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PURIFICATION AND PARTIAL CHARACTERIZATION OF A HEMAGGLUTINATING FACTOR (HAF): A POSSIBLE ADHESIVE FACTOR OF THE DIFFUSE ADHERENT OF *Escherichia coli* (DAEC)

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SUMMARY

The mannose-resistant hemagglutinating factor (HAF) was extracted and purified from a diffuse adherent *Escherichia coli* (DAEC) strain belonging to the classic enteropathogenic *E. coli* (EPEC) serotype (0128). The molecular weight of HAF was estimated to be 18 KDa by SDS-PAGE and 66 KDa by Sephadex G100, suggesting that the native form of HAF consists of 3-4 monomeric HAF. Gold immunolabeling with specific HAF antiserum revealed that the HAF is not a rigid structure like fimbriae on the bacterial surface. The immunofluorescence test using purified HAF on HeLa cells, in addition to the fact that the HAF is distributed among serotypes of EPEC, suggests that HAF is a possible adhesive factor of DAEC strains.

KEYWORDS: *Escherichia coli*; Diffuse adherence; Hemagglutinating factor (HAF); adhesive factor.

INTRODUCTION

Currently, *Escherichia coli* strains that cause gastrointestinal infections in humans are classified on the basis of enteropathogenic mechanisms into at least five categories: enterotoxigenic (ETEC); enteropathogenic (EPEC); enteroinvasive (EIEC); enterohemorrhagenic (EHEC) and enteroadherent or enteroaggregative (EAEC).

Among these *E. coli* strains, EPEC were the first discovered, and although they continue to be a major cause of infant gastroenteritis in many parts of the developing world[4,10], their pathogenic mechanisms have yet to be established. Several authors[1,13,15] have reported that the EPEC show at least two distinct types of adherence pattern to HeLa and HEp-2 cells: one, diffuse adherence (DA), and the other, localized adherence (LA). The properties of *E. coli* strains to adhere in clusters to a localized area of the cell (LA) are highly correlated with enteropathogenesis of EPEC infection[2,15,17].

Thus, DONNENBERG & KAPER[4] proposed the separation of *E. coli* that exhibits diffuse adherence (DA) from the EPEC group, despite belonging to the classic EPEC serotypes.

BILGE et al.[3] characterized a fimbrial adhesin, F1845, that mediates diffuse adherence (DA) of *E. coli* (DAEC) strain C1845 (075:NM), isolated from children with diarrhea.

Recently, YAMAMOTO et al.[18] described a novel hemagglutinin from diarrhea-associated human *Escheri-*

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chia coli (serotypes 073:H33 and 089:H–) that adhered diffusely to HeLa and HEp-2 cells, mediated by 57 KDa outer membrane protein.

In previous experiments[19], we have observed some strains of DAEC belonging to the classic EPEC serotypes (0128) that showed hemagglutinating activity only with human erythrocytes, in the presence of D-mannose. Now, in this study we report the purification and partial characterization of the hemagglutinating factor (HAF) of DAEC strain, and suggestive evidence that HAF is an adhesive factor.

MATERIALS AND METHODS

Bacterial strain

A diffuse adherent *E. coli* (DAEC) (serotype 0128:H$^+$) strain showing mannose-resistant hemagglutinating (MRHA) activity with human erythrocytes[19] was used for this study.

Mannose-resistant microhemagglutination (MRMH) test

MRMH was carried out following the method described by PARRY & PORTER[14]. Briefly, cell-free antigens, diluted in 0.01 M sodium phosphate buffered saline (PBS) pH 7.4 containing 1% D-mannose, were incubated with an equal volume of a 1% suspension of either human, bovine, guinea-pig, horse, chicken or sheep red blood cells (RBC). After 2 hrs at 4°C the reading was performed. Cell-free material that showed MRMH was named as hemagglutinating factor (HAF).

Extraction and purification of hemagglutinating factor (HAF)

The *E. coli* strain was cultivated on CFA agar[6] at 37°C for 18 h. The bacterial cells were then harvested and suspended in 50 mM sodium phosphate buffer (pH 7.4) containing 1 M NaCl. The bacterial cell suspension was then sheared for 5 min on ice by a Sorvall Omnixer. Cell debris was pelleted by centrifugation at 8,000 g for 15 min. The supernatant material precipitated with ammonium sulphate (80% saturation) at 4°C was then dissolved in PBS and applied to a Phenyl-Sepharose column, equilibrated with 2 M ammonium sulphate [$(NH_4)_2 SO_4$] in 0.01 M sodium phosphate buffer (PB). The column was eluted initially with PB containing 2 M ammonium sulphate followed by a decreasing linear gradient of ammonium sulphate (2 M to 0 M) in the same buffer.

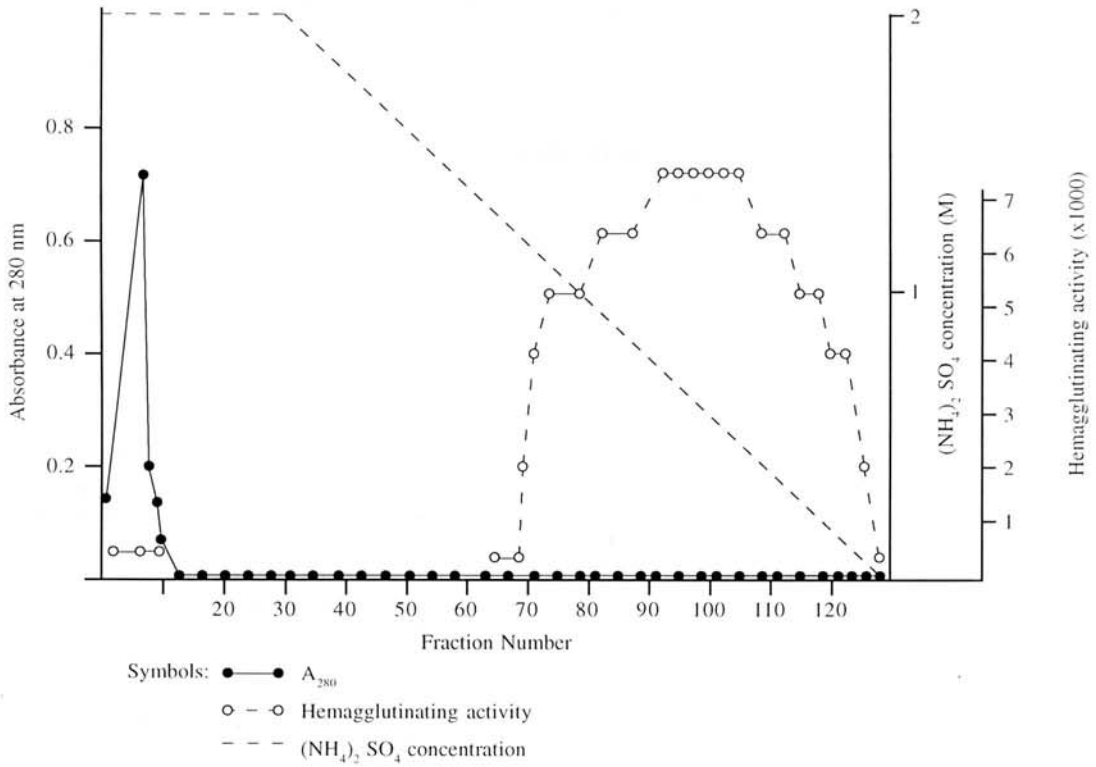


Fig. 1 – Phenyl-Sepharose CL-4B Chromatography of Hemagglutinating Factor (HAF) of DAEC.

Fractions containing HAF identified by the MRMH test with human erythrocytes were collected and concentrated by ultrafiltration (Amicon, USA) using diaflo membrane PM 10. The material thus prepared was applied to a Sepharose CL-4B column equilibrated with 4 M urea in phosphate buffer. The fractions with HAF activity were collected and concentrated, and then fractionated by HPLC (Pharmacia), using a Phenyl-Superose column and a decreasing linear gradient. Protein concentrations were determined by the method of HARTREE[11].

Poliacrylamide gel electrophoresis (PAGE)

SDS-PAGE was performed essentially as described by LAEMMLI[12] using 7% acrylamide. Electrophoresis was performed at a constant current of 25 mA for 3 h.

Sephadex G 100 gel filtration

The molecular weight of the purified HAF was determined by Sephadex G100 gel filtration, using the following standards: albumin (68,000 Da), ovalbumin (45,000 Da), chymotrypsinogen A (25,000 Da) and cytochrome C (12,500 Da) (Sigma).

Anti-HAF antiserum preparation

For the preparation of HAF antiserum, aliquots (1 ml) of purified HAF (100 µg/ml/animal) emulsified in an equal volume of Freund's complete adjuvant (Difco Lab.) were injected subcutaneously into rabbits. A similar booster was given 4 weeks later and animals were bled after further 15 days.

Electron microscopy

Immunolabeling with colloid-gold-labeled goat anti-rabbit antiserum was performed[17]. Briefly, 50 µl of a washed suspension of the *E. coli* strain in PBS containing 0.5% BSA were placed on carbon-coated grids. Excess liquid was removed and each grid was placed face down on specific antiserum diluted to 1/50 in PBS with 0.1% BSA, for 1 hr. After being washed in 3 sucessive drops of PBS the grids were each placed on 50 µl of 10 nm-gold-labeled goat anti-rabbit serum (Auro Probe EM, Jansen Pharm. Piscataway, N.J.) for 30 min. Each grid was washed in 3 drops of distilled water and stained with phosphotungstic acid (PTA). The material was examined under a Zeiss transmission electron microscope operated at 80 KV.

RESULTS

The cell-free HAF preparation was chromatographed on a Phenyl-Sepharose CL-4B column (Figure 1). The HAF was subsequently chromatographed on a Sepharose 4 B column, equilibrated with 4 M urea in phosphate buffer (PB). The hemagglutinating activity with human erythrocytes was eluted in the 2nd peak (Figure 2). Purified HAF was obtained by HPLC with a Phenyl-Superose column, as the final step of purification (Figure 3).

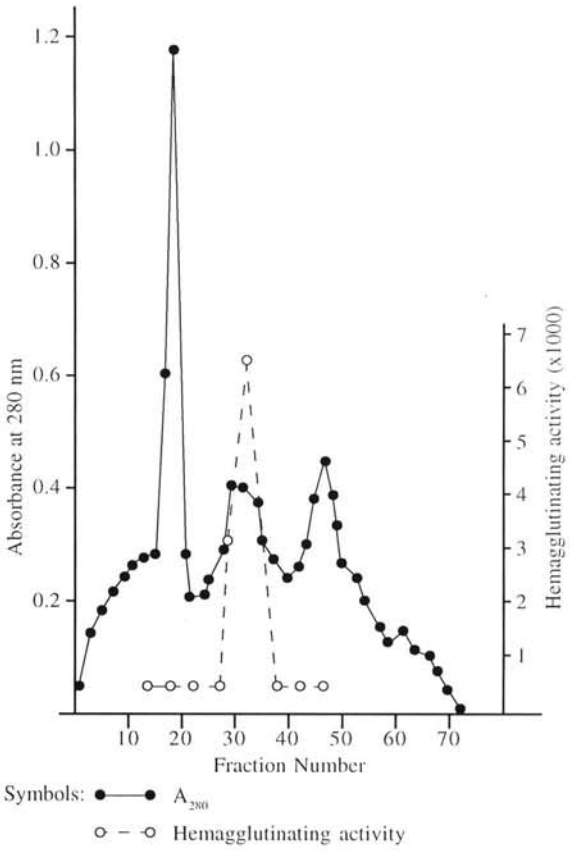


Fig. 2 – Sepharose 4 B Chromatography of Hemagglutinating Factor (HAF) of DAEC.

The HAF prepared as described above was subjected to SDS-PAGE. As shown in Figure 4, only one protein band was obtained, suggesting that the HAF was highly purified. The molecular weight of HAF was estimated to be 18,000 Da. On the other hand, by Sephadex G100 gel filtration the molecular weight of HAF was determinated to be about 66,000 Da (Figure 5).

Electron micrographs of immunolabeling carried out using specific HAF antiserum (Figure 6A) revealed that the HAF is distributed on the bacterial surface. As shown in Figure 6B, the same antiserum did not reveal HAF antigen on EPEC (055), which exhibited a localized adherence pattern.

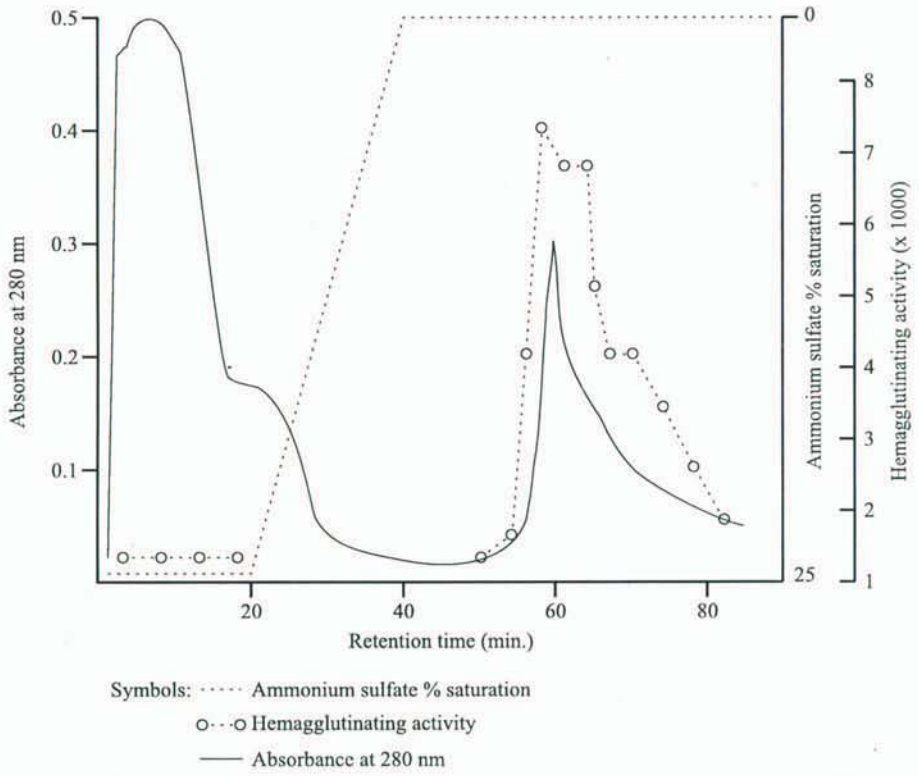


Fig. 3 – Elution profile of Hemagglutinating Factor (HAF) of DAEC on HPLC on a Phenyl-Superose column.

DISCUSSION

A mannose-resistant hemagglutinating factor (HAF) was purified from a strain of *Escherichia coli* belonging to the classical EPEC serotype (0128:H$^+$) which shows the characteristic of diffuse adherence (DA) to HeLa cells. The molecular weight of the HAF subunit as analysed by SDS-PAGE was estimated to be 18 KDa while that of the native protein, analysed by Sephadex G-100 gel filtration, was ca. 66 KDa. These results suggest that HAF is possibly a polymer which consists of 3 or 4 subunits which bind to each other through disulfide bond.

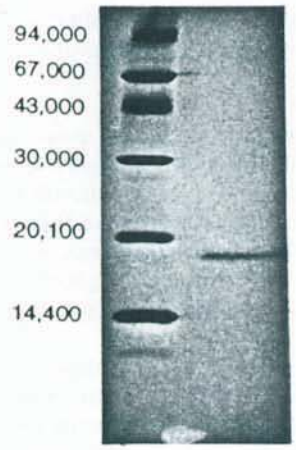


Fig. 4 – SDS-slab PAGE of purified HAF.

BILGE et al.[3] described a fimbrial adhesin (F1845) of a DAEC strain isolated from children with diarrhea in which 14.3 KDa subunits agglutinate, in the presence of D-mannose, human group O erythrocytes. A 57 KDa outer membrane protein, described by YAMAMOTO et al.[18] as possible adhesive factor in diffuse pattern to HeLa and HEp-2 cells, hemagglutinated human, bovine and sheep erythrocytes in presence of D-mannose. However, the purified HAF did not agglutinate bovine, guinea-pig, horse, chicken, or sheep erythrocytes but agglutinated only human group A erythrocytes in the presence of D-mannose (data not shown).

GIRON et al.[7,9] described a fimbrial adhesin, termed bundle-forming pili (BFP), that showed hemagglutinat-

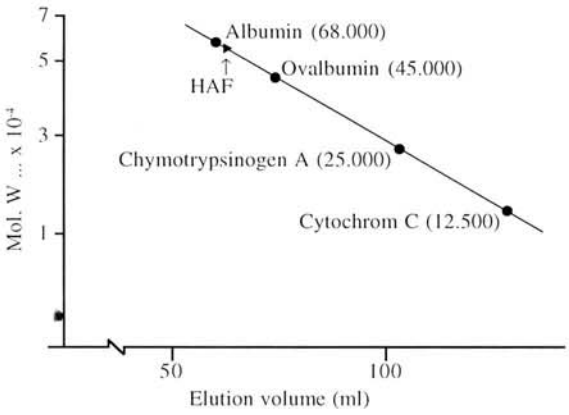


Fig. 5 – Determination of molecular weight of HAF by Sephadex G100 column chromatography.

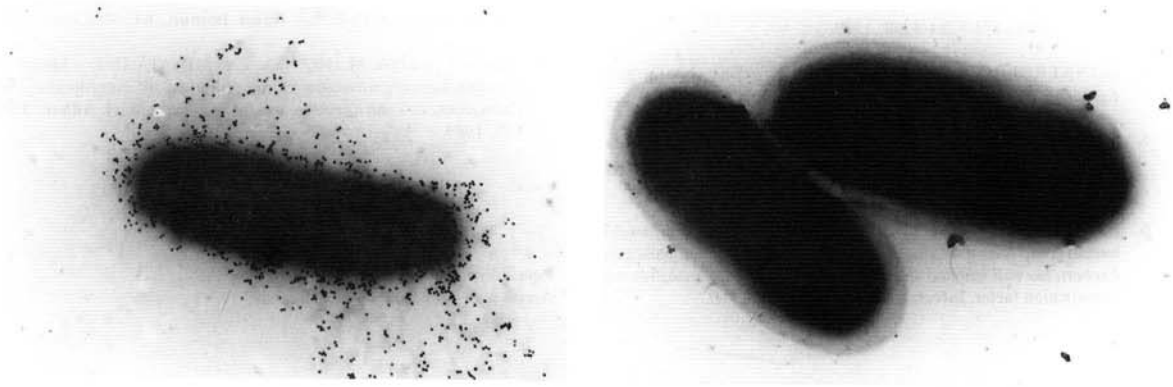
ing activity with human erythrocytes in the presence of mannose, regardless of the ABO blood antigen. Moreover, it did not agglutinate erythrocytes of other species, showing similar behaviour for hemagglutination activity of the HAF[19]. The structure of HAF on the bacterial surface, revealed by a serological method (immunolabeling test), did not show to be a fimbria-like structure (Figure 6A). Furthermore there was no HAF antigen on the bacterial cell surface of *E. coli* (055) of the localized adherence (LA) group (Figure 6B). Thus we think that the molecular mechanisms of adherence of DAEC and EPEC (LA^+) are different.

Recently, DONNENBERG & KAPER[4] suggested that EPEC (LA^+) infection proceeds in a three-stage process. Initially, the BFP mediates an interaction of the bacterium with the epithelial cell. In the second-stage, there is an increase in intracellular calcium levels and in the effacement of microvilli. In the third stage, "intimin" is produced that mediates close attachment to the epithelial cell.

The association of DAEC strains with diarrheal disease is not clear and has been a matter of considerable controversy, but GIRON et al.[8] found evidence for potential enteropathogenesis of DAEC strains, distributed in several serotypes, and isolated from cases of diarrhea in Mexico.

In previous experiments[19] we showed that 15 of 45 *E. coli* strains of several serotypes (0128:K^+; 01:K51; 0164:K^+; 0126:K71; 0136:K78; 0142:K^+ and 08:K^+) hemagglutinated only human erythrocytes, and all these 15 strains also reacted with our specific HAF antiserum. Furthermore, the adherence of the purified HAF to HeLa cells was examined by the immunofluorescence test, as described previously[5], with specific HAF antiserum and fluorescein isothiocyanate-anti rabbit immunoglobulin G conjugate (FITC) (Sigma). The HAF was diffusely located on the HeLa cells (data not shown), suggesting that this protein mediates the DAEC adherence on HeLa cells.

The data suggest that the HAF described in this paper is an adhesive factor in enteropathogenesis, and it may be common in several serotypes of DAEC strains.



A B

Fig. 6 – Electron micrographs of immunogold preparation with specific HAF antiserum.
A) *E. coli* (0126) (DA^+), grown at 37°C.
B) *E. coli* (055) (LA^+), grown at 37°C.

RESUMO

Purificação e caracterização parcial de um fator hemaglutinante (HAF): uma possível adesina de *Escherichia coli* do tipo aderência difusa (DAEC)

O fator hemaglutinante (HAF) foi extraído e purificado a partir de amostra de *Escherichia coli* de aderência difusa (DAEC), pertencente ao sorogrupo 0128 de *E. coli* enteropatogênica clássica (EPEC). O peso molecular do HAF foi estimado em 18.000 Da usando SDS-PAGE e 66.000 Da empregando Sephadex G 100, o que sugere a estrutura do HAF consistir de 3 a 4 subunidades. O emprego da técnica "gold immunolabeling" com antissoro específico anti-HAF revelou que este fator não é uma estrutura do tipo fímbria na superfície da bactéria. O resultado do teste de imunofluorescência usando HAF purificado em células HeLa, além do fato deste fator estar presente entre os sorotipos de EPEC, sugerem que o HAF seja um possível fator de aderência das amostras de DAEC.

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