New Methods for Isolation of K99 Fimbriae from Enterotoxigenic *Escherichia coli*

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K99, K88, F41 and/or FY fimbrial-antigen are detected most frequently in enterotoxigenic *E. coli* isolated from calves and piglets with diarrhea [2, 10]. The diarrhea which occurred in these newborn animals have resulted in a large economic loss in the world.

Recently, studies were concerning fimbria developed in P fimbriae of human uropathogenic *E. coli* and Type 1 fimbriae of various pathogenic *E. coli*. These studies indicated that both P and Type 1 fimbriae are mainly composed of fibrin subunits, which serve fimbrial antigenicity, and adhesin subunits, which bind to the host receptor site [3, 6, 7].

In the case of K99 fimbriae, it showed hemagglutination (HA) activity against equine erythrocytes by recognizing a carbohydrate epitope, N-glycolyl neuraminic acid of glycosphingolipids [12]. It was found that the fimbrial gene cluster consists of 8 different genes, but the adhesin gene and its product have not been identified yet while the major fimbrin subunit was found to be produced by *fan C* gene [13–15]. For development of the effective vaccination by inhibiting the bacteria from attaching to host intestinal mucosa, it is necessary to identify the adhesin subunit of fimbriae. The vaccination of K99 whole molecules against pregnant parents was tried to prevent the infection by maternal immunity, but the effectiveness has not been clarified so far. K99 adhesin molecules will be expected to induce a complete immunological effect.

For the development of identification of K99 adhesin, it is necessary to purify K99 fimbriae conserving HA activity. There are several reports dealing with purification of K99 fimbriae, but none of them checked the HA activity of purified fraction. In this communication, we tried to purify HA active K99 fimbriae using two different methods.

HA tests were performed as follows. Two fold serial dilutions of each fimbrial fraction (50 μ l) were mixed with equal volumes of 1% human or equine erythrocyte suspension in a well of a microplate (Falcon 3911, Becton Dickinson Co., Ltd., NJ, U.S.A.) and incubated at 4°C for 1 hr. HA titer were expressed as reciprocals of the highest dilution of samples that showed HA reaction.

Strain B41 (0101: K-:K99+: F41+) were cultured on Minca plate or in minimal medium for the fimbrial

preparation. Minca and minimal medium were prepared according to De Graaf *et al.* [1]. Approximately 15 mg of the fimbriae-rich protein fraction was obtained from 1.5×10^{11} bacterial cells by heat treatment (65°C, 30 min) in Dulbecco's phosphate buffered saline solution (PBS). The fraction was applied on Sepharose 4B gel filtration column. K99 and F41 fimbrial proteins were eluted together at the first protein peak, and equine erythrocyte HA activity was detected in the same peak (Fig. 1). The second UV absorbance peak was not coincident with the 2nd protein peak determined by Bio-Rad protein assay. This peak showed higher absorbance at 260 nm than at 280 nm (data not shown), indicating degradation of derivatives of nucleic acids. The gel filtration using Sepharose 4B succeeded in removal of contaminants other than fimbriae [8]. In the previous report, K99 fimbrial fraction was prepared by mild homogenization followed by $(\text{NH}_4)_2\text{SO}_4$ precipitation [4], by heat treatment in physiological saline solution after gel filtration [9] or by heat treatment in 2 M urea-containing phosphate buffer [5]. We also followed these methods, but it was revealed that in SDS-PAGE analysis the fimbrial preparations contained many other protein bands than the major fimbrin band (16.5 K).

Next, the K99 fimbrial proteins were separated from F41 fimbrial protein by salting-out chromatography using

Fig. 1. Sepharose 4B gel filtration of the fimbrial fraction from strain B41. A fimbrial preparation (15 mg in protein content) was applied on Sepharose 4B column (2.7×60 cm) equilibrated with PBS and each 9 ml eluate was fractionated. Absorbance at 280 nm (●), protein concentration determined by Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA, U.S.A.) (○) and equine erythrocyte HA activity (▽) were determined in each fraction. SDS-PAGE of each fraction was shown at the upper portion of the figure. K99 fimbrial subunit (16.5 K) and F41 fimbrial subunit (29.5 K) were detected.

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Fig. 2. Separation of K99 and F41 fimbriae by salt fractionation. Sephadex G-25 column (17×450 mm) was equilibrated with 30% saturated $(\text{NH}_4)_2\text{SO}_4$ solution (pH 7.0) and then flowed with 2/3 column volume of 30 to 0% saturated $(\text{NH}_4)_2\text{SO}_4$ linear gradient solution. A fimbrial fraction, 2 ml of fraction 4 shown in Fig. 1 was applied and gradually desalted by elution with PBS. Each 2 ml was fractionated and monitored by absorbance at 280 nm (o) and equine (white bar) and human erythrocyte (black bar) HA assay. SDS-PAGE analysis was done as shown in Fig. 1. Lane 1, fraction 4 in Fig. 1; lane 2, pooled fractions of the first peak; lane 3, pooled fractions of the second peak. Black triangles indicate K99 fimbrial subunit and white bars indicate F41 fimbrial subunit.

Sephadex G-25 column equilibrated with linear gradient of 0–30% saturated $(\text{NH}_4)_2\text{SO}_4$ (Fig. 2). A sample of the fimbriae (fraction 4 in Fig. 1) was applied on the column. The first peak was K99 fimbrial protein which showed equine erythrocyte HA, and the second peak was F41 fimbrial protein which showed human erythrocyte HA in a higher titer than equine erythrocyte HA.

To purify K99 fimbriae more easily, we looked for different K99 fimbriae-positive strains without any other fimbriae. Fimbrial preparation isolated by means of heat treatment from strain H1915 (08:K25:K99+), F41 fimbriae-negative strain [4], showed homogeneous protein band of 16.5 K in SDS-PAGE analysis and showed only

equine erythrocyte HA, not human erythrocyte HA (data not shown). Fimbrial protein from strain H1915 was similarly fractionated using Sepharose 4B column as the first peak, and followed by ultracentrifuged at $230,000 \times g$ for 4 hr. Most of HA activity was precipitated, and the specific activity increased 86 fold in comparison to that of the starting materials (Table 1). We speculated that the increase of total HA activity may be caused by the change of fimbrial form. It is well known that the viral HA activity was increased in the step of purification [11]. Monovalent HA protein is more effective for HA activity than the polymeric form as the virion. Another speculation is the removal of low density inhibitor like lipids. In Fig. 1, fractions of peak of fimbriae showed different specific activities each other, especially fraction of 64–72 ml which was the first fraction of fimbriae showed lower specific activity (0.15 HA unit/ng) compared with the fraction of 90–99 ml giving the highest specific activity (2.0 HA unit/mg). If the inhibitor was eluted at void volume of Sepharose 4B column with fimbriae, it had high molecular weight and low density like micelles of lipids. However, in the case, it was not clear which natural K99 fimbriae was polyvalent form or not and there were low density inhibitor in this preparation or not. It is remained unclarified what did elevate HA activity of K99 fimbriae after the treatment of ultracentrifuge.

To obtain K99 fimbriae with high HA activity, the method using ultracentrifuge was more effective than that of salting out. The specific activity of K99 fimbriae from H1915 by using ultracentrifuge was higher (18.2 HA unit/ng), than that of K99 fimbriae from strain B41 by salting out (0.52 HA unit/ng).

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Table 1. Ultracentrifugation of fimbrial preparation from strain H1915

Sample ^{a)}	Volume (ml)	HA unit /25 μ l	Protein conc. (μ g/ml)	Total HA unit	Total protein (mg)	Specific activity (HA unit/ng)
1	30	2^{10}	180	120×10^4	5.4	0.22
2	1	2^{12}	514	16×10^4	0.5	0.32
3	27	2^6	97	7×10^4	2.3	0.03
4	3	2^{19}	1164	6300×10^4	3.5	18.2

a) A fimbrial fraction of strain H1915 (1) which was obtained by Sepharose 4B gel filtration of strain H1915 (1) which was obtained by Sepharose 4B gel filtration as shown in Fig. 1 was centrifuged at $140,000 \times g$ for 4 hr by using Hitachi ultracentrifuge apparatus 55P-72 equipped with RPS 40T-508 rotor (Hitachi Koki Co., Ltd, Japan). The precipitation was suspended in 1 ml PBS (2) and the supernatant was centrifuged again at $230,000 \times g$ for 4 hr. The supernatant (3) and the suspension of the precipitate in 3 ml PBS (4) were also tested for protein concentration and equine erythrocyte HA activity.

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