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The bovine lactoferrin region responsible for binding to bifidobacteria cell surface protein

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Abstract

Bovine lactoferrin (bLf) is a multifunctional iron-binding glycoprotein secreted mainly in milk and other secretory fluids. Bovine lactoferrin is reported to promote the growth of bifidobacteria and binding of bLf to bifidobacteria cell is thought to be involved. After separation of bLf half molecule and extraction of surface proteins from bifidobacteria, binding profiles were observed by immunoblotting. No binding was appeared when bLf C-lobe was being reacted with cell surface proteins on PVDF membrane. Conversely, a 50-kDa band was appeared when reacted with either intact bLf or nicked bLf. This result strongly suggests that binding region could be N-lobe. Moreover, blot, probed with nicked bLf, reacted with anti-lactoferricin antibody also produced a 50-kDa band that indicates the binding occurred at lactoferricin region of bLf molecule. Interestingly, despite absence of binding, bLf C-lobe can stimulate the growth of bifidobacteria.

Introduction

Lactoferrin (Lf), a multifunctional iron-binding transferrin family glycoprotein is secreted mainly in milk and other secretory fluids, e.g. tears, saliva etc. and is also found in the granules of the neutrophils as reviewed by Shimazaki [1]. This 80 kDa protein is composed of a single polypeptide chain of about 690 amino acid residues and is folded into two homologous (~ 40% sequence identity) lobes, representing its N- and C-terminal halves and connected by a short “hinge” peptide of 10-15 residues. 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 [2]. There are striking conservation between these two lobes in respect of their iron retention ability (C-lobe bind iron more tightly) [3] and biological functions (some functions of Lf are thought to be involved in the N-lobe) [4, 5]. N- and C-lobes also possess unique binding regions for microbial membranes [6]. The participation of N-and C-lobes in binding to cell surface receptors has also been reported [5, 7, 8]. Bovine lactoferrin (bLf) C-lobe is also reported to promote the contractile activity of collagen gels more prominently than native bovine lactoferrin or it's N-lobe [9].

Bifidobacteria, one of the predominant bacterial groups that exist in the human gut and play important roles in maintaining human health throughout the life span of the

individual [10], are appeared to require iron for their growth and apparently shown to produce no siderophores [11]. Studies also have shown that Lf may indeed provide iron to bifidobacteria [12]. In contrast, Petschow *et al.* [13] suggested that growth promotion of *Bifidobacterium* spp. *in vitro* is independent of the iron saturation level of Lf and binding of Lf to bifidobacterial cells may be involved. Lactoferrin binding proteins have been identified in many Gram-positive and Gram-negative bacteria but most of their functional roles have not been extensively and definitively determined [14]. We also previously reported lactoferrin binding protein in bifidobacteria [15, 16]. It would be valuable to identify the bLf region responsible for binding to bifidobacteria in order to elucidate the molecular analysis of bLf effects on bifidobacteria growth. Consequently, the main aim of this study was to identify the binding profiles of bLf half molecule with surface proteins extracted from bifidobacteria.

Materials and Methods

Bacteria

Two strains of bifidobacteria (*B. infantis* JCM 7007 and *B. longum* JCM 7054) were used in this study. Strains were purchased from Japan Collection of Microorganisms (JCM). All strains were maintained as frozen stocks at – 80°C in sterile MRS broth (Merck, Darmstadt, Germany) containing 20% glycerol and 0.05% L-cysteine.HCL.

For further use, each bacterium was reactivated by two consecutive subcultures in MRS broth containing 0.05% L-cysteine.HCL under anaerobic condition at 37°C.

Separation of bLf half molecule

Bovine lactoferrin half molecule was separated according to Shimazaki et al. [17]. Lactoferrin was kindly supplied by Morinaga Milk Company Ltd. (Zama, Japan) as lyophilized form and then iron-saturated as described by Shimazaki and Hosokawa [18]. Partial proteolysis of bLf by trypsin occurred in 0.05 M Tris-HCl buffer, pH 7.8, containing 0.02 M CaCl₂ at 37°C for 2 h. The tryptic digestion mixture of bLf was applied on a Carboxymethyl Toyopearl 650 (Tosoh, Tokyo) column in the refrigerator. The unabsorbed parts were washed out with 0.08 M sodium phosphate buffer (pH 6.8) and the absorbed parts were then eluted with 0.08 M sodium phosphate 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 sodiumdodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and the bLf C-lobe was recognized by Western blotting using mouse anti-lactoferrin C-lobe antibody.

Extraction of bifidobacteria cell surface proteins

Bifidobacteria cell surface proteins were extracted as described by Fang and Oliver

(19) and Almeida et al. (20). Bifidobacteria strains were grown in MRS containing 0.05 % L-cysteine.HCl under anaerobic condition for 16 h at 37°C. Bacterial cells were harvested and washed three times with sterile phosphate buffered saline (PBS, pH 7.4) by centrifugation at $4,000 \times g$ for 10 min. Cell surface proteins were extracted by incubating with 0.2 % SDS (30 mg moist weight of cell pellets per 100 μ L of 0.2 % SDS (w/v) for 1 h at 37°C with intermittent mixing). Extraction mixtures were centrifuged at $12,000 \times g$ for 10 min and the supernatants (cell surface proteins) were collected and stored at -20°C for further analysis.

Identification of binding region of bLf to bifidobacteria cell surface protein

Binding region of bLf to bifidobacteria surface proteins was identified by immunoblotting. The extracted surface proteins from bifidobacteria were separated by SDS-PAGE (10%) according to Laemmli [21] and were either stained with coomassie brilliant blue (CBB) R-250 or transferred onto polyvinylidene-difluoride (PVDF) membrane. The blots were then blocked for 90 min with 3% bovine serum albumin (BSA) dissolved in PBS containing 0.5% (v/v) Tween 20 (PBST). After removal of excess amount of blocking reagent, the blots were either probed with intact bLf, bLf C-lobe or nicked bLf (5 μ g/mL) for 6 h at 4°C. After five 15-min washes with PBST, blots were further probed for overnight at 4°C with either rabbit anti-bLf antibody

(Fujirebio, Inc., Tokyo) or mouse anti-lactoferricin antibody [22] (in the case of nicked bLf only) at a dilution of 1:5000 . After five 15-min washes with PBST, blots were then incubated for 1 h at room temperature with horseradish peroxidase (HRP) conjugated either goat anti-rabbit or anti-mouse IgG (Wako chemicals, Tokyo) diluted 1/5000. After a final five 15-min washes with PBST, the activity of HRP on blots was visualized using 3,3'-diaminobenzidinetetrahydrochloride (DAB) as substrate.

Effects of bLf C-lobe on the growth of Bifidobacterium strains

Bifidobacterium strains were grown under anaerobic condition in MRS broth (Merck, Darmstadt, Germany) containing 0.05% L-cysteine.HCl at 37°C with the addition of bLf C-lobe or without adding protein (control). Protein solution was prepared by dissolving in sterilized PBS (pH 7.2) followed by filter sterilization (pore size 0.20 µm). The protein concentration was estimated by spectrophotometric analysis ($A_{280}:A_{465}$) using extinction coefficient $A_{280}^{1\%,1\text{cm}} = 15.1$ for holo-type and 12.7 for apo-type bLf as reviewed by Shimazaki (1). Two-fold serially diluted protein solution was added into fresh medium to achieve a final concentration of 4, 2, 1, 0.5 or 0.25 mg/ml. The medium was then inoculated with reactivated *Bifidobacterium* strain. For control cultures, PBS was added instead of protein solution. After 16 h incubation under anaerobic conditions, bacterial growth was monitored spectrophotometrically at

660 nm with 10 times dilution of the cultured medium. The effect was expressed as relative growth promotion level (%) and calculated using the formula as described by Saito et al. [23]:

Results are given as 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 and Discussion

Bovine lactoferrin half molecule was separated by generating bLf fragments (lane 1, Fig. 1b) with trypsin, which was then applied on Carboxymethyl Toyopearl 650 (Tosoh, Tokyo) column. 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 bLf C-lobe by western blot (Fig. 1c). Remaining fragments were eluted with peak-2 and -3 as shown in Fig. 1a. SDS-PAGE analysis showed multiple bands for peak-2 (lane 4, Fig. 1b) whereas two distinct band with an estimated molecular mass of around 52 and 36 kDa, respectively were observed for peak-3. The fractions eluted as peak-3 was termed as 'nicked bLf' in this study. Theoretically this 36 kDa band represents 80% of N-lobe and 52 kDa represents fragment containing entire C-lobe and a part of N-lobe. A summary of bLf half molecule separation is shown in Fig. 1d.

Although, we reported previously bLf binding protein in the membrane associated fraction of bifidobacteria [15, 16]; recently, we detected and purified bLf-binding protein (bLf-BP) in the surface proteins of bifidobacteria (data yet to be published) and the estimated molecular weight is different from that of our previous result [15, 16]. This may be caused by the difference of extraction method from bacteria. The region of bLf responsible for binding to bifidobacteria cell surface proteins was evaluated by immunoblotting as shown in Fig. 2. Bifidobacteria cell surface proteins were extracted and analyzed by SDS-PAGE (Fig 2 A). After transfer proteins onto PVDF membrane and probed with bovine lactoferrin C-lobe resulting no bands (Fig. 2 C) whereas around a 50 kDa band was appeared when probed either with intact bovine lactoferrin or nicked bovine lactoferrin (Fig. 2 B and D). Analogous band of 50-kDa was also appeared when blot, probed with nicked bLf, was reacted with mouse anti-lactoferricin (monoclonal) antibody.

The effect of bLf C-lobe on the growth of bifidobacteria in MRS medium was investigated, and the results are shown in Fig. 3. The concentration of bLf C-lobe was adjusted to 0.25, 0.5, 1.0, 2.0 or 4.0 mg/mL and the relative growth promotion level was expressed as the ratio of the absorbance in the presence of bLf C-lobe to the control absorbance value after 16 h of cultivation. A statistically significant ($P < 0.05$)

dose-dependent growth stimulating effect by bLf C-lobe was observed for both the strain tested. However, no statistical differences was found between 0.25 and 0.5 mg/ml and 1.0 and 2.0 mg/ml concentration in *B. longum* and between 0.5 and 1.0 mg/ml concentration in *B. infantis*. It should be noted that no inhibitory effect was observed even at high concentration (4 mg/ml). Comparison of growth responses between two strains showed a significant difference ($P < 0.05$) at each concentration as indicated by asterisks mark in Fig. 3.

The present findings indicate that not bLf C-lobe but may be N-lobe bind with bifidobacteria cell surface proteins. In *Moraxella* and *Neisseria* spp., lactoferrin-binding proteins are reported to play role in iron acquisition from lactoferrin [24] and binds to both domains of the human lactoferrin C-lobe [25]. This different result may be due to the differences in characteristics between *Moraxella* and *Neisseria* spp. and of *Bifidobacterium* spp. Studies have shown that bifidobacteria needs iron for their growth, but iron uptake mechanism of bifidobacteria is not clear yet. Through binding, utilization of iron from Lf by bifidobacteria may be one possible mechanism behind the Lf growth stimulation effects on bifidobacteria. Since Lf N-lobe site is reported to bind iron more weakly than C-lobe [3], it can be explained that bifidobacteria binds with this site of Lf as a means of iron acquisition. However, this

explanation seems not to be reasonable because growth of bifidobacteria was also stimulated by bLf C-lobe in a dose-dependent fashion (Fig. 3), even bLf C-lobe did not bind with bifidobacteria (Fig. 2 C). The present findings suggest several possible explanations. The C-lobe used in the study was being iron-saturated; as a result one possible explanation is that growth of bifidobacteria was promoted by utilizing this iron, and the iron uptake mechanism of bifidobacteria is not related with the binding with iron-binding protein. This idea is supported by Miller-Catchpole *et al.* [12] who studied effect of C-terminal fragment of human lactoferrin on the growth of *Bifidobacterium breve*. Another explanation is bifidobacteria may hydrolyze protein by secreting enzymes and the resulting peptides may promote the growth. Liepke *et al.* [26] identified bifidogenic peptides from human milk in which lactoferrin-derived peptides were being reported.

The binding ability of nicked bLf strongly suggests the binding site as bLf N-lobe (Fig. 3 D). In addition, since bLf-BP was appeared when we used anti-lactoferricin antibody (Fig. 3 E), it can be said that binding occurred at lactoferricin region. Binding with N-lobe of lactoferrin especially at lactoferricin region generates another possible explanation. Lactoferrin is known to have bactericidal, fungicidal, and antiviral activity as well as antitumor, anti-inflammatory and immunoregulatory

properties as described in several review articles [27, 28, 29, 30]. Most of these activities reside in the N-domain of lactoferrin [31]. This domain is also termed as antimicrobial domain due to release of lactoferricin, a more potent antimicrobial peptide, by pepsin digestion [6, 32] and lactoferrampin, a second stretch of N1 domains reported as another novel antimicrobial peptide [33]. Therefore, bifidobacteria may make protection against antimicrobial activity of lactoferrin by binding with this domain. This explanation is supported by the lactoferrin behavior towards bifidobacteria growth that shows lactoferrin does not inhibit the growth of bifidobacteria rather stimulates.

Conclusion

Lactoferrin is composed of two homologous lobes of which N-lobe is termed as antimicrobial region and C-lobe is reported to bind iron more tightly. The bovine lactoferrin region responsible for binding with bifidobacteria surface protein was suggested to be N-lobe. Although no binding was appeared with bovine lactoferrin C-lobe, bifidobacteria showed growth responses against C-lobe in a dose-dependent fashion. This result indicates that bifidobacteria either utilize iron from C-lobe by other mechanism rather than binding with protein or may hydrolyze protein by secreting enzymes and the resulting peptides may promote the growth. Binding with nicked bovine lactoferrin suggests that, since bovine lactoferrin N-lobe has been recognized as

antimicrobial domain, bifidobacteria may make protection against antimicrobial activity of lactoferrin by binding with this domain.

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Figure legends

Fig. 1 Separation profiles of bovine lactoferrin (bLf) half molecule. Tryptic digestion mixture of bLf was applied on a Carboxymethyl Toyopearl 650 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 -3) with 0.08 M sodium phosphate buffer (pH 7.4) containing 0.5 M NaCl. Tryptic digestion mixture of bLf (lane-1), intact blf (lane-2), 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) were recognized as bLf C-lobe by western blot (c). Summary of separation profile is shown in a tabular form (d).

Fig. 2 Identification of bovine lactoferrin (bLf) region responsible for binding with surface proteins of bifidobacteria. Cell surface proteins, extracted from *B. longum* JCM 7054 (lane 1) and *B. infantis* JCM 7007 (lane 2) were analysed by SDS-PAGE (A). Bovine lactoferrin-binding protein (bLf-BP) was recognized by western blot probing either with intact bLf (B), bLf C-lobe (C) or nicked bLf (D) followed by further probing with rabbit anti-bLf antibody. Probing with mouse anti-lactoferricin antibody in nicked bLf (E) was also carried out. The western blot has the same arrangement of

lanes as in the SDS-PAGE. Absence of any band while probing with bLf C-lobe (C) and presence of around a 50 kDa band while probing with nicked bLf (D) indicate the responsible binding region could be N-lobe. Probing with monoclonal antibody also indicates that the lactoferricin region could be the binding sites. M indicates prestained protein markers that were used to estimate the molecular weights.

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

(a)

(b)

(c)

(d)

Peak number. in chromatography analysis	Lane number in SDS-PAGE analysis	Number of bands	Estimated molecular mass (kDa)	Status of separated molecule
Peak-1	3	1	45	bLf C-lobe
Peak-2	4	multiple	Not estimated	No status
Peak-3	5	2	52	Nicked bLf
			36