A Chromatographic Method for the Purification of K99 Pili from Enterotoxigenic *Escherichia coli*

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Details of a relatively inexpensive method for the purification of K99 pili in their native conformation are reported. The method involved sequential precipitation of K99 pili with $(\text{NH}_4)_2\text{SO}_4$, followed by precipitation in 12% (w/v) mannose or sorbitol solution. The crude pili preparation was adsorbed with Bio-Gel A-5m and subjected to sequential gel filtration on Bio-Gel A-5m equilibrated with Tris/EDTA/ NaN_3 /NaCl buffer and KSCN/KCl solution, respectively. The K99 containing peak was subjected to sequential ion-exchange chromatography on DEAE-Bio-Gel A equilibrated with 0.02 M-Tris/HCl, pH 8.6 containing 0.05 M-NaCl and DEAE-Sephadex A-50 equilibrated with 0.05 M-phosphate buffer, pH 7.2. The purified pili yielded a single band on SDS-PAGE with an estimated molecular weight of 13 000. Attempts to purify pili by other methods evaluated, viz. MgCl_2 precipitation and chromatofocusing were unsuccessful. While the amino acid composition of purified K99 pili was similar to that reported previously the N-terminal amino acid was apparently blocked.

INTRODUCTION

A substantial proportion of strains of enterotoxigenic *Escherichia coli* isolated from field cases of calf and lamb scours, and some of porcine origin have been found to possess pili designated K99 (Guinée *et al.*, 1976; Moon *et al.*, 1976; Myers & Guinée 1976; Sivaswamy & Gyles, 1976). Purification of this antigen is necessary for the evaluation of its immunogenic potential in the prevention of colibacillosis. An earlier investigation revealed that K99 pili were composed primarily of two protein subunits, having molecular weights of 22 500 and 29 500 (Isaacson, 1977). However, recently we found that K99 pili purified from the crude KSCN extracts of enterotoxigenic *E. coli* were different in that (a) they were composed of single subunits, 13 000 in molecular weight and (b) while the sulphur-containing amino acids, cysteine and methionine were absent, phenylalanine and tyrosine were present (Altmann *et al.*, 1982). Otherwise the purified pili resembled those described by Isaacson (1977) in sedimentation coefficient, diameter and approximate length (determined ultrastructurally). These results concur, at least partially, with those reported recently by De Graaf *et al.* (1981) for K99 pili purified by the use of dissociating agents such as urea and sodium deoxycholate. However, the diameter of pili purified in this way was smaller than that reported previously (Isaacson, 1977; Altmann *et al.*, 1982) thus suggesting a loss of their native conformation. The method developed for the purification of K99 pili in this laboratory (Altmann *et al.*, 1982) was expensive in that it involved the use of preparative isoelectric focusing, which requires highly specialized equipment and

expensive chemicals, viz. pharmalytes. A relatively inexpensive preparative method for obtaining purified K99 pili in their native conformation is described here which should enable the immunogenic properties of purified K99 in experimental colibacillosis to be evaluated.

METHODS

Bacteria. Enterotoxigenic *Escherichia coli* (C1443) which was kindly provided by Dr J. Craven of the Veterinary Research Laboratory, 'Attwood' Westmeadows, Victoria, Australia, had the serotype O20:KX106:K99⁺, H⁻ (determined by Mr Ian Links, Regional Veterinary Laboratory, Wagga Wagga, N.S.W., Australia) and produced both heat-stable and heat-labile enterotoxins.

Growth conditions. Enterotoxigenic *E. coli* were grown in Minca medium (Guinée *et al.*, 1976) at 37 °C in a batch fermenter (New Brunswick) in 10 l lots, with aeration at the rate of 3 l min⁻¹.

Extraction of K99 pili. Crude preparations of K99 pili were obtained by extraction with 3 M-KSCN for 30 min at 22 °C as described previously (Altmann *et al.*, 1982).

Assays of K99 activity. K99 activity was assayed by double diffusion in 0.85% (w/v) Agarose C (Pharmacia) using specific K99 antiserum and/or haemagglutination of horse red blood cells using phosphate-buffered saline as a diluent (Morris *et al.*, 1980). The specific activity in precipitating units or hemagglutinating units and the yield of K99 were calculated as described previously (Altmann *et al.*, 1982).

Preparation of antisera. Merino sheep were immunized with crude KSCN extract (5 mg protein per animal at each injection) or purified K99 pili (2.5 mg protein per animal at each injection) emulsified in incomplete Freund's adjuvant. Four intramuscular injections were given at monthly intervals when the animals were bled and then the sera were stored at -20 °C.

Assay for heat-labile toxin. The heat-labile enterotoxigenic activity was assayed using the y-1 adrenal tumour cell line (Donta *et al.*, 1974).

Analytical SDS-PAGE. SDS-PAGE was carried out in 10% (w/v) acrylamide with standard proteins in the range 68000 to 12100 Dal as described previously (Altmann *et al.*, 1982).

Absorption with Bio-Gel A-5m. Absorption of K99-enriched preparations was carried out with Bio-Gel A-5m equilibrated with TEAN buffer (0.05 M-Tris, 0.001 M-Na₂EDTA, 0.003 M-NaNO₃, 0.2 M-NaCl, pH 7.5) containing 10% (w/v) (NH₄)₂SO₄ (Altmann *et al.*, 1982).

Chromatography. Molecular sieve chromatography was carried out on a column (1.6 × 70 cm) of Bio-Gel A-5m equilibrated with TEAN buffer (Clements & Finkelstein, 1979) followed by re-chromatography on the same gel equilibrated with 3 M-KSCN solution saturated with KCl, pH 8.2 (KSCN/KCl solution). Ion-exchange chromatography was carried out on columns (1.6 × 3.5 cm) of DEAE-Bio-Gel A equilibrated with 0.02 M Tris/HCl and 0.05 M-NaCl, pH 8.6 and DEAE-Sephadex A-50 equilibrated with 0.05 M-sodium phosphate buffer, pH 7.2.

Chemical analysis. Protein concentration was estimated using the Bio-Rad protein kit. The method used for the detection of DNA was that of Ceriotti (1952), RNA was detected by the method of Mukkur & Pyliotis (1981). The lipopolysaccharide (LPS) content was estimated by using 2-keto-3-deoxyoctonate as a standard as described previously (Mukkur & Pyliotis, 1981; Altmann *et al.*, 1982). Estimation of total carbohydrates was made using anthrone reagent according to Mokrasch (1954), with glucose as a standard.

Immunoelectrophoresis. Immunoelectrophoresis was carried out using a standard procedure in 0.85% (w/v) Agarose C. Antisera used for development were sheep anti-K99 and anti-KSCN extracts.

Electron microscopy. Crude and purified K99 preparations were negatively stained in 2% (w/v) sodium phosphotungstate and examined in a Philips EM301 electron microscope (Altmann *et al.*, 1982).

Determination of sedimentation coefficient. The S_{20,w} of purified K99 (1.0 mg ml⁻¹) was determined in a Beckman analytical ultracentrifuge equipped with a multiplex scanner. Centrifugation was carried out at 37020 r.p.m. in a Titanium rotor (An G-Ti) at 20 °C.

Determination of N-terminal amino acid and amino acid composition. The method attempted for the determination of the N-terminal amino acid was the dansylation procedure as recommended by Rosmus & Deyl (1971); ribonuclease was processed in parallel as a control. The amino acid composition of purified K99 pili was determined following hydrolysis with 6 M-HCl at 110 °C, *in vacuo* as described previously (Altmann *et al.*, 1982) using a JLC-6AH amino acid analyser.

RESULTS

Precipitation of K99 pili with ammonium sulphate

KSCN-extracted crude K99 pili were subjected to (NH₄)₂SO₄ precipitation (50% final saturation) as described earlier (Altmann *et al.*, 1982) with the modification that the precipitate obtained at 14% saturation was discarded since it did not possess haemagglutinating activity. This procedure resulted in 2.3- to 2.5-fold purification of K99 activity (Table 1). While RNA and

Table 1. *Purification of K99 from enterotoxigenic E. coli (C1443)*

Step	Total protein (mg)	Total K99 (P.U.)*	Specific activity (P.U. mg ⁻¹)	Purification (fold)	Yield (%)	Total K99 (H.U.)†	Specific activity (H.U. mg ⁻¹)	Purification (fold)	Yield (%)
Crude KSCN extract	30.3	22400	739	0	100	819200	27036	0	100
(NH ₄) ₂ SO ₄ precipitation	12.0	20800	1733	2.3	92	819200	68267	2.5	100
Aggregation in 12 % (w/v) mannose	6.6	14336	2172	2.9	64	532480	80678	3.0	65
Bio-Gel A-5m chromatography	3.0	10400	3467	4.7	46	409600	136533	5.1	50
DEAE (Bio-Gel A and Sephadex A-50) chromatography	0.86	6400	7441	10.1	29	298188	346731	12.8	36

* Precipitin units.

† Haemagglutination units.

Fig. 1. SDS-PAGE of: A, crude KSCN extract of (100 g) enterotoxigenic *Escherichia coli* (C1443); B, enriched K99 obtained following gel filtration on Bio-Gel A-5m equilibrated with KSCN-KCl solution (50 µg); and C, purified K99 (50 µg).

DNA were still detectable, LPS was absent. On SDS-PAGE, the minimum number of bands staining with Coomassie blue was nine (Fig. 1*a*).

Precipitation of K99 pili in 12% mannose or sorbitol

The $(\text{NH}_4)_2\text{SO}_4$ precipitated pili were dissolved in 0.05 M-phosphate buffer, pH 7.2 and dialysed extensively against 12% (w/v) mannose or sorbitol at 4 °C. The precipitate formed was removed by centrifugation at 27 750 *g* for 30 min and dissolved in phosphate buffer. This step

Fig. 2. Elution profiles showing gel filtration of the $(\text{NH}_4)_2\text{SO}_4$ fractionated pili on Bio-Gel A-5m; (a) column equilibrated with TEAN buffer and (b) re-chromatography of peak A-1 with KSCN-KCl solution.

resulted only in a slight increase in purification of K99 (Table 1) but traces of DNA and RNA were removed completely. However, the heat-labile enterotoxigenic activity was still detectable and no reduction in the number of bands detected by SDS-PAGE was observed.

Molecular sieve chromatography

Crude pili prepared as above were mixed with Bio-Gel A-5m (Altmann *et al.*, 1982) and the supernatant solution subjected to gel filtration on Bio-Gel A-5m equilibrated with TEAN buffer when two peaks (A-1 and A-2) were eluted (Fig. 2a). K99 activity was confined to peak A-1, the ascending portion of which was pooled and re-chromatographed using the same gel equilibrated with KSCN/KCl solution, when two peaks B-1 and B-2 were eluted (Fig. 2b). Fractions were first dialysed against 0.15 M-KCl, pH 8.2, then against phosphate-buffered saline and tested for the presence of K99 activity. The latter was found to be localized to peak B-1 which was pooled and concentrated by pervaporation. No resolution was achieved if phosphate buffer or phosphate buffered saline were used as eluting buffers. The total purification was increased to approximately fivefold (Table 1), although the pili preparation was still heterogeneous as judged by the detection of a minimum of two very faint contaminating bands on SDS-PAGE (Fig. 1b). Introduction of this step resulted in the removal of the heat-labile enterotoxin as reported previously (Clements & Finkelstein, 1979; Altmann *et al.*, 1982).

Ion-exchange chromatography

The preparation obtained by gel filtration on Bio-Gel A-5m was then subjected to ion-exchange chromatography on DEAE-Bio-Gel A equilibrated with 0.02 M-Tris/HCl, pH 8.6 containing 0.05 M-NaCl. On elution with the latter buffer, two peaks were eluted (Fig. 3a) of which only the first peak (peak C-1) possessed K99 activity. An additional peak (peak C-3) was eluted by 0.02 M-Tris/HCl, pH 8.6 containing 1.0 M-NaCl but it contained no K99 activity. Although peak C-1 contained a single major band on SDS-PAGE, a faint contaminating band with a molecular weight of approximately 40000 could be detected on overloading the gel. This

Fig. 3. Discontinuous elution profile showing ion-exchange chromatography of (a) the K99-rich fraction obtained by gel filtration (peak B-1, Fig. 2b) on DEAE-Bio-Gel A equilibrated with 0.02 M-Tris/HCl, pH 8.6 containing 0.05 M-NaCl. (b) Peak C-1 eluted in (a) on DEAE-Sephadex A-50 equilibrated with phosphate buffer. The second buffer in both instances consisted of the initial eluting buffer containing 1.0 M-NaCl.

contaminant was removed by chromatography on DEAE-Sephadex A-50, when pure K99 pili were eluted with the equilibrating buffer (Peak D-1; Fig. 3b).

K99 pili purified by this method yielded a single band on SDS-PAGE with a molecular weight of approximately 13000 (Fig. 1c). While no DNA, RNA, LPS or heat-labile enterotoxin were detectable in the purified K99 pili preparation, the amount of carbohydrates measured by the anthrone method was estimated to be $50 \mu\text{g (mg protein)}^{-1}$.

N-terminal amino acid analysis and amino acid composition

The *N*-terminal amino acid was shown to be blocked by the failure to detect an *N*-terminal dansylated amino acid. The amino acid composition of purified K99 pili was not significantly different from that reported previously (Altmann *et al.*, 1982).

Ultrastructural, immunochemical and biological properties

As reported previously (Altmann *et al.*, 1982), the average length of purified pili was 160 nm and the average diameter, 7.4 nm. Further, K99 pili purified by the method described in this investigation fulfilled all the criteria of purity described previously (Altmann *et al.*, 1982). Immunoelectrophoresis of crude KSCN extract yielded a single arc on development with specific anti-K99 and anti-KSCN extract antisera (Fig. 4) and the $S_{20,w}$ of purified K99 pili was determined to be 12.0.

DISCUSSION

Recently a new method for the extraction and purification of K99 pili was reported (Altmann *et al.*, 1982). Briefly, the method involved extraction of pili with 3 M-KSCN followed by $(\text{NH}_4)_2\text{SO}_4$ precipitation, Bio-Gel A-5m absorption, and preparative isoelectric focusing. This was an effective though expensive method and it was considered appropriate to develop a relatively inexpensive method for the purification of K99 pili. Initial attempts made to purify

Fig. 4. Immunoelectrophoresis of the crude K99 extract of enterotoxigenic *E. coli* (C1443) (wells 2 and 3) and purified K99 (wells 1 and 4). The antisera used for development were (a) antiserum raised against purified K99 and (b) antiserum against crude K99 extract.

K99 pili by the MgCl_2 precipitation method of Brinton (1965) were not successful. Since preparative isoelectric focusing had proved effective, the potential of chromatofocusing in achieving the same objective was evaluated. Again, no significant resolution was achieved. Treatment of crude pili preparations with various dissociating agents such as 6 M-guanidine HCl, 8 M-urea and SDS (Sequal grade) resulted in significant losses of K99 activity thus limiting their usefulness. It should be noted, however, that de Graaf *et al.* (1981) recently reported the isolation of pure pili using urea and sodium deoxycholate. The pili thus purified were 5 nm in diameter differing from the value (7.4 ± 0.6 nm) reported here and by other investigators (Isaacson, 1977; Altmann *et al.*, 1982) and suggesting that the deoxycholate-treated pili did not resume their native conformation. The molecular weight of K99 pili purified in this investigation was approximately 13000 which, while in agreement with that reported earlier (Altmann *et al.*, 1982), is 5000 to 6000 lower than that recently reported by de Graaf *et al.* (1981). The possibility that proteolysis occurred during the purification of K99 pili described here has not been ruled out. However, the possible existence of K99 antigenic subtypes or the existence of molecular heterogeneity as reported for colonization antigens I and II (Wevers *et al.*, 1980) is suggested by the finding that the *N*-terminal amino acid was blocked, whereas de Graaf *et al.* (1981) found *N*-terminal asparagine. It will be interesting to compare the immunochemical and biological properties of K99 pili purified according to the method of de Graaf *et al.* (1981) with those prepared according to the method reported here because the quaternary structure of K99 pili reported in this paper has apparently not undergone any gross conformational change.

In general, the ultrastructural, biological and physicochemical properties of K99 pili purified in this investigation were similar to those reported earlier (Altmann *et al.*, 1982). Three points that must be emphasized with regard to the method reported here are that (a) the enterotoxigenic *E. coli* must be washed extensively (at least three times) with phosphate-buffered saline until the absorbance of the supernatant obtained upon centrifugation is less than 0.05 at 280 nm, (b) the buffer used for molecular sieve chromatography sequentially on Bio-Gel A-5m must be TEAN (to facilitate removal of heat-labile enterotoxin activity) and KSCN/KCl and (c) the samples must be extensively dialysed against appropriate buffers before subjecting them to chromatography. The K99 purified in this investigation apparently represented a homogeneous product because (a) a single band was detected on SDS-PAGE, (b) a single boundary was observed on analytical ultracentrifugation and (c) immunoelectrophoresis of the crude KSCN extract yielded a single arc on development against K99 antiserum prepared by inoculation of sheep with K99 purified by the method described in this investigation. Although the results reported here represent data obtained in a typical experiment using enterotoxigenic *E. coli* C1443, similar results have been repeatedly obtained using the K99 reference strain, B41. It should be possible now to test the potential of purified pili as protective immunogens with a view to understanding their contribution in the pathogenesis of colibacillosis in lambs, calves and piglets.

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