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ORIGINAL RESEARCH PAPER

Bovine lactoferrin region responsible for binding to bifidobacterial cell surface proteins

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Abstract Bovine lactoferrin promotes bifidobacterial growth. Its binding to bifidobacteria is thought to be responsible for such action. After separating the bovine lactoferrin half molecule and extraction of surface proteins from bifidobacteria, binding profiles were observed by immunoblotting. No binding appeared when lactoferrin C-lobe was reacted with the cell surface proteins on a polyvinylidene difluoride membrane. Conversely, a 50-kDa band appeared when the surface proteins were reacted with either intact or nicked bovine lactoferrin. This result strongly suggests that the binding region could be lactoferrin N-lobe. Interestingly, despite the absence of binding, C-lobe enhanced bifidobacterial growth.

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Introduction

Lactoferrin is a multifunctional iron-binding transferrin glycoprotein secreted mainly in milk and other secretory fluids, as reviewed by Shimazaki (2000). This 80-kDa protein is folded to two homologous (~40% sequence identity) lobes, representing its N- and C-terminal halves. Each lobe has two domains (N1 and N2, C1 and C2) and can bind a single ferric ion concomitantly with one bicarbonate ion very tightly (Baker et al. 2002). There is a distinct difference between these two lobes with respect to their iron retention ability (the C-lobe binds iron more tightly than the N-lobe; Ward et al. 1996) and biological functions (some functions of lactoferrin are thought to involve N-lobe; van Berkel et al. 1997; Rochard et al. 1989). The N- and C-lobes also possess unique binding regions for microbial membranes (Bellamy et al. 1992). The N- and C-lobes participate in the binding of bovine lactoferrin to eukaryotic cell surface receptors (Sitaram and Mcabee 1997). Bovine lactoferrin C-lobe also promotes the contractile activity of collagen gels more prominently than native lactoferrin or its N-lobe (Takayama et al. 2002).

Bifidobacteria, one of the predominant bacterial groups existing in the human gut and play important

roles in maintaining human health throughout the life span of an individual (Mitsuoka 1990), require iron for growth but apparently produce no siderophores (Bezkorovainy and Kot 1998). Lactoferrin may though provide iron to bifidobacteria (Miller-Catchpole et al. 1997). In contrast, Petschow et al. (1999) suggested that growth promotion of *Bifidobacterium* spp. in vitro is independent of the iron saturation level of lactoferrin and that binding of lactoferrin to bifidobacterial cells may be involved instead. Lactoferrin-binding proteins in bifidobacteria have also been detected (Kim et al. 2004). It would be of great value if the lactoferrin region responsible for binding to bifidobacterial cells can be identified so that the effects of lactoferrin on bifidobacterial growth can be elucidated at the molecular level. In this regard, the main aim of this study was to identify the binding profiles of the lactoferrin half molecule with surface proteins extracted from bifidobacteria.

Materials and methods

Bacteria

Two strains of *Bifidobacterium infantis* JCM 7007 and *B. longum* JCM 7054, from Japan Collection of Microorganisms (JCM, Tokyo), were used.

Separation of bovine lactoferrin half molecule

The bovine lactoferrin half molecule was separated according the procedure of Shimazaki et al. (1993). Briefly, after partial proteolysis of iron-saturated lactoferrin by trypsin, the digestion mixture was applied to a CM-Toyopearl 650 M (Tosoh, Tokyo) column (9 cm diam × 15 cm) under refrigerated condition. The unabsorbed parts were washed out with 0.08 M sodium phosphate buffer (pH 6.8) and the absorbed parts were then eluted with the same buffer (pH 7.4) containing 0.5 M NaCl. Samples from different peaks were collected and dialyzed to remove salt. After freeze-drying, samples were stored at 4°C for further analysis. Collected samples were also analyzed by SDS-PAGE and the bovine lactoferrin C-lobe was identified by Western blotting using mouse anti-lactoferrin C-lobe antibody.

Extraction of bifidobacterial cell surface proteins

Bifidobacterial cell surface proteins were extracted as described by Almeida et al. (2006). After being grown for 16 h, bacteria were harvested and washed three times with sterile phosphate buffered saline (PBS, pH 7.4) by centrifugation at 4,000*g* for 10 min. Cell surface proteins were extracted by incubating with 0.2% SDS [30 mg moist wt of cell pellets per 100 µl of 0.2% SDS (w/v) for 1 h at 37°C with intermittent mixing]. Extraction mixtures were centrifuged at 12,000*g* for 10 min and the supernatants (cell surface proteins) were collected and stored at −20°C for further analysis.

Identification of lactoferrin region responsible for binding to bifidobacterial cell surface proteins

The region responsible for binding to bifidobacterial cell surface proteins was identified by immunoblotting. The extracted surface proteins from bifidobacteria were separated by SDS-PAGE (10%), and were either stained with Coomassie Brilliant Blue (CBB) R-250 or transferred onto a PVDF membrane. The blots were then blocked for 90 min with 3% (v/v) bovine serum albumin dissolved in PBS containing 0.5% (v/v) Tween 20 (PBST). After removing excess amount of the blocking reagent, the blots were either probed with intact lactoferrin, lactoferrin C-lobe or nicked lactoferrin (5 µg/ml) for 6 h at 4°C. After five 15-min washes with PBST, the blots were further probed overnight at 4°C with rabbit anti-bovine lactoferrin antibody at a 1:5000 dilution. Then, the blots were again subjected to five 15-min washes with PBST and incubated for 1 h at room temperature with horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG at a 1:5000 dilution. After the final five 15-min washes with PBST, HRP activity on the blots was visualized using 3,3′-diaminobenzidinetetrahydrochloride (DAB) as substrate.

Effects of C-lobe on growth of Bifidobacterium strains

Bifidobacterium strains were grown under anaerobic condition in MRS broth (Merck) containing 0.05% L-cysteine. HCl at 37°C with or without (control) the addition of lactoferrin C-lobe. Protein solution was prepared by dissolving separated C-lobe in sterilized

PBS (pH 7.2) followed by filter sterilization (pore size, 0.20 μm). Two-fold serially diluted protein solution was added to fresh medium to give 4, 2, 1, 0.5 or 0.25 mg/ml. The medium was then inoculated with a reactivated *Bifidobacterium* strain. For control cultures, PBS was added instead of the protein solution. After 16 h incubation under anaerobic conditions, growth was monitored as the OD_{660} with 10 times dilution of the cultured medium. The effect was expressed as relative growth promotion level (%) and calculated using the following formula-

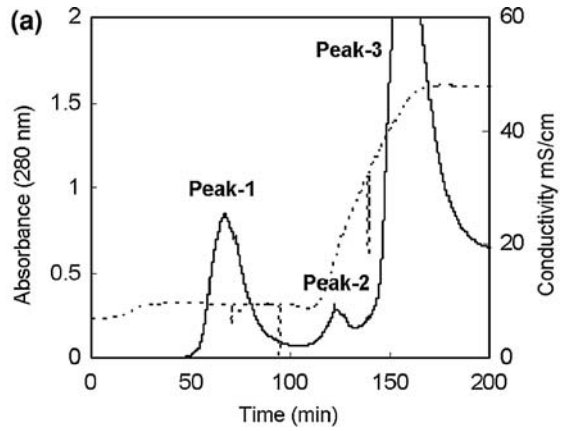
$$\text{Relative growth promotion level } (\%) = OD_{660}\ (\text{protein added})/OD_{660}\ (\text{control}) \times 100$$

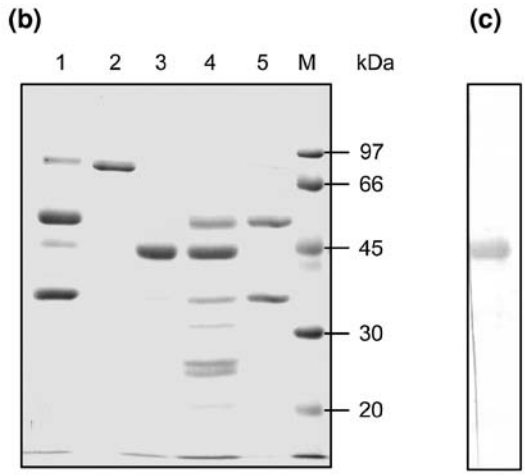
Results are expressed as the mean relative growth promotion level (%) of triplicate assays. Differences among the means were determined by Duncan's Multiple Range Test (DMRT) and $P < 0.05$ was considered statistically significant.

Results

The bovine lactoferrin half molecule was separated by generating lactoferrin fragments through trypsin digestion, which was then applied to a CM-Toyopearl 650 M column (lane 1, Fig. 1b). As shown in Fig. 1a, the unabsorbed parts (peak-1) had an estimated molecular mass of around 43 kDa (lane 3, Fig. 1b) and were recognized as lactoferrin C-lobe by Western blotting (Fig. 1c). The remaining fragments were eluted with peak-2 and peak-3, as shown in Fig. 1a. SDS-PAGE analysis showed multiple bands for peak-2 (lane 4, Fig. 1b), whereas two distinct bands with estimated molecular masses of around 52 and 36 kDa, respectively, were observed for peak-3 (lane 5, Fig. 1b). Fractions eluting as peak-3 were termed 'nicked lactoferrin'. Theoretically, the 36-kDa band represents 80% of the N-lobe and the 52-kDa band represents the fragment containing the entire C-lobe and part of N-lobe. A summary of the separation procedure for the lactoferrin half molecule is shown in Fig. 1d.

The lactoferrin region responsible for binding to bifidobacterial cell surface proteins was evaluated by immunoblotting (Fig. 2) Bifidobacterial cell surface proteins were extracted and analyzed by SDS-PAGE (Fig. 2a). After transferring the proteins onto a PVDF





(d)

Peak number in chromatography analysis	Lane number in SDS-PAGE analysis	Number of bands appeared	Estimated molecular mass (kDa)	Status of separated molecule
Peak-1	3	1	43	bLf C-lobe
Peak-2	4	multiple	not estimated	No status
Peak-3	5	2	52 36	Nicked bLf

Fig. 1 Separation profiles of bovine lactoferrin half molecule. Tryptic digestion mixture of lactoferrin was applied to a CM-Toyopearl 650 M column (**a**). The unabsorbed parts (peak-1) were washed out with 0.08 M sodium phosphate buffer (pH 6.8) and the absorbed parts were eluted (peak-2 and peak-3) with the same buffer (pH 7.4) containing 0.5 M NaCl. Tryptic digestion mixture of lactoferrin (*lane-1*), intact lactoferrin (*lane-2*), and elutes from peak-1 (*lane-3*), peak-2 (*lane-4*) and peak-3 (*lane-5*) were analyzed by SDS-PAGE (**b**). The unabsorbed parts (peak-1, *lane-3*) were recognized as lactoferrin C-lobe by Western blotting (**c**). Summary of the separation profile is shown in a tabular form (**d**)

membrane and probing with lactoferrin C-lobe, there were no resulting bands (Fig. 2c). On the other hand, a band at approx. 50 kDa appeared when the proteins were probed with either intact lactoferrin or nicked lactoferrin (Fig. 2b and d). Although, we previously reported the detection of lactoferrin-binding proteins in the membrane-associated fraction of bifidobacteria (Kim et al. 2004), we have recently purified lactoferrin-binding proteins in the surface proteins of these bacteria (unpublished data). The estimated molecular weight (50 kDa) of these proteins was different from that of our previous results (Kim et al. 2004). This may be due to the difference in the extraction method used for bacteria.

Furthermore, the effect of lactoferrin C-lobe on bifidobacterial growth in MRS medium was investigated, and the results are shown in Fig. 3. The concentration of lactoferrin C-lobe was adjusted to 0.25, 0.5, 1, 2 or 4 mg/ml and thier effects on growth were measured after 16 h. A statistically significant ($P < 0.05$) dose-dependent growth-stimulating effect

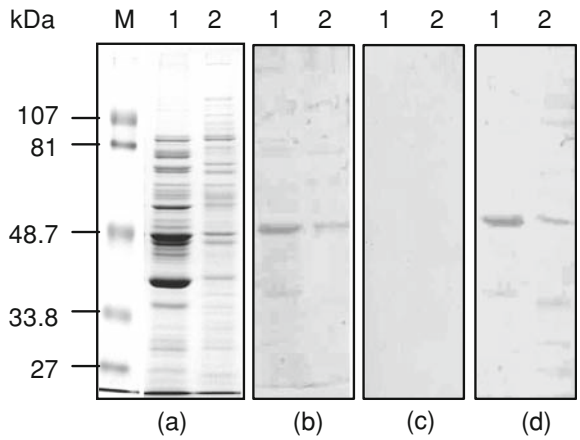


Fig. 2 Identification of bovine lactoferrin region responsible for binding to bifidobacterial cell surface proteins. Cell surface proteins extracted from *B. infantis* JCM 7007 (*lane 1*) and *B. longum* JCM 7054 (*lane 2*) and were analyzed by SDS-PAGE (**a**). Bovine lactoferrin-binding proteins were identified by Western blotting probed either with intact lactoferrin (**b**), lactoferrin C-lobe (**c**) or nicked lactoferrin (**d**), followed by further probing with rabbit anti-bovine lactoferrin antibody. The Western blot has the same arrangement of lanes as that in the SDS-PAGE analysis. The absence of any bands during the probing with lactoferrin C-lobe (**c**) and the presence of an approximately 50-kDa band during probing with nicked lactoferrin (**d**) indicate that the region responsible for binding to cell surface proteins could be N-lobe. M indicates prestained protein markers used for estimating the molecular weights

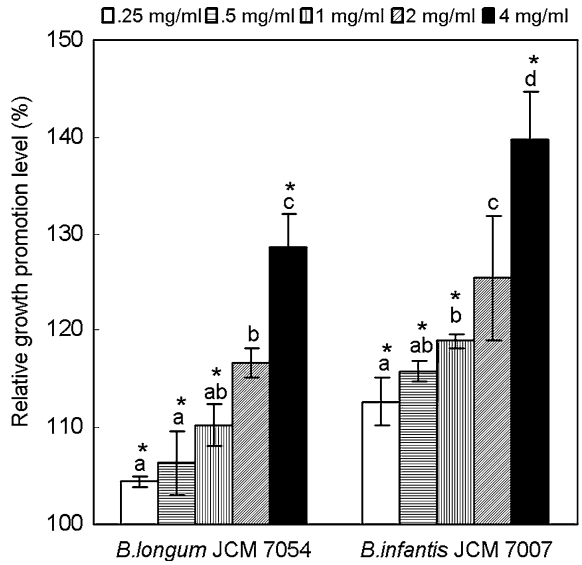


Fig. 3 In vitro effects of bovine lactoferrin C-lobe on growth of *Bifidobacterium* strains. Bacteria were grown in MRS medium with or without the addition of lactoferrin C-lobe at various concentrations. Relative growth promotion level was expressed as the ratio of the absorbance at 660 nm in the presence of lactoferrin C-lobe to the control absorbance after 16 h of cultivation at 37°C under anaerobic condition. The average absorbances of the control were 0.36 for *B. longum* and 0.5 for *B. infantis*. The experiment was repeated three times in triplicates. Results are expressed as the average of triplicate assays. The same letters on the bars indicate no significant differences, whereas different letters indicate significant differences ($P < 0.05$) between effects of different concentrations on the growth of respective strains. Asterisks indicate significant differences ($P < 0.05$) between growth responses of bacterial strains at specific concentrations

by lactoferrin C-lobe was observed for both strains tested. However, no significant differences were found between 0.25 and 0.5 mg/ml and 1 and 2 mg/ml in *B. longum* or between 0.5 and 1 mg/ml in *B. infantis*. Growth responses of two strains also differ significantly ($P < 0.05$) at any concentration (except 2 mg/ml). No inhibition was observed even at 4 mg/ml. A comparison of growth responses between two strains showed a significant difference ($P < 0.05$) at each concentration, as indicated by asterisks in Fig. 3.

Discussion

The present findings indicate that the N-lobe and not the C-lobe of lactoferrin may be the region responsible for binding to bifidobacterial cell surface proteins. In

Moraxella and *Neisseria* spp., lactoferrin-binding proteins are reported to play an important role in iron acquisition from lactoferrin (Bonnah and Schryvers 1998) and they bind to both domains of human lactoferrin C-lobe (Wong and Schryvers 2003). These different results may be due to differences in characteristics between *Moraxella* and *Neisseria* spp. and of *Bifidobacterium* spp. Bifidobacteria need iron for growth; however, the mechanism underlying the iron uptake of bifidobacteria is not clear at present. The utilization of iron from lactoferrin by bifidobacteria through binding may be one possible mechanism behind the lactoferrin growth stimulatory effects. Since the N-lobe region of lactoferrin was reported to bind iron more weakly than the C-lobe region (Ward et al. 1996), it can be deduced that bifidobacteria bind to this region for iron acquisition. However, this may not be feasible because bifidobacterial growth was also enhanced by the lactoferrin C-lobe in a dose-dependent manner (Fig. 3) even when the C-lobe did not bind with bifidobacterial cell surface proteins (Fig. 2c).

This result indicates that the lactoferrin-binding protein in bifidobacteria may not be involved with the mechanism by which lactoferrin promotes the growth of bifidobacteria rather play different role. However, growth promotion by the lactoferrin C-lobe suggests the following possible explanations. One is that the lactoferrin C-lobe becomes iron-saturated and this may promote bifidobacterial growth through the utilization of accumulated iron. This idea suggests that the iron uptake mechanism of bifidobacteria is not related to the binding of lactoferrin to iron-binding proteins. This idea is supported by Miller-Catchpole et al. (1997) who studied the effects of the C-terminal fragment of human lactoferrin on the growth of *B. breve*. Another possible explanation is that bifidobacteria may hydrolyze proteins by secreting enzymes and the resulting peptides may promote growth. Liepke et al. (2002) identified bifidogenic peptides from human milk in which lactoferrin-derived peptides were reported.

The binding ability of nicked lactoferrin (Fig. 2d) strongly suggests that lactoferrin N-lobe is the binding site of bifidobacterial cell surface proteins. The binding with N-lobe indicates another possible role of lactoferrin-binding protein in bifidobacteria, and can be explained as follows. Lactoferrin has bactericidal, fungicidal and antiviral activities as well as antitumor, anti-inflammatory and immunoregulatory properties (see Gonzáles-Chávez et al. 2008). Most of these activities reside in the N-domain of lactoferrin. This domain is also termed as the antimicrobial domain due to the release of lactoferricin, a more potent antimicrobial peptide, by pepsin digestion (Bellamy et al. 1992) and lactoferrampin, a second stretch of N1 domains reported as another novel antimicrobial peptide (van der Kraan et al. 2004). Therefore, bifidobacteria may achieve protection against the antimicrobial activity of lactoferrin by binding with this domain. This is supported by the behavior of lactoferrin toward bifidobacterial growth in that it does not inhibit growth but promotes it instead.

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