Biological Function of Lactoferrin in Milk

Shimazaki · Kei-ichi
Dariy Science Laboratory, Graduate School of Agriculture, Hokkaido University, Japan

Abstract

Lactoferrin is an iron-binding glycoprotein and its bacteriostatic and bactericidal effects on Gram-positive and Gram-negative bacteria have been well-known. However, certain kind of lactic acid bacteria are resistant against its antibacterial effects. Moreover, it is reported that lactoferrin promotes the growth of bifidobacteria by *in vitro* and *in vivo* experiments. In this experiment, lactoferrin-binding protein was found both in the membrane and cytosolic franctions of *Bifidobacterium*. *Bifidobacterium* was grown in anaerobic conditions in MRS broth containing cysteine, gathered by centrifugation and processed by sonication. The lactoferrin-binding proteins on the PVDF-membrane transferred after SDS-PAGE were detected by far-western method using biotinylated lactoferrin and streptavidin-labeled horse radish peroxidase. Observation in growth effects of lactoferrin on *Bifidobacterium* suggested that there is a relation between the presence of lactoferrin-binding proteins on the cells and their growth.

I. Introduction

Milk contains several specific antimicrobial factors, like immunoglobulins, complement and lymphocytes, as well as many non-specific factors, such as lactoferrin, lactoperoxidase, lysozyme, xanthine oxidase, superoxide dismutase, oligosaccharides, folate binding protein, lipid-antivirus factor, etc. (van Hooijdonk, et al., 2000). These secretory proteins play important roles for protection of mucosal surfaces from infection. Immunoglobulins (IgG, IgM and secretory IgA) act by a specific mode of action involving antigen-antibody reactions. The other proteins are non-specific protective factors, and their antimicrobial mechanisms of action differ from each other. Lysozyme is an enzyme which hydrolyzes peptidoglycan in the cell wall resulting in the bacteriolysis of Gram-positive bacteria. However, very little of this enzyme is present in cow's milk. Lactoperoxidase, a heme-containing protein, catalyzes an oxidation reaction involving hydrogen peroxide (H_2O_2) and functions as a component of the host defense system. Lactoperoxidase, thiocyanate ion (SCN^-) and H_2O_2 generate hypothiocyanite ion ($OSCN^-$), a potent bacteriostatic or bactericidal substance.

Lactoferrin was initially called the "red protein" or lactosiderophilin due to its high similarity to transferrin or siderophilin in their structure and iron-binding property. Lactoferrin has attracted the attention of many researchers because it shows so many biological functions related to the host defense system of humans and other animals, as reviewed in many articles (Sanchez, et al., 1992, Brock, 1995, Nuijens, et al., 1996, Shimazaki, 2000). These include antimicrobial and antiviral activities, immunomodulatory and anti-inflammatory properties, antitumor and antimetastatic activities, cell growth-promoting activities, regulation of granulopoiesis, regulation of iron absorption in the gut, and antioxidant activity including inhibition of superoxide and hydroxyl radical formation.

Bifidobacteria, anaerobic and Gram-positive bacteria, are known to contribute beneficially to human health as

natural predominant microflora in the intestinal tract (Mitsuoka, 1990). Biological functions of bifidobacteria are reported such as production of lactic acid and acetic acid, reduction of cholesterol in serum, amelioration of diarrhea or constipation, elimination of procarcinogens, vitamin B synthesis, improved adhesive ability and activation of immune systems, such as enhancement of NK cells, cytotoxic T lymphocytes activities, promotion of antibody production and activation of macrophages. On the other hand, many substances are known as the growth-stimulating factors of bifidobacteria (Mitsuoka, 1990, Crittenden, 1999) They are oligosaccharides such as lactulose or N-acetylglucosamine, food protein hydrolysates such as casein or ovalbumin and glycoproteins and glycopeptides isolated from κ-casein. Moreover, lactoferrin is reported to be one of the growth promoting factors for bifidobacteria (Hentges, et al., 1992, Petschow and Talbott, 1991, Petschow, et al., 1999, Roberts, et al., 1992, Sanders, 1998, Wharton, et al., 1994). In this report, growth promotion effects of lactoferrin on *Bifidobacterium* has been studied with the findings of lactoferrin-binding protein on the cell.

II. Materials and Methods

1. Bifidobacteria

Bifidobacterium bifidum Bb-11, *Bifidobacterium bifidum* ATCC 15696, *Bifidobacterium breve* ATCC 15700, *Bifidobacterium longum* ATCC 15707 and *Bifidobacterium infantis* ATCC 15697 were used. Bifidobacteria were grown in anaerobic conditions in MRS broth containing 0.05% Cysteine·HCl.

2. Estimation of growth promotion effects of lactoferrin on Bifidobacterium

After twice passages of preincubation of the test organisms, the activated culture was freshly inoculated into MRS broth and incubated anaerobically at 37℃. Lactoferrin solution was filter-sterilized and was added to the autoclaved medium. The growth of bifidobacteria was monitored by measuring the optical density at 660 nm in defined time intervals. Each sample was measured in triplicate. Transferrin and ovotransferrin were studied with their effects, too.

3. Binding assay by far western blot

Bifidobacterum cultured were corrected by centrifugation and they were treated to obtain membrane and cytosolic fractions by the method of sonication and freeze-thaw followed by the treatment with lysis buffer containing protease inhibitor, Triton X-100 and CHAPS. Protein components contained in cellular membrane fraction was separated by SDS-PAGE and transferred onto PVDF membrane. After blocking the membrane by BSA solution, the membrane was incubated with biotinylated lactoferrin and the membrane was finally reacted with streptavidin-labeled horseradish peroxidase. The peroxidase reaction on the membrane was detected with enhanced chemiluminescence method.

4. Competitive inhibition assay

Competitive inhibition assay was carried out by far western blot using biotinylated bovine transferrin and biotinylated ovotransferrin.

5. Proteins and chemicals

Bovine lactoferrin was kindly supplied by Morinaga Milk Co. (Zama, Japan). Human lactoferrin, bovine transferrin, ovotransferrin, N-hydroxysuccinimidebiotin and FITC-labeled avidin were products of Sigma Chemical Co. (St. Louis, Mo, USA). Streptavidin-labeled horseradish peroxidase was a product of Nichirei Co. (Tokyo, Japan). Biotinylation procedure of proteins were carried out with reaction of N-hydroxysuccinimide biotin dissolved in dimethylsulfoxide. Prestained Protein Marker was a product of BioLabs Inc. (MA, USA).

III. Results and Discussion

The effect of growth stimulation by transferrin family proteins supplementation was compared with four strains of bifidobacteria, *Bifdobacterium bifidum*, *Bifdobacterium longum*, *Bifdobacterium breve and Bifdobacterium infantis*. Growth promotion effects of lactoferrin on three of four strains of bifidobacteria were observed as shown in Table 1. However, growth of *Bifdobacterium longum* was not stimulated by the addition of lactoferrin (upto 1 mg/ml). Growth patterns of bifidobacteria showed concentration dependence of lactoferrin. It seemed that apo- and holo-types of lactoferrin did not affect bifidobacteria growth. Bovine transferrin showed similar tendencies of growth stimulating effects on bifidobacteria. Moreover, it was observed that hydrolysates of lactoferrin by trypsin or pepsin kept their growth promotion effects on bifidobactria (submitted).

Antibacterial effect has been one of the well-known function of lactoferrin and it is due to the ability to sequester environmental iron ions (Arnold, et al., 1980) or to release lipopolysaccharide (Ellison, 1994). However, it has been reported that certain kinds of lactic acid bacteria show resistance against lactoferrin (Arnold, et al., 1980) and bifidobacteria is one of such lactoferrin-resistant bacteria (Hentges, et al., 1992, Petschow and Talbott, 1991, Petschow, et al., 1999). It has been reported that the increase of the intestinal bifidobacteria counts in mice (Hentges, et al., 1992) and infants (Roberts, et al., 1992, Kawase and Teraguchi, 1996, Yamauchi, et al., 1999) fed infant formula containing bovine lactoferrin. Our results shown in Table 1 support these ideas.

In order to find mechanisms to explain such growth promotion effects of lactoferrin, lactoferrin-binding protein on membrane fraction of bifidobacteria has been performed. For this purpose, far-western analysis was used with biotinylated proteins and streptavidin-labeled horseradish peroxidase. Lactoferrin-binding bands were observed in the membrane fraction of bifidobacteria except *Bifidobacterium* longum. One example of the lactoferrin-binding band detected by far-western analysis is shown in Figure 1. Molecular weight of the lactoferrin-binding protein was estimated to be 69 kDa according to the electrophoretic mobilities. The patterns of lactoferrin-binding protein showed the same profiles when the membrane fraction was treated with and without 2-mercaptoethanol for SDS-PAGE.

Table 1. Effects of bovine lactoferrin on the growth of *Bifidobacterium* spp.

Bifidobacterium spp.	lactoferrin
Bifidobacterium bifidum ATCC 15696	Yes
Bifidobacterium infantis ATCC 15697	Yes
Bifidobacterium breve ATCC 15700	Yes
Bifidobacterium longum ATCC 15707	No

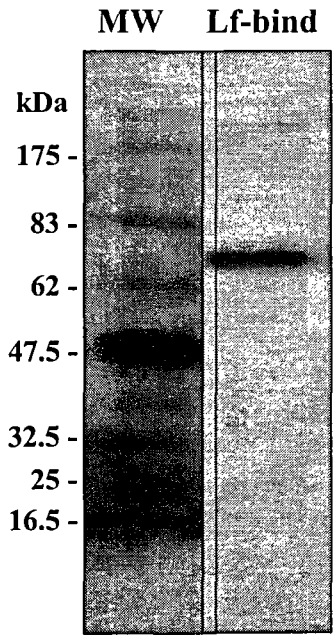

Figure 1. Detection of lactoferrin-binding protein of membrane fraction of *Bifidobacterium bifidum* ATCC 15696 by SDS-PAGE followed by far-western blot analysis. MW means molecular weight standard proteins.

Moreover, bovine transferrin and ovotransferrin were used to see the binding components of bifidobacteria and similar binding patterns were observed, i.e., the same electrophoretic mobility of their binding proteins. These results are summarized in Table 2. Each protein showed their specific interaction against binding proteins of bifidobacteria judged by the results of competitive inhibition assay. Iron content of lactoferrin did not influence on the binding properties.

Generally, lactoferrin-binding protein is expected to play biological roles for the growth promoting effects, because such binding proteins have been found on the cell surface of many kinds of microorganisms (Gray-Owen and Schryvers, 1996) and on mammalian cells of various tissues (Brock, 1995, Iyer and L¯nnerdal, 1993). In cases of bacterial lactoferrin-binding proteins, iron acquisition of bacteria mediated by the binding of lactoferrin is one of their possible roles. In this report, bovine lactoferrin-binding proteins is detected in the membrane associated proteins and in the cytosolic proteins of *Bifidobacterium* spp. However, biological function of the lactoferrin-binding proteins described above is not resolved yet. As it is known that lactoferrin can interact with many kinds of substances (Brock,

Table 2. Binding of bovine lactoferrin, bovine transferrin and ovotransferrin on membrane fraction of *Bifidobacterium* spp.

	lactoferrin	transferrin	ovotransferrin
Bifidobacterium bifidum Bb-11	Yes	Yes	Yes
Bifidobacterium bifidum ATCC 15696	Yes	Yes	Yes
Bifidobacterium infantis ATCC 15697	Yes	Yes	Yes
Bifidobacterium breve ATCC 15700	Yes	Yes	Yes
Bifidobacterium longum ATCC 15707	No or little	No or little	No or little

1995) including proteins and DNA (He and Furmanski, 1995), it maybe possible to propose the idea that the lactoferrin and binding protein complex can interact with the promotion region of the gene, or the regulatory sequence of DNA, or the gene regulatory protein.

The ability of lactoferrin to stimulate the growth of bifidobacteria may indicate that lactoferrin plays indirect roles in host defense system by promoting the development of a more favorable intestinal flora. Based on the above results, we are now resolving some problems. One of them is to determine the binding site or amino acid residues of lactoferrin molecule against *Bifidobacterium*. The other one is to resolve the question if the binding protein of membrane fraction of *Bifidobacterium* has biological roles as receptor or not. Such research may develop the studies of prebiotics effects of lactoferrin in vivo and in vitro and it can be expected the application of lactoferrin for functional foods.

Acknowledgments

The authors are grateful to Morinaga Milk Industry, Nutritional Laboratory and Dr.H.Kumura and Dr.T.Tanaka for their helpful advises and technical supports.

IV. References

1. Arnold, R. R., Brewer, M., and Gauthier, J. J. 1980. Bactericidal activity of human lactoferrin: sensitivity of a variety of microorganisms. Infect. Immun. **28**: 893-898.
2. Brock, J. 1995. Lactoferrin: a multifunctional immunoregulatory protein? Immunol. Today **16**: 417-419.
3. Crittenden, R. G. 1999. Prebiotics. in *Probiotics: A Critical Review*. Horizon Scientific Press, Wymondham, pp. 141-156.
4. Ellison, R. T. 1994. The effects of lactoferrin on gram-negative bacteria. in *Lactoferrin. Structure and Function.* (Hutchens, T. W., Rumball, S. V., and L¯nnerdal, B., Eds), Plenum Publishing Co., New York, pp. 71-90.
5. Gray-Owen, S. D., and Schryvers, A. B. 1996. Bacterial transferrin and lactoferrin receptors. Trends Microbiol. **4**: 185-191.
6. He, J., and Furmanski, P. 1995. Sequence specificity and transcriptional activation in the binding of lactoferrin to DNA. Nature **373**: 721-724.
7. Hentges, D. J., Marsh, W. W., Petschow, B. W., Thal, W. R., and Carter, M. K. 1992. Influence of infant diets on the ecology of the intestinal tract of human flora-associated mice. J. Pediat. Gastroent. Nutr. **14**: 146-152.
8. Iyer, S., and LSYMBOL 175 \f "Math A"nnerdal, B. 1993. Lactoferrin, lactoferrin receptors and iron metabolism. Eur. J. Clin. Nutr. **47**: 232-241.
9. Kawase, K., and Teraguchi, S. 1996. Physiological functions of lactoferrin. Rakunoukagaku Shokuhin no Kenkyu **45**: A75-81.
10. Mitsuoka, T. 1990. Bifidobacteria and their role in human health. J. Ind. Microbiol. **6**: 263-267.
11. Nuijens, J. H., Van Berkel, P. H. C., and Schanbacher, F. L. 1996. Structure and biological actions of lactoferrin. J. Mammary Gland Biol. Neoplasia **1**: 285-295.
12. Petschow, B. W., and Talbott, R. D. 1991. Response of *Bifidobacterium* species to growth promoters in human and cow milk. Pediat. Res. **29**: 208-213.
13. Petschow, B. W., Talbott, R. D., and Batema, R. P. 1999. Ability of lactoferrin to promote the growth of *Bifidobacterium*

spp. in vitro is independent of receptor binding capacity and iron saturation level. J. Med. Microbiol. **48**: 541-549.
14. Roberts, A. K., Chierici, R., Sawatzki, G., Hill, M. J., Volpato, S., and Vigi, V. 1992. Supplementation of an adapted formula with bovine lactoferrin: 1. Effect on the infant faecal flora. Acta Paediat. **81**: 119-124.
15. Sanchez, L., Calvo, M., and Brock, J. H. 1992. Biological role of lactoferrin. Arch. Dis. Child. **67**: 657-661.
16. Sanders, M. E. 1998. Overview of functional foods: Emphasis on probiotic bacteria. Int. Dairy J. **8**: 341-347.
17. Shimazaki, K. 2000. Lactoferrin: A marvelous protein in milk? Anim. Sci. J. (Chikusa Gakkai-ho) **71**: 329-347.
18. Van Hooijdonk, A. C., Kussendrager, K. D., and Steijns, J. M. 2000. In vivo antimicrobial and antiviral activity of components in bovine milk and colostrum involved in non-specific defence. Br. J. Nutr. **84 Suppl 1**: S127-134.
19. Wharton, B. A., Balmer, S. E., and Scott, P. H. 1994. Faecal flora in the newborn. Effect of lactoferrin and related nutrients. Adv. Exp. Med. Biol. **357**: 91-98.
20. Yamauchi, K., Teraguchi, S., and Hayasawa, H. 1999. Potential for usefulness of bovine lactoferrin in human beings (in Japanese). Milk Science **48**: 227-232.