New Binding Assay and Preparative Trial of Cell-Surface Lectin from *Lactobacillus acidophilus* Group Lactic Acid Bacteria

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**ABSTRACT**

To select *Lactobacillus acidophilus* group bacteria as a probiotic yogurt starter, we designed a new screening method that measures the binding activity of surface layer protein to rat colonic mucin, which contains sugar chains similar to those in human colonic mucin. The B1 subgroup (*Lactobacillus gasseri*), which is the dominant strain in the human intestinal tract, showed the highest binding activity to rat colonic mucin among all the subgroups of *L. acidophilus*. The binding activity of the surface layer protein was also shown to be significantly reduced after periodate oxidation of the rat colonic mucin. This new screening method is useful for rapid selection of *L. acidophilus* strains that have high adhesion to the human intestinal tract. Lectin-like proteins that were bound to rat colonic mucin were isolated from the surface layer proteins with a rat colonic mucin-coated membrane and were analyzed by SDS-PAGE. A few main bands together with several minor bands were observed on the electrophoretograms obtained from the strains tested. It is possible that those lectin-like proteins contribute to adhesion of the bacterial cell to human colonic mucosa by binding specifically to carbohydrate portions.

(Key words: *Lactobacillus acidophilus*, lectin, rat colonic mucosa, surface layer protein)

Abbreviation key: Fuc = L-fucose, Gal = D-galactose, GalNAc = N-acetyl-D-galactosamine, GlcNAc = N-acetyl-D-glcosamine, NeuAc = N-acetylneuraminic acid, RCM = rat colonic mucosa, SLP = surface layer proteins.

**INTRODUCTION**

*Lactobacillus acidophilus* group lactic acid bacteria are used in the manufacture of several kinds of fermented milk, acidophilus milk, and beverages. They are considered to play an important role in maintaining the health of their host (11) through providing a barrier against pathogenic bacteria or improving the intestinal environment. Adhesion of the bacteria is an important first step in the colonization of and probiotic contribution to the human gastrointestinal tract. The adhesion mechanism of *L. acidophilus* to the human gastrointestinal tract has been partially studied (1, 3, 10, 15). *Lactobacillus gasseri* (B1 subgroup) is the dominant strain among the *L. acidophilus* group bacteria found in the human gastrointestinal tract (2). Mukai et al. (8) and Yamada et al. (14) have confirmed the presence of lectin-like proteins in the surface layer protein (SLP) of *L. acidophilus* through hemagglutination assay. Such proteinaceous components in the SLP may contribute to cell adhesion through their binding to carbohydrate portions of the colonic mucous layer that is common to all animal species.

Slomiany et al. (12) have reported eight kinds of acidic and neutral O-glycosidic (mucin-type) sugar chains combining to rat colonic mucin (RCM), which were very similar in structure to those isolated from human colonic mucin (9). The most representative sugar chain from human colonic mucin is shown below.

\[
\text{GalNAc}_\alpha 1-3-\text{Gal}_\beta 1-3-[\text{Fuc}_\alpha 1-2]-\text{GlcNAc}_\beta 1-3-\text{Gal}_\beta 1-4-\text{GlcNAc}_\beta 1-3-[\text{NeuAc}_\alpha 2-6]-\text{GalNAc}\ldots\text{Threonine or Serine}
\]

where \text{Fuc} = L-fucose, \text{Gal} = D-galactose, \text{GalNAc} = N-acetyl-D-galactosamine, \text{GlcNAc} = N-acetyl-D-glcosamine, and \text{NeuAc} = N-acetylneuraminic acid.

Recently, Takahashi et al. (13) reported that conventional hemagglutination assay is not a good method for selecting *L. acidophilus* strains that have high adhesion to the human intestinal tract, and they introduced a new screening method using polystyrene beads coated with RCM. *Lactobacillus acidophilus* strain SBT2062 was shown by this method to actively bind to RCM, and its binding to human colonic mucosa was confirmed through histochemical staining using Carnoy’s-fixed human colon tissues. However, this method could not be used to screen B-group strains of *L. acidophilus* be-
Fig. 1. Comparison of the binding rate of surface layer proteins from 35 strains of *Lactobacillus acidophilus* group strains to rat colonic mucosa (RCM). Black bars (■) represent the type strain in each subgroup. The experiment was performed in triplicate (n = 3). Letters in parenthesis represents the origin of each strain. (a): human feces; (b): human intestine; (c): unknown; (d): commercial yogurt; (e): sour milk; (f): human eye; (g): chicken crop; (h): human blood; (i): chicken feces; (j): cattle waste-corn fermentation; (k): pig feces. Superior T in the strain name represents type strain.

cause of the high nonspecific binding of SLP to polystyrene beads. We describe herein a modified screening method using an RCM-coated microtiter plate for evaluating adhesiveness of *L. acidophilus* group bacteria to human intestinal tracts. This method was also used for the isolation of lectin-like proteins from the SLP of *L. acidophilus* bacteria that are related to the adhesion of the bacteria to human colonic mucosa.

**MATERIALS AND METHODS**

**Bacteria**

Thirty-five strains of *L. acidophilus* group lactic acid bacteria, which were classified according to the recent system (5, 7), were used in this study. Their names and origins are shown in Figure 1. Ten strains were purchased from the Japan Collection of Microorganisms (JCM; Wako Pure Chemical Industries, Ltd., Osaka, Japan). Four F strains were provided by T. Fujisawa (4), and the other strains were isolated mainly from human feces at the Technical Research Institute of Snow Brand Milk Products Co., Ltd. (SBT; Kawagoe, Japan) or in our laboratory (LA).

**Materials**

Ten-week-old, male Wister rats were kindly provided by the Laboratory of Animal Reproduction (Graduate School of Agricultural Science, Tohoku University, Sendai, Japan). Unless otherwise specified, all reagents were purchased from Wako Pure Chemical Industries, Ltd.

**Preparation of Biotinylated Surface Layer Protein from *L. acidophilus* Group Lactic Acid Bacteria**

The SLP was prepared according to previous methods (13, 14) with slight modifications. The SLP (1 mg), dissolved in 0.1 M Na_2HPO_4 solution containing 0.15 M NaCl (pH 9.0), was mixed with a Biotinyl reagent [2 mg of D-Biotinyl-ε-aminocaproic acid N-hydroxysuccinimide ester (Boehringer Mannheim GmbH, Boehringer, Germany) in 40 µl of dimethylsulfoxide] and was stirred at 4°C for 16 h. The biotinylated SLP solution was dialyzed against 0.1 M Na_2HPO_4·KH_2PO_4 containing 0.15 M NaCl (PBS, pH 7.4) at 4°C for 24 h. The protein content was adjusted to 20 µg/ml with a Micro BCA protein assay reagent kit (Pierce, Rockford, IL). Bovine
Preparation of RCM

The RCM was prepared from the colon of Wister rats by the method described in previous papers (13, 14) with modifications. The mucosal layer was scraped from the large intestine with a slide glass, and it was then defatted with Folch’s solvent (CHCl₃:CH₃OH; 2:1, vol/vol). The residue obtained by centrifugation (12,000 rpm, 20 min, 4°C) was rinsed two times with solvent and then rinsed three times with diethyl ether. The defatted sample was incubated for 2 h in 6 M guanidine hydrochloride solution at 37°C with gentle shaking. After centrifugation at 12,000 rpm for 20 min, the supernatant was exhaustively dialyzed against distilled water at 4°C and then lyophilized. The sample was dissolved in 4.0 M guanidine hydrochloride solution (20 ml) and subjected to gel permeation chromatography on a Toyopearl HW-50 column (2.6 x 72 cm; TOYOPEARL, Tokyo, Japan) at an elution rate of 1 ml/min. The material eluting at the void volume (about 150 ml) was pooled, dialyzed against distilled water, and then lyophilized (RCM).

Microtiter Plate Assay using RCM

The wells of a microtiter plate (96-well, flat-bottomed type; Sumitomo Bakelite Co. Ltd., Tokyo, Japan) were incubated with 75 µl of RCM solution (50 µg/ml of 6 M guanidine hydrochloride solution) for 16 h at 4°C. The solution was removed by aspiration, and the wells were refilled to 100 µl of blocking buffer (PBS containing 0.05 % Tween 20 and 1 % BSA, pH 6.8). After incubation for 2 h, the microtiter plate was washed four times with 300 µl of blocking buffer. The wells were incubated for 1 h with 50 µl of biotinylated SLP solution (10 µg/ml of blocking buffer). The binding reaction was stopped by aspirating the solution, and the plate was washed two times with blocking buffer (300 µl). Next, 60 µl of horseradish peroxidase-conjugated streptavidin (3000× dilution with blocking buffer; Zimed Laboratories Inc., San Francisco, CA) was added to the wells. After incubation for 1 h, the reaction was stopped by removing the horseradish peroxidase-conjugated streptavidin solution, and the plate was washed as described above. The wells then were immersed in 60 µl of tetramethylbenzidine color-developing reagent (Sumitomo Bakelite Co. Ltd.). After 10 min, the reaction was stopped by the addition of a stop solution (60 µl). The sample solution (100 µl) was then transferred to a new well, and the absorbance of the solution was measured at 450 nm by a microtiter plate reader (Model 450; Bio-Rad Laboratories, Inc., Heracules, CA). All steps were performed at room temperature except coating the microtiter plate with RCM.

Periodate Oxidation of RCM

Wells of a microtiter plate were coated with RCM according to the method described above. The RCM on the wells was oxidized with 0.1 M periodic acid solution (75 µl) for 12 h at 4°C, during which time overoxidation was prevented. The reaction was stopped by aspirating the solution, and the plate was washed four times with a blocking buffer (300 µl) and then used for the binding assay analysis.

Preparation of Mimic Human Intestinal Mucous Membrane and Isolation of Lectin-like Proteins from SLP

An RCM solution (500 µl of 6 M guanidine hydrochloride at 25 mg/ml) was applied to an Immobilon™-P Transfer Membrane (PVDF membrane, 25 cm²; Millipore Corporation, Bedford, MA) and then dried. The RCM-coated membrane (mimics human intestinal mucous membrane) was washed three times with 10 ml of PBS and was blocked three times with blocking reagent (10 ml of PBS containing 0.2 % polyvinylpyrrolidone-40). The RCM-coated membrane was reacted with an SLP solution (40 mg/40 ml of PBS containing 0.1 % Tween 20) for 1 h with gentle shaking. The binding reaction was stopped by transferring the membrane into PBS, and the membrane was then washed three times with the same buffer. The specifically bound proteins on the membrane were recovered by extraction with 0.1 M formic acid-ammonia buffer (pH 2.5). The treatment was repeated 10 times. The whole extract was desalted by a Micro Acilizer G1 (Asahi Kasei Co. Ltd., Tokyo, Japan) and then lyophilized (RCM-binding protein). All steps were performed at room temperature.

SDS-PAGE

The SDS-PAGE was performed according to the method used by Laemmli (6) with a 10% (wt/vol) separating gel. After electrophoresis at 20 mA for 1.5 h, the gel was stained with a commercial kit (Quick CBB; Wako Pure Chemical Industries, Ltd.). A low molecular weight kit (Daichi Pure Chemicals Co., Ltd., Tokyo, Japan) supplied the marker protein for calibration.

RESULTS AND DISCUSSION

Binding Assay of Surface Layer Protein from L. acidophilus to Rat Colonic Mucin

The binding activity of the biotinylated SLP, which was prepared from 35 strains of the L. acidophilus
group, with RCM was investigated by microtiter plate assay (Figure 1). The SLP from all strains except SBT264 (A3) bound to the RCM at varying rates. The binding activity was estimated from the difference in absorbance measured between wells coated and not coated with RCM. Nonspecific binding was estimated to be negligible (less than ODdiff = 0.1). Although the binding activity of each strain differed, the binding tendency within each subgroup was similar (subgroup specificity). The B-group strains generally showed a higher level of binding activity than did the A-group strains; the B1 subgroup showed the highest activity, and the A3 subgroup had the lowest binding value. The B1 subgroup is known to be a dominant lactic acid bacteria among the *L. acidophilus* group found in human gastrointestinal tracts. This screening method showed that three strains, JCM1025 (OD450 = 0.799), SBT267 (0.775), and LA2 (0.771) bound strongly to the RCM and that SBT2062 (0.532) demonstrated the strongest binding of all the A-group strains. A previous screening method using polystyrene beads (13) also showed that SBT2062 has the highest level of binding activity of all the A-group strains of *L. acidophilus*.

The changes in the binding activity of five kinds of SLP, which are shown in Figure 1, were compared before and after periodate oxidation of the RCM (Figure 2). For all of the SLP tested, the binding activity to RCM was significantly reduced (about 55 to 77%) after oxidation treatment. The periodate treatment destroyed a part of vicinal hydroxyl group (—OH) in a nonreducing end unit of GalNAc and a branch unit in the backbone structure of Fuc and NeuAc in the common sugar chain of RCM (see Introduction). An internal unit linking with β1-3 linkage remained unchanged. The marked decrease in binding activity to the RCM after oxidation strongly suggests that the RCM binding proteins in the SLP bound to the sugar portion of the RCM in a lectin-like manner. The lectin-like proteins in these strains must contribute to adhere to the colonic mucus layer in their host's intestinal tracts. The remaining binding activity after periodate oxidation suggests that there are some lectin-like proteins that recognize the internal sugar structure rather than the external sugars (GalNAc, Fuc, and NeuAc).

We concluded that the method used is quite useful for selection of *L. acidophilus* strains that have high adhesion to the human intestinal tract.

### Isolation of Lectin-like Proteins from the Surface Layer Protein of Bacteria

The lectin-like proteins in the SLP that bind to the RCM-coated membrane were recovered by extraction with an acidic buffer and analyzed by SDS-PAGE. About 1 mg of crude lectin was recovered from 10 mg of SLP by this method, and a trace amount of nonspecifically bound proteins was present on the membrane after blocking treatment. Figure 3 shows the electrophoretic pattern of the lectin-like proteins and whole SLP obtained from three bacterial strains. The amount of nonspecifically bound proteins on the PVDF membrane was only slight (data not shown). The bound components displayed a few main bands together with several minor bands, and these patterns were different in all three tested strains. Lectin-like proteins isolated from the SLP of the LA2 strain displayed three main bands (about 60, 30, and 25 kDa) together with a num-

![Figure 3. The SDS-PAGE of lectin-like proteins bound on rat colonic mucosa (RCM)-coated membrane from three *Lactobacillus acidophilus* strains. Lane A, marker proteins; Lanes B, D, and F, whole surface layer proteins (SLP) from each strain; Lanes C, E, and G, lectin-like proteins isolated from the SLP of each strain.](image-url)
number of minor bands. Lectin-like proteins from the SLP of strains SBT267 and LA187 migrated into two main bands (about 50 kDa and 30 kDa). These main bands were not found in the bound components on the RCM-coated membrane previously oxidized with periodate (data not shown). Thus, we consider these proteins to play a key role in the cell adhesion of lactic acid bacteria to the human colonic mucous surface.

Further work on the isolation, characterization and cloning of lectin-like proteins from *L. gasseri* LA2 is now in progress.

**ACKNOWLEDGMENTS**

This study was partially supported by a Grant-in-Aid for Exploratory Research to T. Itoh (No. 09876069) from the Ministry of Education, Science and Culture of Japan (Tokyo).

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