibits heparin-binding ability.

In water, this peptide consists of mainly β-sheet(ca. 50%) and unordered structures, as determined by examining circular dichroic spectra. When heparin was added into the lactoferricin solution, the circular dichroic spectra changed and this alteration was found to be reversible. Therefore, it may be concluded that the interaction between the peptide and heparin led to the reversible spectral change due to a conformational change in the peptide.

Inhibition of tumor metastasis

The effectiveness of bovine lactoferrin and lactoferricin B in inhibition of tumor metastasis has been examined in experimental and spontaeous metastasis models using syngeneic mice (Yoo et al., 1997). The subcutaneous administration of bovine apo-lactoferrin(1 mg/mouse) or lactoferricin B(0.5 mg/mouse) one day after tumor inoculation significantly inhibited liver and spleen metastasis of L5178Y-ML25 lymphoma cells and lung metastasis of B16-BL6 cells, whereas human apo-lactoferrin and bovine holo-lactoferrin at the dose of 1 kg/mouse did not. Furthermore, both bovine apo-lactoferrin and lactoferricin B, but not human apo-lactoferrin or bovine holo-lactoferrin, inhibited the number of tumor-induced blood vessels and suppressed tumor growth on day 8 after tumor inoculation in an in vivo model. In a long-term analysis of tumor growth for up to 21 days after tumor inoculation, single administration of bovine apo-lactoferrin significantly suppressed the growth of B16-BL6 cells throughout the examination period, but lactoferricin B showed inhibitory activity only during the early period(8 days).

In a spontaneous metastasis model(Yoo et al., 1994), multiple administration of either bovine apo-lactoferrin or lactoferricin B significantly inhibited lung metastasis of B16-BL6 cells, however only bovine apo-lactoferrin exhibited an inhibitory effect on growth of the primary tumor as assessed at the time of primary tumor amputation(on day 21 after tumor inoculation). The results suggest that bovine apo-lactoferrin and lactoferricin B inhibit tumor metastasis through different mechanisms, and that the inhibitory activity of bovine lactoferrin on tumor metastasis may be related to the property of iron-saturation. Further studies are now in progress to elucidate more fully the mechanism of the effects of bovine apo-lactoferrin and lactoferrincin B on tumorigenesis, and to examine the potential for application of these materials as therapeutic agents.

In addition to the inhibitory effect of lactoferricin B on tumorigenesis, we recently found that lactoferricin B induced apoptosis in culture of human leukemia cells and this apoptosis-inducing activity was related to the production of intracellular reactive oxygen species(Yoo et al., 1997). Furthermore, analysis of the biochemical mechanism associated with apoptosis induction revealed that lactoferricin B regulated the cell cycle(G1 arrest) and activated CPP 32/Yama(Caspase-3) protease in tumor cells undergoing apoptosis.

Practical applications of lactoferrin

Lactoferrin is now used in formulated milk powder for infants as it is expected to contribute to the babies' defense system in the gut against harmful microorganism, i.e., inhibition of the growth of *Enterobacteriaceae* and promotion of the growth of *bifido-bacteria*. Also, inhibition of bacterial translocation in the gut has been reported recently(Teraguchi et al., 1995). Lactoferrin saturated with excess iron ions is expected to be useful as an iron supplement for women to prevent anemia. Cosmetics for facial skin containing lactoferrin are on the market, which are expected to be advantageous because of its anti oxidation effects, its suppression of inflammation and its protective effect for somatic cells. Chewing gum containing lactoferrin and lactoperoxidase is now on sale.

It is reported that oral administration of lactoferrin to cats suffering from stomatitis improved clinical signs of pain and inflammation in 68% of the animals treated. This therapy was also effective for cats suffering from intractable stomatitis due to feline immunodeficiency virus infection(Sato et al., 1996) and the effect seems to be attributable to inhibition of secondary bacterial infection or activation of the immune system by lactoferrin. Lactoferrin is not only being used in treatment of cats and dogs but also other animals such as an elephant in the

zoo. Lactoferrin is being used in the fish farming industry to prevent or immunoglobulins isolated from bovine milk by membrane treatment is commercially available for use as a feed additive or calf milk replacer. It is expected to be effective to decrease the occurrence of diarrhea and promote the growth of young calves.

Conclusions

Lactoferrin shows many kinds of biological activities in vivo and in vitro, it is very difficult to explain all of its mechanisms of action without any inconsistency. On the other hand, the multifunctionality of lactoferrin has attracted the interest of many researchers in various fields from basic academic research to applied commercial research, in areas ranging from agriculture and food to pharmacology and medicine. Because of its ability to contribute to host defense mechanisms, considerable potential exits for the expanded use of lactoferrin in many foods, animal feedstuffs and medicine.

Since 1992, four international symposia have been held to discuss advances in lactoferrin research. One of them was the International Dairy Federation(IDF) Seminar on "Indigenous antimicrobial agents of milk－Recent developments" held in Uppsala, Sweden in 1993(published by IDF, 1994). Others were the International Symposium on Lactoferrin held in Honolulu, Hawaii in 1992("Lactoferrin : Interactions and Biological Functions" Humana Press Inc., 1997). The third International Conference on Lactoferrin was held in Le Touquet, France in May, 1997. The next International lactoferrin Conference is going to be held in Sapporo, Japan in 1999.

Acknowledgments

I would like to thank Dr. Y.－C.Yoo and Prof. I. Axuma, Inst. Immunol. Sci, Hokkaid Univ., Sapporo, and Dr. Y. Omata and Prof. A. Saito, Depart. of Vet. Physiol. and Res. Center Protozoan Mol. Immunol., Obihiro Univ., Obihiro, for collaboration on lactoferrin research.

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