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Purification and Analysis of Colonization Factor Antigen I, Coli Surface Antigen 1, and Coli Surface Antigen 3 Fimbriae from Enterotoxigenic *Escherichia coli*

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Enterotoxigenic *Escherichia coli* fimbriae are immunogenic and play a key role in intestinal colonization. Native colonization factor antigen I, coli surface antigen 1, and coli surface antigen 3 fimbriae were purified by a common method involving shearing, differential centrifugation, gel filtration, and density gradient ultracentrifugation. The compositions and N-terminal sequences were determined. Coli surface antigen 3 possesses two N-terminal isoforms, one of which matches the published DNA sequence, except for the previously proposed signal sequence cleavage point.

To overcome the peristaltic defense mechanism, many enterotoxigenic *Escherichia coli* (ETEC) strains utilize fimbriae to adhere to the gut of infected animals and humans (5). Antifimbrial antibody can confer immunity to infection, so purification and characterization of ETEC fimbriae have facilitated the formulation of advanced vaccines (6, 11). ETEC having colonization factor antigen II (CFA/II) express coli surface antigens CS1, CS2, and CS3 in permutations which correlate with the serotype and biotype of the *E. coli* host (3, 15, 17). In this paper we report the results of biochemical studies on colonization factor antigen I (CFA/I), CS1, and CS3.

Fimbriae were purified from the following ETEC strains: H10407 (O78:H11; heat-labile and heat-stable enterotoxin positive; a source of CFA/I) (12), 60R75 (a mutant with a $CS1^+$ $CS3^-$ phenotype), and E9034A (O8:H11; a source of CS3) (12). The last two strains were kindly provided by B. Rowe, Division of Enteric Pathogens, Central Public Health Laboratory, London, England.

Bacteria were grown in aluminum pans (28 by 47 by 2 cm, for a total area of 1,316 cm^2) containing 1 liter of colonization factor antigen agar (1% Casamino Acids, 0.15% yeast extract, 0.005% $MgSO_4$, 0.0005% $MnCl_2$ 2% agar [pH 7.4]) overnight at 37°C as described elsewhere (4, 18). Bacterial cells were harvested, suspended in 20 ml of phosphate-buffered saline at pH 7.2, and subjected to shearing on ice for 90 s in an Omnimixer (Ivan Sorvall, Inc., Norwalk, Conn.) at setting 5. The sheared homogenate was clarified by centrifugation at 8,000 × *g* for 30 min and then at 39,000 × *g* for 90 min. The supernatant was centrifuged at 190,000 × *g* for 1.5 h in an L8-80 ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.) to pellet fimbriae, which were then suspended in 0.1 M Tris–0.2 mM EDTA.

The gel filtration step utilized the large sizes of native CFA/I, CS1, and CS3 to produce improved yields of nondenatured fimbriae. A column (length, 30 cm; internal diameter, 2.5 cm; Spectrum Bio-Instruments, Inc., Los Angeles, Calif.) was packed with Bio-Gel A 1.5 M (Bio-Rad Laboratories, Richmond, Calif.) to a height of 25 cm and equilibrated in phosphate-buffered saline–0.05% NaN_3. Samples (of 7-ml volume) were loaded and run at 45 ml/h, and the void volume (45 ml) was collected in 3-ml fractions, assayed for protein (13), analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10, 14, 16, 18), and assayed for fimbriae by electron microscopy (12, 18). Fractions containing fimbriae were mixed with cesium chloride (final density, 1.3273 g/cm^3) and sodium *N*-lauryl sarcosine (final concentration, 1.32%), loaded into ultracentrifuge tubes (14 by 89 mm; 13.2 ml per tube) in an SW41 Ti swinging-bucket rotor, and centrifuged in a Beckman L8-80 ultracentrifuge for 44 h at 177,000 × *g*. Protein bands were collected by tube puncture, dialyzed, and assayed for fimbriae by SDS-PAGE and electron microscopy. Fimbrial filaments were separated from subunits by ultracentrifugation. High-yield batches produced approximately 500 and 250 μg of CS1 and CS3, respectively, from each 1,316-cm^2 pan, a notable increase over the 150 μg obtained previously (12). Although depolymerized fimbriae can reassemble, it may be advantageous to purify nondenatured filaments to ensure that the antigenic structure remains unaffected by purification. This is particularly important since with some fimbriae the actual adhesin subunit is distinct from the structural subunit. Furthermore, the method described above standardizes the purification of native CFA/I, CS1, and CS3.

Compositional analysis (Table 1) was done by the Waters PICO-Tag method (2), and amino-terminal protein sequencing (Table 2) was carried out on a model 477A protein sequencer (Applied Biosystems) with a model 120A analyzer in accordance with manufacturer protocols.

Analysis of CFA/I. CFA/I migrated in SDS-PAGE with a relative molecular mass of 15,000, in agreement with sequencing and other reports (7, 18). The sequence and composition information were compatible with previously published data (8).

Analysis of CS1. Purified CS1 migrated in SDS-PAGE with an apparent relative molecular mass of 16,800 (Fig. 1). The amino acid content of CS1 from strain 60R75 (Table 1) was 44% hydrophobic. Amino-terminal sequence analysis of CS1 (Table 2) revealed a high degree of similarity with CFA/I.

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TABLE 1. Amino acid composition of ETEC fimbriae

Amino acid	No. (%) of residues/mol in:			
	CS1	CS3[a]	CS2[b]	CFA/I
Alanine	14 (8.2)	13 (9.0)	16 (9.7)	19 (12.9)
Arginine	1 (0.6)	1 (0.7)	4 (2.4)	1 (0.7)
Asparate or asparagine	23 (13.5)	23 (15.9)	19 (11.5)	12 (8.2)
Glutamate or glutamine	9 (5.3)	6 (4.1)	17 (10.3)	11 (7.5)
Glycine	11 (6.4)	8 (5.5)	13 (7.9)	10 (6.7)
Cysteine	0 (0)	0 (0)	0 (0)	0 (0)
Histidine	3 (1.8)	2 (1.4)	2 (1.2)	1 (0.7)
Isoleucine	9 (5.3)	9 (6.2)	10 (6.1)	5 (3.4)
Leucine	17 (9.9)	15 (10.3)	13 (7.9)	12 (8.2)
Lysine[c]	9 (5.3)	6 (4.1)	10 (6.1)	8 (5.4)
Methionine	1 (0.6)	0 (0)	3 (1.8)	3 (2.0)
Phenylalanine	3 (1.8)	3 (2.1)	4 (2.4)	2 (1.3)
Proline	9 (5.3)	5 (3.4)	7 (4.2)	7 (4.8)
Serine	20 (11.7)	16 (11.0)	13 (7.9)	17 (11.6)
Threonine	19 (11.1)	27 (18.6)	18 (10.9)	15 (10.2)
Tryptophan		1 (0.7)		1 (0.7)
Tyrosine	3 (1.8)	1 (0.7)	3 (1.8)	4 (2.7)
Valine	20 (11.7)	9 (6.2)	12 (7.3)	19 (12.9)
Total	171	145	164	147
Relative molecular mass	16,800	15,300	17,400	15,000
Hydrophobicity (%)[d]	44.6	37.9	41.2	48.2

[a] Calculated from the N terminal (Table 2) and DNA (1) sequences.
[b] Data are from reference 8.
[c] Includes hydroxylysine.
[d] Percentages of Pro, Ala, Val, Met, Ile, Leu, Tyr, and Phe.

Among the first 19 N-terminal residues (approximately 11% of the primary sequence) were five positions (4, 5, 6, 14, and 15) which differed between CFA/I and CS1. The difference at position 4 (Asn in CFA/I and Thr in CS1) is a conservative change of two polar but uncharged residues. At position 5 the substitution of Ile (in CFA/I) for Glu (in CS1) represents a change from a strongly hydrophobic residue (Ile) to Glu, a negatively charged, acidic amino acid. The substitution of Thr (in CFA/I) for Ser (in CS1) at position 6 is a conservative one. At position 14 polar, uncharged Thr (in CS1) is substituted for the structurally similar but hydrophobic Val (in CFA/I), and at position 15 both Ile and Val have similar structures and hydropathy. The amino acid composition of CS1 showed that the number and proportion of hydrophobic amino acids were similar to those in CFA/I.

Analysis of CS3. Purified CS3 migrated as a pair of bands with relative molecular masses of 14,500 and 15,500, as previously noted (12). As with other ETEC fimbriae, the amino acid compositions of CS1 and CS3 showed a high degree of hydrophobicity. The CS3 N terminus was not homologous with those of other ETEC fimbriae. Sequence analysis (23 cycles) revealed 16 positions in which a single type of amino acid residue was detected and 7 positions in which two amino acid residues were detected. Had the preparation contained a mixture of two totally unrelated proteins, two amino acids would have been detected at most or all positions. The two apparent isoforms detected by N-terminal sequencing were present in approximately equimolar amounts. Unlike CS1 and CFA/I, purified CS3 did not migrate as a tight band in SDS-PAGE. The presence of two overlapping isoforms which do not exactly comigrate may explain this result. We are currently purifying these species to determine the significance of the second component. Position 8 of the second isoform (CS3a) may possess the only Met residue, resulting in <1 Met per mole being detected in compositional analysis (Table 1).

TABLE 2. N-terminal sequences of ETEC fimbriae

Fimbriae	Sequence
CFA/I	VEKNITVTASVDPVIDLLQ
CS2[a]	AEKNITVTASVDPVIDLLQ
CS1	VEKTESVTASVDPTVDLLQ
CS3	AAGPTLTKELALNVLSPAALDAT
CS3a	AVGPTKDMSLGANVLSPAALDAT

[a] As reported by Klemm et al. (9).

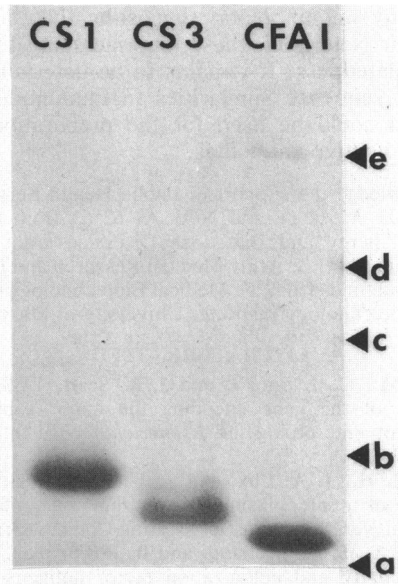

FIG. 1. Coomassie blue R250-stained SDS-polyacrylamide gel (12.5% [wt/vol] total acrylamide) of fimbriae purified by differential centrifugation, exclusion chromatography, and isopycnic gradient centrifugation. The relative molecular masses of CS1 (16,800), CS3 (14,500 and 15,500), and CFA/I (15,000) concur with previous reports (7, 12, 18). The relative molecular mass markers used were SDS-7 (Sigma Chemical Co., St. Louis, Mo.). Markers were 14,200 (a), 20,100 (b), 24,000 (c), 29,000 (d), and 36,000 (e).

The N-terminal sequence of one CS3 isoform (CS3 in Table 2) corresponds exactly to the recently reported DNA sequence (1), and the consensus sequence is shown in Table 2 as the CS3 N terminus. Our results show that the mature processed polypeptide amino terminus corresponds to the Ala residue at position 23 of the precursor polypeptide and confirm the sequence of the subsequent 22 residues of the mature protein. Although Boylan et al. (1) suggested an alternative processing site, the characteristics of processing sites described by von Heijne (19) are associated with the cleavage site indicated by our data. We therefore conclude that CS3 is processed by cleavage between amino acid residues 22 and 23 after the ATG initiation codon.

CS1, CS2, and CFA/I fimbriae possess similar morphologies and filament diameters (6 to 7 nm) as well as N termini. Polyclonal rabbit antisera raised to CFA/I and CS1 do not cross-react in denaturing Western blots (immunoblots) or

native colony immunoblots, suggesting that, although related in their N termini, these fimbriae share little, if any, antigenic relatedness. It remains to be determined if there are peptide sequence similarities in immunodominant regions which could be used for the preparation of cross-reactive, protective antibodies.

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