INFECTION AND IMMUNITY, Sept. 1980, p. 877-883
0019-9567/81/090877-07$02.00/0

Vol. 33, No. 3

Purification, Characterization, and Partial Covalent Structure of *Escherichia coli* Adhesive Antigen K99

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Received 9 February 1981/Accepted 12 May 1981

The adhesive antigen K99 of enterotoxigenic *Escherichia coli* strains of calf origin was isolated and purified. The K99 fimbriae were removed from the cells by heat treatment, concentrated by precipitation with ammonium sulfate, and purified by gel filtration on Sepharose CL-4B and treatment with deoxycholate. The purified K99 antigen was composed of protein subunits with a molecular weight of 18,500 and had an isoelectric point of 9.5. The N-terminal amino acid sequence, as well as the composition of the C-terminal part of the K99 protein subunits, was determined.

One of the well-known causes of diarrhea is an infection with noninvasive enterotoxigenic *Escherichia coli* strains. The pathogenicity of these strains is mainly based on two virulence factors: (i) the ability to colonize the small intestine, and (ii) the production of enterotoxins which cause the actual diarrhea. The colonization of the small intestine by enteropathogenic *E. coli* strains, or of the urinary tract by uropathogenic strains, is mediated by cell surface antigens (adhesins) which enable the bacteria to adhere to the brush border of epithelial cells. These adhesive antigens, also called fimbriae or pili, are long, threadlike proteinaceous structures (0.5 to 3 μm in length) made up of 100 or more identical subunits. Recently the isolation of several of these fimbriae from enterotoxigenic strains of *E. coli* was described; the K88 antigen was isolated from porcine enterotoxigenic strains (18, 22), the K99 antigen was isolated from bovine and ovine enterotoxigenic strains (12, 20), the colonization factor antigens CFA/I and CFA/II were isolated from human strains (3–5, 14), and the 987P antigen was isolated from porcine strains (7). Several of these adhesion antigens have been characterized as to their molecular weight, chemical composition, and N- and C-terminal amino acid sequence. In this paper we describe the isolation and purification of the K99 antigen as part of a study of the structure-function relationship of fimbrial proteins. It was found that the purified K99 antigen differed from the preparation earlier described by Isaacson (12), both in molecular weight and amino acid composition. The N- and C-terminal amino acid sequence of the K99 protein subunits was not homologous with those of any of the other characterized adhesion antigens.

MATERIALS AND METHODS

Bacterial strains and media. The K99 antigen was isolated from two different K99-positive *E. coli* strains: F57 and 1474. Strain F57 is a nalidixic acid-resistant *E. coli* K-12 W3110 strain that harbors a K99 plasmid containing an insertion of transposon Tn*901* coding for ampicillin resistance. The K99 plasmid originated from the K99 reference strain, *E. coli* B41 (25). Strain 1474 is a K99-positive *E. coli* K-12 strain obtained from R. E. Isaacson.

Minca medium, a minimal medium, or Trypticase soy broth (TSB; BBL Microbiology Systems, Cockeysville, Md.) were used for the cultivation of the bacteria. Minca medium contained KH_2PO_4, 1.36 g; $Na_2HPO_4 \cdot 2H_2O$, 10.1 g; glucose, 1.0 g; Casamino Acids (Difco Laboratories, Detroit, Mich.), 1 g; and trace salts solution, 1 ml, as described by Guinée et al. (9) but supplemented with 1 g of yeast extract (Oxoid Ltd., London, England) per liter. Minimal medium contained KH_2PO_4, 3 g; $Na_2HPO_4 \cdot 2H_2O$, 7.5 g; NaCl, 0.5 g; NH_4Cl, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.12 g; $CaCl_2 \cdot 2H_2O$, 11 mg; $FeSO_4 \cdot 7H_2O$, 5 mg; and glucose, 5 g per liter. For the cultivation of strain F57, all media were supplemented with 100 μg of ampicillin per ml.

Buffers. The buffers used were phosphate buffer (50 mM, pH 7.2), phosphate-urea buffer (phosphate buffer containing 2 M urea from Merck & Co., Inc., Rahway, N.J.), and phosphate-deoxycholate (DOC) buffer (phosphate buffer [pH 7.5] containing 0.5% (wt/vol) sodium DOC from BDH Chemicals, Poole, England).

Preparation of ultrasonic extracts. For determination of the K99 antigen in culture samples, the cells were collected by centrifugation and suspended to a standard concentration of 5×10^8 cells per ml in phosphate buffer supplemented with 0.01% Tween 80

(Serva). Three milliliters of the suspension was then ultrasonicated for 5 min at 75W and 4°C (Branson Sonic Power Co., Stamford, Conn.; type B12, setting 4.5). Under these conditions, a maximal lysis of the cell suspension was obtained concomitant with a maximal value for the detectable amount of K99 antigen. The ultrasonic extracts were used directly for K99 determination by an enzyme-linked immunosorbent assay (ELISA).

Preparation of K99 antiserum. Antiserum against the K99 antigen was obtained from the National Institute for Public Health (Bilthoven, The Netherlands). The antiserum was prepared by immunization of rabbits with purified K99 antigen, prepared by preparative electrophoresis of an ultrasonic extract of *E. coli* strain H416[O101:K(A)?:NM] as described by Guinée et al. (9).

Determination of the K99 antigen. The amount of K99 antigen in ultrasonic extracts, culture supernatants, and isolated K99 preparations was determined by an ELISA exactly as described by Mooi et al. (19) for the determination of the K88 antigen, using disposable polystyrene microtiter trays (Cooke). Routinely, the trays were read by eye by two individuals. Purified K99 antigen, prepared as described in this paper, was used as a standard. The minimal concentration of K99 antigen which still showed a brownish-pink coloring in the ELISA was about 3 ng/ml.

Polyacrylamide gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis on 11% acrylamide slab gels and preparation of samples were done by the procedure described by Lugtenberg et al. (17).

Isoelectric focusing. Isoelectric focusing was performed in a 110-ml focusing column, using Ampholine, pH 3.5 to 10 (LKB Instruments, Inc., Rockville, Md.).

Amino acid analysis. Amino acid analysis was performed on a Durrum D-500 amino acid analyzer. Duplicate samples were hydrolyzed for 24, 48, and 72 h at 110°C in 6 N HCl containing 0.1% phenol. Furthermore, samples of performic acid-oxidized protein were hydrolyzed for 24 h to determine cysteine as cysteic acid. For serine and threonine, values extrapolated to zero time were used, whereas the values for 72-h hydrolysis were used for valine and isoleucine.

Determination of the N-terminal sequence. The primary structure of the N-terminal part of the K99 antigen was determined by manual Edman degradation as described by Klemm et al. (15). Identification of phenylthiohydantoin (PTH) amino acid derivatives was performed as described by Klemm (14). The primary structure of the N-terminal part of the K99 antigen was also determined by automatic Edman degradation on a Beckman 890C sequenator. The repetitive yield was 91%. PTH amino acid derivatives were identified by high-performance liquid chromatography as described by Frank and Strubert (6).

Carboxypeptidase Y digestion. Digestion of 20 nmol of K99 antigen with carboxypeptidase Y was performed as described by Gaastra et al. (8).

Electron microscopy of purified K99 antigen. A lyophilized preparation of the K99 antigen was dissolved in 0.1 M phosphate buffer (pH 7.3) in a concentration of 1 mg/ml. This solution was then diluted 10 times, and $MgCl_2$ was added to a final concentration of 10 mM. The preparation was sprayed on carbon-coated grids and stained with uranyl acetate (1% wt/vol). The micrographs were taken with a JEM 100B electron microscope operating at 80 kV.

Chemical analysis. Protein was determined by the biuret method (10), using bovine serum albumin as standard. Carbohydrate was determined by the phenol method (10), using glucose as standard.

RESULTS

Production of the K99 antigen. In a previous paper we stated that a minimal salt medium or a semisynthetic Minca medium was the most suitable medium to obtain a high production of the K99 antigen by wild-type *E. coli* strains of various serotype (2). In complex media, K99 production is strongly reduced, probably because of the presence of free alanine which was shown to repress the biosynthesis of the K99 antigen (1). Isolation of the K99 antigen from most *wild-type E. coli* strains is hampered, however, by the presence of excessive amounts of capsular antigens produced by these strains. The K99 antigen was found to be firmly associated with these capsular antigens, and the aggregates could only be dissociated by using strongly denaturating conditions (data not shown). Therefore, a K99-positive *E. coli* K-12 exconjugant, strain F57, was used for isolation and purification of the K99 antigen. This laboratory strain has a rough phenotype.

First, the extent of K99 production by strain F57 was determined after overnight growth in the minimal salt medium, in semisynthetic Minca medium, and in TSB. The amount of K99 antigen, determined in the ultrasonic extracts, was 1.5, 1.5, and 0.1 μg/ml, respectively, using purified K99 antigen as a standard.

Isolation of the K99 antigen. To isolate the K99 antigen from the cells, a culture of strain F57 was grown in minimal medium until the stationary growth phase. The cells were then collected by centrifugation, and the pellet was suspended in phosphate-urea buffer to an optical density of 100 measured at 660 nm. One half of this suspension was heated for 20 min at 60°C, the other half was homogenized for 20 min in a Sorvall Omni-Mixer (maximal setting). After these treatments, the suspensions were centrifuged for 15 min at 30,000 × *g*, and the amount of K99 antigen in supernatants and pellets was determined (Table 1). Heat treatment removed most of the antigen from the cells (about 90%), whereas treatment in the Omni-Mixer detached about 50% of the antigen. A prolonged shearing or a repeated treatment in the Omni-Mixer did not improve this result considerably. The urea present in the extraction buffer could be replaced by 1 M NaCl, but extraction with NaCl

TABLE 1. *Extraction of the K99 antigen from whole cells*[a]

Procedure	K99 antigen concn (μg/ml)	
	Supernatant	Pellet
Heat extract	1.8	0.2
Shearing	0.9	0.9

[a] After extraction, the supernatants and the pellets were diluted or suspended in phosphate-urea buffer to the volume of the initial suspension. Samples of these supernatants or cell suspensions were then further diluted 100-fold in phosphate buffer. Cell suspensions were ultrasonicated, and the amount of K99 antigen was determined by the ELISA.

was shown to be a little less efficient and reproducible. Furthermore, a part of the cells suspended in phosphate-NaCl buffer was lysed during both extraction procedures, whereas phosphate-urea buffer did not cause detectable cell breakage.

The purity of the isolated antigen preparations was investigated by analysis of the supernatant fluids on SDS-polyacrylamide gels (Fig. 1). Both extracts contained a prominent protein band with a molecular weight of 18,500 in the lower half of the gel, as well as various other polypeptides. The number of other polypeptides was smaller in the heat extract than in the extract obtained by shearing.

Purification of the K99 antigen. An overnight culture of strain F57 was inoculated into 6 liters of minimal medium and cultivated in a Microferm fermentor (New Brunswick Scientific Co., New Brunswick, N.J.) at 37°C under vigorous aeration. The culture was harvested in the late logarithmic growth phase (optical density of 1.6 at 660 nm), and the cells were extracted by heat treatment in phosphate-urea buffer as described above. The K99 antigen was then precipitated from the heat extract by adding ammonium sulfate (60% saturation) and stirring for 2 h at 4°C. The precipitate was collected by centrifugation, suspended in 4 ml of phosphate-urea buffer, and dialyzed against the same buffer for 16 h at 4°C.

For purification, the concentrated K99 preparation was fractionated on a Sepharose CL-4B column equilibrated in phosphate-urea buffer. As shown in Fig. 2, the extract eluted from the column in two fractions. Only the first fraction (tube 11-21) gave a positive reaction in the ELISA. Analysis of this fraction on an SDS-polyacrylamide gel (inset of Fig. 2) showed that the fraction was heterogeneous, containing only one major polypeptide and several minor polypeptides.

Several attempts were made to separate the major polypeptide from the other proteins by ion-exchange chromatography. To that purpose, the material eluted as the first fraction from the Sepharose CL-4B column was dialyzed against distilled water, lyophilized, and then applied to either diethylaminoethyl or carboxymethyl Sephadex columns, using different buffers with variable ionic strength. All these attempts, however, had no success. Complete purification of the major polypeptide present in the crude preparation was, however, achieved by dissolving the lyophilized material in an excess of SDS followed by three cycles of gel filtration on Sephacryl S200 in the presence of SDS (Fig. 1). The volume in which the K99 eluted from this column indicated that the K99 fimbriae were disaggregated into their subunits. These purified subunits showed a rather weak reaction with K99 antiserum. Complete purification of native K99 antigen was obtained by the treatment of crude K99 preparations with sodium DOC, as recently described by Korhonen et al. (16). To that purpose, the K99-containing fraction eluted from the Sepharose CL-4B column was dialyzed against phosphate buffer and then supplemented with DOC to a final concentration of 0.5% (wt/vol). This suspension was dialyzed for 72 h against phosphate-DOC buffer, and the DOC-insoluble material was then removed by centrifugation. The supernatant was dialyzed for

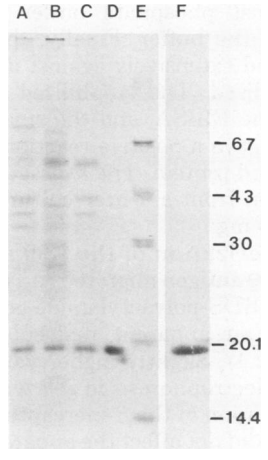

FIG. 1. *SDS-polyacrylamide gel electrophoresis of K99 preparations. (A) The heat-extract; (B) the extract obtained by shearing; (C) the K99 preparation after gel filtration on Sepharose CL-4B; (D) purified K99 antigen; (E) reference proteins; (F) purified K99 subunits obtained by gel filtration in the presence of SDS. The apparent moleular weight of the marker proteins is indicated on the right. Equivalent amounts of protein were applied to each slot.*

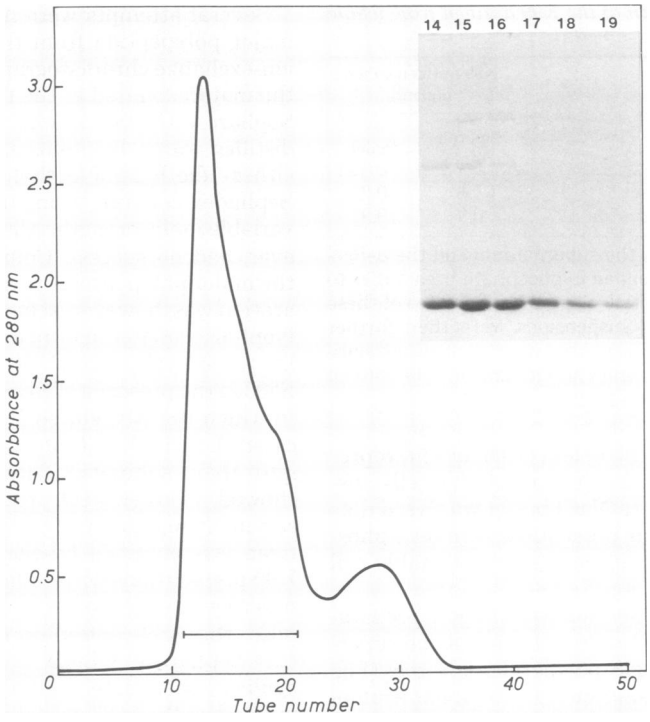

FIG. 2. *Fractionation of a crude K99 antigen preparation on a Sepharose CL-4B column (1.5 by 90 cm) equilibrated in phosphate-urea buffer. After application of the concentrated sample (4 ml), the column was eluted with the same buffer (v = 10 ml/h). The bar indicates the fraction that gave a positive reaction in the ELISA. The inset shows an analysis of this fraction on an SDS-polyacrylamide gel. The numbers refer to the tube numbers.*

48 h against phosphate buffer with several changes of the buffer. Finally, the suspension was dialyzed extensively against distilled water and lyophilized. The lyophilized material was tested in the ELISA, and the smallest amount which still gave a positive reaction was about 3 ng of purified protein. The average yield of pure K99 antigen from a 1-liter culture (about 10^{12} cells) was 3 mg.

Characterization of the K99 antigen. The purified K99 antigen migrated as a single protein band upon SDS-polyacrylamide gel electrophoresis with an apparent molecular weight of 18,500 (Fig. 1). Slightly higher values were obtained by electrophoresis in gels with 15% acrylamide. Omission of the β-mercaptoethanol from the system did not affect the apparent molecular weight.

Isoelectric focusing of the purified antigen revealed only one fraction with an isoelectric point of 9.5.

Chemical analysis of the K99 antigen on samples containing 2 mg of K99 antigen per ml of phosphate buffer showed 97% of protein and about 2% of carbohydrates.

The amino acid composition of the purified antigen is given in Table 2 and is compared with the composition of a K99 preparation isolated by Isaacson (12). As can be seen from this table, there is little similarity between the amino acid composition of both preparations. Furthermore, the analysis demonstrated a preponderance of amino acids with apolar side chains.

The N-terminal sequence of the first 22 residues of the K99 antigen subunit is shown in Fig. 3 and compared with the N-terminal sequence of other fimbrial proteins isolated from *E. coli*. As can be seen, there was no homology between the N-terminal amino acid sequence of the K99 antigen subunit and the other fimbrial proteins. The composition of the C-terminal part of the K99 protein, determined after digestion with carboxypeptidase Y, also has no resemblance to the C-terminal sequences as known from other fimbrial proteins (Fig. 4). In the electron micrograph of a lyophilized K99 antigen preparation (Fig. 5), the K99 antigen can be seen as fimbria-like threads that seem to have a helix structure.

DISCUSSION

In our studies of the production of the K99 antigen by *E. coli* strains of different serotypes,

	1	5	10	15	20
K99	Asn-Thr-Gly-Thr-Ile-Asn-Phe-Asn-Gly-Lys-Ile-Glu-Thr-Ala-Thr-Ser-X-Ile-Glu-Pro-Ala-Val-				
K88	Trp-Met-Thr-Gly-Asp-Phe-Asn-Gly-Ser-Val-Asp-Ile-Gly-Gly-Ser-Ile-Thr-Ala-Asp-Gly-Tyr-Gly-				
CFA/I	Val-Glu-Lys-Asn-Ile-Thr-Val-Thr-Ala-Ser-Val-Asp-Pro-Val-Ile-Asp-Leu-Leu-Gln-Ala-Asp-Gly-				
Type 1	Ala-Ala-Thr-Thr-Val-Asn-Gly-Gly-Thr-Val-His-Phe-Lys-Gly-Glu-Val-Val-Asn-Ala-Ala-				

FIG. 3. *The N-terminal amino acid sequence of the K99 protein subunit. The N-terminal sequences of other E. coli fimbrial proteins are given for comparison (8, 11, 14).*

TABLE 2. *Amino acid composition of the K99 protein subunit*

Amino acid	No. of residues	
	This report[a]	Isaacson[b]
Asp	23.2	25.5
Thr	18.9	20.2
Ser	14.6	11.8
Glu	9.9	7.2
Pro	4.7	2.6
Gly	18.9	15.2
Ala	18.0	46.4
Cys	3.4	2.0
Val	8.0	13.6
Met	3.1	3.3
Ile	8.7	13.1
Leu	9.4	7.5
Tyr	7.3	—[c]
Phe	8.6	—
His	3.2	3.4
Lys	8.9	9.4
Arg	5.7	4.2
Trp	ND[d]	2.9
Hydroxylysine	—	2.8

[a] The number of residues was calculated assuming a molecular weight of 18,500 for the K99 protein subunits.

[b] Values reported by Isaacson (12).

[c] —, None.

[d] ND, Not determined.

grown under various conditions, we used an ELISA as a semiquantitative method to measure the extent of K99 production (2). During these studies, the necessity to standardize and quantitate the ELISA became apparent. Therefore, we started to isolate a pure K99 preparation to be used as a standard. Following the isolation procedure described by Isaacson (12), we were unable to obtain a pure preparation. Meanwhile, the need to obtain a pure K99 preparation became the more urgent when the identification of the K99 antigen was obscured by a publication of Morris et al. asserting that the K99 antigen isolated from the wild-type *E. coli* reference strain B41 was composed of both an anionic and a cationic component (22).

The isolation and purification of the K99 antigen turned out to be more difficult than the procedure we described for the isolation of a pure K88 antigen (18). One of the main problems encountered during the isolation of the K99

K99	-(Ser,Val)-(Phe,Thr)-(Leu,Met,Tyr)-COOH
K88	-Asn-Val-Ala-Ile-Thr-Tyr-Tyr-COOH
CFA/I	-Met-(Val,Thr,Gly)-Ser-Leu-COOH

FIG. 4. *The amino acid composition of the C-terminal part of the K99 protein subunit. The C-terminal structures of other E. coli fimbrial proteins are given for comparison (8, 14).*

antigen was the interaction of the hydrophobic K99 fimbriae with other components from the surface of *E. coli*, regardless of whether they were proteins or polysaccharides. This interaction invariably appeared to be rather strong and could only be disturbed by drastic methods, such as incubation with SDS. Since we wanted to preserve the full antigenic activity of the native K99 antigen, the use of strong detergents was not a suitable way to purify the antigen although a pure preparation of K99 protein subunits could be obtained. Incubation of a crude preparation of K99 antigen with DOC turned out to be the best and mildest way to obtain a pure and native K99 antigen. The application of DOC for the isolation of pure pili has recently been described by Korhonen et al. (16).

Analysis of the purified K99 antigen on SDS-polyacrylamide gels showed that the antigen is composed of subunits with an apparent molecular weight of 18,500. This result is different from the data published by Isaacson, who reported that the antigen was composed of two different subunits: a major component with a molecular weight of 22,500 and a minor component of 29,500 (12). Probably, the minor component represents another K99 plasmid-specific protein that copurifies with the K99 antigen in the procedure used by Isaacson. The difference in the molecular weight of the actual K99 subunits (22,500 versus 18,500) could not be explained. Variation in the percentage of acrylamide or in buffer systems caused only a minor deviation in the apparent molecular weight of 18,500. Therefore, we decided to isolate the K99 antigen from *E. coli* strain 1474, used by Isaacson, but according to the procedure described in this paper. It turned out that the K99 antigen isolated from strain 1474 was composed of subunits with molecular weights similar to those of the K99 isolated from strain F57, and the preparation contained no impurities. Furthermore, a

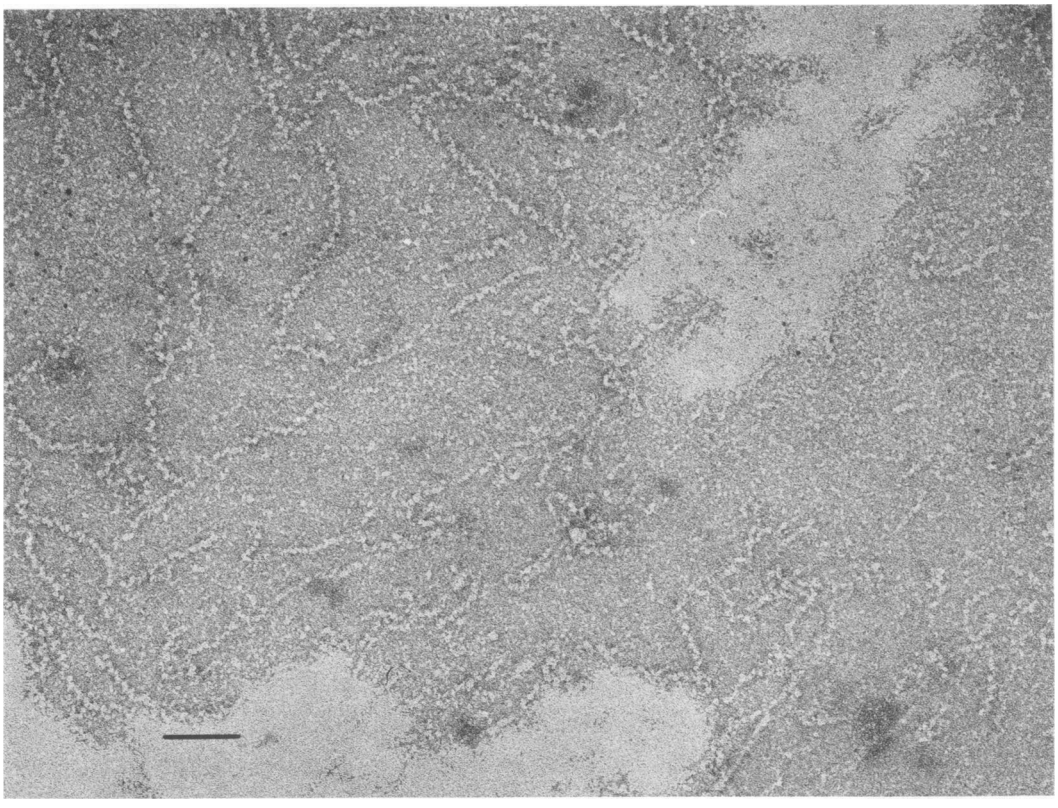

FIG. 5. *Electron micrograph of purified K99 fimbriae. The bar represents 46.5 nm. The fimbriae are approximately 5 nm in diameter and seem to have a helix structure.*

molecular weight of 18,500 is in good agreement with the most probable molecular weight calculated on the basis of the data of the amino acid analysis (18,400).

Isoelectric focusing of the pure K99 antigen revealed an isoelectric point of 9.5. This result is in good agreement with the isoelectric point of about 10, previously determined by Isaacson (12) but quite different from that of 4.2. reported by Morris et al. (21) and determined with cell-free K99 antigen isolated from *E. coli* B41. The explanation for a difference in the isoelectric point of the K99 antigen as reported in the literature (12, 13, 21, 22) was recently given by Morris et al. (23). *E. coli* strains from the O9 and O101 serogroups, including reference strain B41, were shown to possess two adhesive antigens. The cationic antigen (isoelectric point of about 10) was identified as K99; the second antigen (isoelectric point of 4.2) is a hitherto unknown adhesive antigen. Comparison of the N-terminal and C-terminal sequence of the K99 subunit with those of other fimbrial proteins of enterotoxigenic *E. coli* strains showed that, apart from residues 7 through 10 of K99 and residues 6 through 9 of K88, there is probably no homology between the primary structures of the fimbrial proteins characterized so far. It is therefore not yet possible to assign a particular function, such as subunit-subunit interaction or adhesion to epithelial cells, to a certain part of the antigen subunit. It should be noted that the K99 subunit, in contrast to other fimbrial proteins (4, 14, 18, 24) contains cysteine. However, since we did not obtain a different molecular weight for the K99 subunit upon SDS-polyacrylamide gel electrophoresis with and without β-mercaptoethanol, the possibility of intersubunit S-S bridges can be ruled out.

ACKNOWLEDGMENTS

We gratefully acknowledge the assistance of Pia Klaasen-Boor, Jelle Thole, and Peter Molenaar. We are indebted to P. A. M. Guinée and W. H. Jansen for supplying the antisera used in this study and to Wilma Bergsma-Schutter and Jan van Breemen of the Biochemical Laboratory of the Rijksuniversiteit in Groningen for the electron micrographs of the purified K99 antigen.

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