

Extraction, purification and identification of surface adhesion ligand from *Lactobacillus*

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ABSTRACT

The objective of this experiment was to purify and analyze adhesion protein from the surface of *Lactobacillus*. The surface adhesion ligand of *Lactobacillus* was isolated with of LiCl method and $(\text{NH}_4)_2\text{SO}_4$ method, followed by sephadex chromatography, analyzed with discontinuous native polyacrylamide gel electrophoresis, and tested with a binding assay with intestinal epithelial cells. We get the adhesion matter by two methods: ammonium sulfate and LiCl extraction. Further purification with sephadex chromatography produced 3 components. The second component of eluted from chromatography had adhesion enhancing ability compared with the control. A single band was present in discontinuous native—poly-acrylamide gel electrophoresis analysis and molecular weight was determined to be 43 - 66 KDa. The purified adhesion ligand can improve the number of *Lactobacillus* adhering to the intestinal epithelial cells.

Keywords: *Lactobacillus*; Adhesion Ligand; Purify; Molecular Weight

1. INTRODUCTION

Under normal physiological conditions, the lumen surface of animal small intestine is colonized by a large amount of bacteria, forming a bacterial biofilm. The biofilm is very important for animal immunity, defense, and nutrient absorption. When animals suffer from diseases such as diarrhea, the normal interaction between the host and bacteria is destroyed, causing disturbance of gastrointestinal microflora. The adhesion of normal bacteria to epithelium is mediated by the interaction between adhesion factor (ligand) from normal bacteria and the receptor from the surface of epithelial cells. Using advanced protein isolation, purification and identification technologies, we successfully isolated a adhesion ligand from

Lactobacillus that colonizes the gastrointestinal tract of chickens, determined the molecular weight. These results provided material and technical resources for the study of the structure of *Lactobacillus* adhesion ligand, its gene, and the mechanism of adhesion. These results also provided useful information for the development of animal microecology.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Intestinal Epithelial Cells

CaCo-2 cells were purchased from Shanghai Institute of Cell Biology.

Cell culture: cells were cultured with DMEM (pH 7.0 - 7.2, Invitrogen) supplemented with 10% calf serum (Beijing Dingguo Biotechnology Inc.), 1000 IU/L penicillin, 100 $\mu\text{g/L}$ streptomycin, in an incubator with CO_2 maintained at 5%. When sufficiently attached to the culture flask, cells were washed with phosphate buffered solution (pH 7.4), digested with digestion solution (0.02% EDTA:D-Hanks solution = 10:1, without Ca^{2+} and Mg^{2+}) for 20 - 30 min. The digestion was stopped before cells detaching from the culture flask by inverting the flask for 3 min. Digestion solution was removed, and 10% DMEM (DMEM containing 10% calf serum) was added to the flask, mixed thoroughly, split to new flasks.

2.1.2. *Lactobacillus*

Lactobacillus (SDnA) was isolated from SPF chicken, provided by Microbiology Laboratory of Shandong Agriculture University.

2.1.3. Protein Chromatography System

REC5004V₁₈ system was purchased from Pharmacia (Sweden).

2.1.4. Spectrophotometer

UV-2000 spectrophotometer was purchased from Unico (Shanghai) Instrument Inc.

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2.2. Methods

2.2.1. Culture of *Lactobacillus*

MRS medium was used.

2.2.2. Extraction of Adhesion Ligand

2.2.2.1. Saturated Ammonium Sulfate Method

Supernatant (5000 ml) of *Lactobacillus* culture was collected after spinning 5000 rpm at 4°C for 15 min, mixed with saturated (NH₄)₂SO₄ solution [1,2] until the final concentration was 30%. After setting overnight at 4°C, the solution was spun 8000 - 10,000 rpm at 4°C for 10 min. The pellet was dissolved in 0.01 mol/L phosphate buffered solution (pH 7.4), dialyzed in pH 7.4 phosphate buffered solution to remove ions of (NH₄)₂SO₄. Then, the sample was concentrated to 1 ml with PEG-6000, measured for OD₂₆₀ and OD₂₈₀ to calculate the concentration. The preparation was stored at -20°C until use [3,4].

2.2.2.2. LiCl Method

Suspension of overnight anaerobic bacterial culture was transferred to 500 ml of fresh MRS medium, cultured until OD₆₀₀ was 1.0. Bacteria were collected after spinning 6000 rpm at 4°C for 10 min, washed twice with 10 mM phosphate buffered solution (pH 7.4), cold dried. The dried bacteria were mixed with LiCl (20 ml 5 M LiCl per 0.1 g dried bacteria), shaken for 3 h at 37°C, spun 6000 rpm at 4°C for 10 min. Then, the supernatant was thoroughly dialyzed with phosphate buffered solution (pH 7.4), concentrated with PEG at 4°C, then stored at -20°C until use.

A blank MRS medium was used as control, extracted the same way as described above.

2.2.3. Adhesion Ligand Purification (Sephadex Chromatography)

Sephadex 50 was swollen with water, slowly packed into a column (1.2 × 100 cm). The resin was then equilibrated with phosphate buffered solution (0.01 mol/L, pH 7.2). Then, ligand preparations (1 ml, containing 30 mg) were loaded slowly into the column, eluted with phosphate buffered solution (0.01 mol/L, pH 7.2) at 0.2 ml/min. each elution peak was collected separately and frozen until use.

2.2.4. Identification of Adhesion Ligand

2.2.4.1. Adhesion Test

Lactobacillus suspension (1000 µl) was incubated with the primary extract (200 µl) or the chromatography elutant (200 µl), at 37°C for 24 h (bacterial cells were not broken). Cells were cultured on coverslips which were placed in 24-well plates (after 2 - 3 days of attachment). Then,

bacteria (200 µl) that were treated with the primary extract or chromatography elutants were added to the cell culture. Three wells were used for each ligand extract. After incubation at 37°C for 24 h, coverslips were picked up and washed 6 times with phosphate buffered solution (pH 7.4), dried naturally, fixed with methanol, stained with Gram stain, and examined under microscope. The number of bacteria attached to each of 50 cells was counted. Two control groups were set up. Control 1 was 200 µl of bacterial suspension that was shaking washed 5 - 6 times with phosphate buffered solution. Control 2 was 200 µl of normally cultured bacterial suspension. A preparation is considered *Lactobacillus* surface adhesion ligand if it has greater adhesion compared with control 1, and contained protein band that was similar adhesion as in control 2 [2].

2.2.4.2. Discontinuous Native PAGE

The primary and fine extracts were analyzed for molecular weight [5].

2.2.5. Statistics Analysis

Statistics between treatment were assessed utilizing with the ANOVA procedure and Duncan's multiple range test was used for multiple comparisons. analyze the treatment effects adherence to the cells.

3. RESULTS

3.1. Extraction of Adhesion Ligand

The adhesion ligand was isolated with two methods. The ammonium sulfate method produced 2 ml of adhesion ligand preparation, with a protein concentration of 40 mg/ml extracted from 5000 ml *Lactobacillus* culture supernatant. The LiCl produced a preparation containing 31 mg/ml protein 2 ml.

3.2. Electrophoresis Results of Primary Adhesion Ligand Extracts

Electrophoresis results of primary adhesion ligand extracts were shown in **Figures 1** and **2**.

3.3. Effect of *Lactobacillus* Ligand Extraction on Attachment

Lactobacillus ligand extracts were tested for their ability to interfere with the attachment of the bacterium to intestinal epithelial cells. The numbers of bacterial cells attached to epithelial cells as influenced by the extracts were shown in **Table 1**.

Compared with control 1, ligand prepared with ammonium sulfate method and with LiCl method significantly increased the ability of *Lactobacillus* adhering to

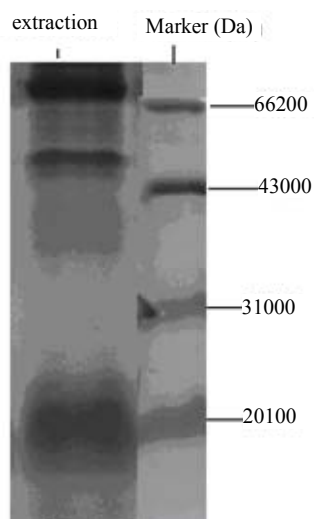


Figure 1. Native gel electrophoresis results of adhesion ligand extracted from *Lactobacillus* with 30% saturated ammonium sulfate.

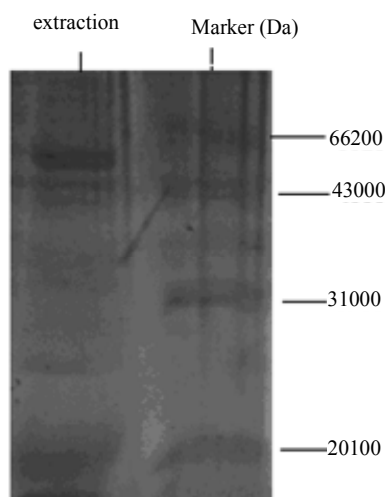


Figure 2. Native gel electrophoresis results of surface adhesion ligand extracted with LiCl from *Lactobacillus*.

Table 1. Effect of adhesion ligand preparation on the attachment of *Lactobacillus* to intestinal epithelial cells.

Method	Number of <i>Lactobacillus</i> adhered (entries/cell)
30% (NH ₄) ₂ SO ₄	20.70 ± 1.48 ^a
LiCl	14.30 ± 1.87 ^b
Control 1	3.00 ± 0.61 ^c
Control 2	19.80 ± 1.97 ^a

Note: Values that were marked with different superscripts were significantly different from each other ($P < 0.01$).

intestinal epithelial cells. Compared with control 2, the numbers of attached bacteria were similar, suggesting that the primary extract contained the ligand, but need

further purification. The ammonium preparation displayed greater adhesion ability.

3.4. Purification of Adhesion Ligand

After sephadex 50 chromatography, three broad peaks were clearly seen (Figures 3 and 4). Each peak was collected in 5 ml elutant.

3.5. Adhesion Test

After sephadex 50 chromatography purification, the adhesion ligand preparations were tested for their influence on the attachment of *Lactobacillus* to intestinal epithelial cells. Results were shown in Tables 2 and 3.

Table 1 showed that the second peak elutant of the ammonium sulfate extract increased the ability of *Lactobacillus* adhering to intestinal epithelial cells, compared with control 1, but had similar adhesion ability compared with control 2. These results suggest that the peak 2 elutant is the purified adhesion ligand.

The peak 2 elutant of the LiCl extract displayed a

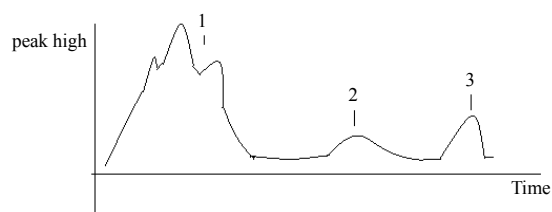


Figure 3. Chromatography profile of adhesion ligand from *Lactobacillus* prepared with 30% saturated ammonium sulfate.

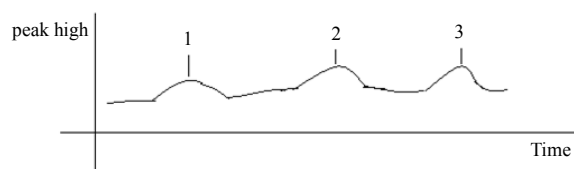


Figure 4. Chromatography profile of adhesion ligand from *Lactobacillus* prepared with LiCl.

Table 2. Effect of adhesion ligand preparations obtained by ammonium sulfate extraction followed by chromatography on the number of *Lactobacillus* adhered to intestinal epithelial cells.

Peak	Number of <i>Lactobacillus</i> adhered (entries/cell)
1	7.40 ± 1.23 ^b
2	20.70 ± 1.45 ^a
3	6.70 ± 1.45 ^b
Control 1	4.00 ± 0.76 ^b
Control 2	21.89 ± 1.32 ^a

Note: Values that were marked with different superscripts were significantly different from each other ($P < 0.01$).

Table 3. Effect of adhesion ligand preparations obtained by LiCl extraction followed by chromatography on the number of *Lactobacillus* adhered to intestinal epithelial cells.

Peak	Number of <i>Lactobacillus</i> adhered (entries/cell)
1	6.80 ± 1.56 ^a
2	19.60 ± 1.89 ^b
3	5.70 ± 1.35 ^a
Control 1	5.00 ± 0.98 ^a
Control 2	21.89 ± 1.32 ^b

Note: Values that were marked with different superscripts were significantly different from each other ($P < 0.01$).

significantly higher ability to increase the adhesion of *Lactobacillus* to intestinal epithelial cells, compared with control 1, but a similar ability, compared with control 2. These results suggest the peak 2 contained the purified adhesion ligand.

Native gel electrophoresis analysis of the elutants isolated from ammonium sulfate extract and LiCl extract indicated that the active ingredient was in the second peak.

3.6. Molecular Weights of the Two Adhesion Ligand Isolates

Molecular weights of the two adhesion ligand isolates were shown in **Figure 5**. The two adhesion ligand preparations were subjected to discontinuous native polyacrylamide gel electrophoresis analysis and molecular weights of the ligand preparations were determined to be 43 - 66 KDa.

4. DISCUSSION

Results from the current study demonstrated that chicken *Lactobacillus* adhesion ligand has a molecular weight 43 - 66 KDa. This molecular weight is similar to that (about 45 KDa) reported by Greene (1994) [6], and that (30 - 70 KDa) reported by Henriksson (1996) [1], but very different from that (14 - 23 KDa) reported by Zheng (1999) for *Bifidobacterium* [2]. Avall-Jaaskelainen (2003) reported that the adhesion protein from *Lactococcus lactis* is 111 Kda [7]. These reports indicate that the molecular weights of adhesion proteins from *Lactococcus*, *Bifidobacterium* and *Lactobacillus* are very different [8-13]. Further studies are necessary in this field.

It is difficult and time-consuming to purify surface adhesion ligand from chicken *Lactobacillus* with column chromatography. A large column chromatography system is needed for the preparation. Very little amount of purified protein can be obtained from one run. Thus a simple and effective method is needed. The current study showed that the 25% ammonium sulfate method pro-

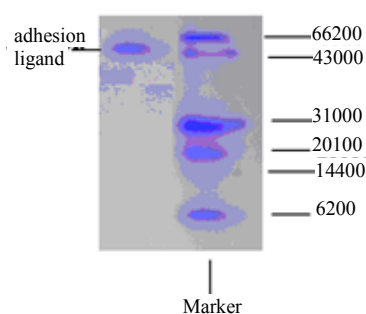


Figure 5. Discontinuous native gel electrophoresis of adhesion ligand from *Lactobacillus A*.

duced more and purer surface adhesion ligand than did LiCl method. Future work may employ the ammonium sulfate method to prepare purer protein.

The current study used sephadex 50 to isolate protein, substituting the cumbersome molecular sieve and ion exchange chromatography method. The method used in this study ideally purified the surface adhesion ligand of *Lactobacillus* from primary extract.

It is important to determine whether there is adhesion protein from normal bacteria that interact with intestinal epithelial cells. This will help the further application of this protein to enhance the ability of normal bacteria to attach to intestinal epithelial cells, help the elucidation of mechanism involved in adhesion.

In the adhesion test of this study, bacteria were incubated at 37°C for 24 h. Electron microscopy analysis indicated that the bacterial cells were intact after incubation, imposed no adverse effect on the results of the experiment.

5. CONCLUSION

We got the *Lactobacillus* adhesion ligand adhering to the intestinal epithelial cells by ammonium sulfate and LiCl extraction, sephadex chromatography purification methods. The purified adhesion ligand molecular weight was 43 - 66 KDa.

6. ACKNOWLEDGEMENTS

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