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Purification and Partial Characterization of a K99-Antigen Associated Adhesin in *Escherichia coli* (637 Strain)

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The K99-antigen associated adhesin in *Escherichia coli* (637 Strain) has been purified to homogeneity by using conventional chromatographic procedures. Sodium deoxycholate was used in the precipitation steps to avoid hydrophobic interactions between the fimbriae and other membrane-associated components.

Homogeneity of the purified adhesin was assessed by electrophoresis, isoelectrofocusing, analytical gel filtration and immunoprecipitation against K99 specific antiserum, being homogeneous in all cases.

The purified adhesin is composed of protein sub-units with a molecular weight of 18,900 ± 950 daltons. No sugars were detected in the molecule. The molecular weight of the adhesin was higher than 2×10^6 daltons, and its isoelectric point was estimated to be about 9.45.

Bacterial fimbriae are filamentous non-flagellar appendages of the bacterial surfaces whose significance for survival and pathogenity is uncertain (19, 12). Some fimbriae — or pili — were shown to enable the bacteria to attach themselves to and colonize certain mammalian surfaces and have been termed «colonization factors». The study of these factors has increased considerably receptly following the discovery that the binding of bacteria to cells is the first step in the production of infections by the majority of the pathogenic bacteria (1).

One of the colonization factors, found in *Escherichia coli* strains isolated from calves, lambs and piglets with diarrhoea, is the K99 antigen. This factor is encoded by a transmissible plasmid (18). At present, there is great controversy concerning the molecular identity of the antigenic and adherent properties associated with the K99 (10, 15) and, in contrast to other colonization factors, purified K99 antigen

does not effectively inhibit the adhesion of K99-positive bacteria nor completely protect the animals vaccinated with this antigen (14).

Previous studies on the K99 antigen and adhesin expression in K99-positive wild strains and exconjugants showed that the two molecules are different in expression and genetic control (submitted for publication).

This paper deals with the purification and biochemical separation of the molecules responsible for the antigenicity and adherence encoded by the K99 plasmid.

Materials and Methods

Purification of the K99 antigen and adhesin. Cultures of the K99-positive strain *E. coli* 637 (obtained from J. Blanco and E. González, Departamento Microbiología, Facultad de Biología, Universidad de Santiago, Spain) were produced in erlenmeyer flasks in a total volume of 8 litres of Minca medium (9). The incubation took place at 37° C, statically, for 24 hours. At the end of the culture period, the bacteria were separated by centrifugation at 12.000 × *g* for 10 min at 4° C. The pellet was suspended in phosphate-buffered saline (PBS) in a total volume of 70 ml.

Fimbriae were extracted from the bacteria by agitation of the suspension in a Sorvall Omnimixer homogenizer, at a setting of 10, for a total of 10 min at 2 min intervals. The vessel of the homogenizer was mantained immersed in a mixture of methanol and ice. The suspension was then centrifuged at 8.000 × *g* for 15 min at 4° C, and the pellet was discarded.

Solid ammonium sulphate was then added to the supernatant to a 50 % saturation and, after standing overnight at 4° C, it was centrifuged at 12.000 × *g* for 20 min at 4° C. The precipitate was suspended in PBS and dialyzed against the same buffer for 48 hours. The dialyzate was centrifuged at 8.000 × *g* for 10 min at 4° C and sodium deoxycholate (DOC) was added to the supernatant to a 0.5 % concentration. This was then dialyzed against 12 volumes of a 0.5 % DOC solution for 48 hours. The dialyzate was centrifuged again as above and the supernatant was dialyzed against 20 volumes of PBS for 48 hours.

The dialyzate was then chromatographed in a Sepharose CL-4B column (1.5 × × 100 cm) equilibrated in PBS and eluted with the same buffer. The active fractions were then mixed and concentrated by precipitation with ammonium sulphate to a 70 % saturation. After centrifugation and dialysis against 0.01 M Phosphate buffer (pH 7.2) - 0.1 M NaCl (PB), the material was applied to a QAE-Sephadex A-50 column (2.5 × 35 cm) equilibrated with PB. Elution was carried out with a 500 ml lineal gradient of NaCl (0.1 M to 2.0 M) in PB.

The peaks in the chromatographic steps were detected with a 280 nm spectrophotometric flow detector.

Analytical methods. Total protein measures were made by the dye-fixation method (2). Sugar content in the purified fimbriae was estimated by the phenol-sulphuric acid method (7).

The purification procedure was followed by polyacrylamide gel electrophoresis according to LAEMLI (3) but omitting the inclusion of SDS in the solutions, by agarose gel electrophoresis as described by VAN ARKEL *et al.* (21), and by immunoprecipitation with anti-K99 serum (8). The immune serum was generously supplied by Dr. P.A.M. Guinée (Rijks Instituut voor de Volkgezondheid, Nederland).

Purity was assessed by isoelectrofocusing in agarose gels (3) and by analytical gel filtration in Sepharose CL-4B columns. These two methods allowed, respectively, the determination of the iso-

electric point and the molecular weight of the fimbriae.

Sub-unit molecular weight was determined by SDS-polyacrylamide gel electrophoresis (13).

Adhesive activity was measured by haemagglutination of sheep red blood cells in Microtiter plates (20).

Results

The purification procedure described allowed the K99- associated adhesin to be obtained as a homogeneous material as well as its separation from the molecules responsible for the K99 antigenicity. Table I shows the activities and recoveries obtained at each purification step. The purified adhesin has a specific activity of 80,629.9 units per mg, and the total recovery was 43.3 %.

Figure 1 shows the elution patterns obtained in the Sepharose CL-4B and in the QAE-Sephadex A-50 columns. The adhesin was eluted in peak 3 in the ion-exchange column, whereas the K99 antigen was eluted in peak 2 in the same column. This last purification step is the only one in which the K99 antigenic activity is separated from the adhesive activity (fig. 1 *B*).

Figure 3 shows the electrophoretic analysis of the active fractions in each purification step. As can be seen, only one band is obtained when peak 3 of the

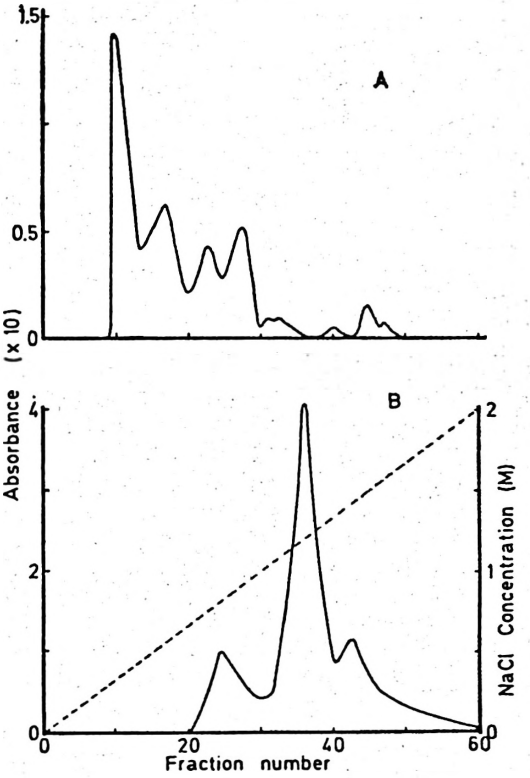

Fig. 1. *Diagrammatic representation of the elution patterns of the Sepharose CL-4B (A) and the QAE-Sephadex A-50 (B) columns.* Size and elution conditions are as explained in the text. Absorbance was measured at 280 nm. (---) NaCl gradient.

QAE-Sephadex A-50 column is analyzed. This band does not enter the polyacrylamide gels, and can only be well ana-

Table I. *Purification of the K99-antigen associated adhesin in* E. coli *637.*

Step	Total protein (mg)	Specific activity *	Units **	Recovery (%)
PBS extract	13.1	1,545.0	20,224.0	100.0
Ammonium sulphate 50 %	4.3	3,545.7	20,480.0	101.3
Sodium deoxycholate	1.5	13,993.9	14,336.0	70.9
Sepharose CL-4B	1.0	30,318.3	11,082.8	54.8
QAE-Sephadex A-50	0.1	80,629.9	8,757.0	43.3

* Expressed as units per mg of protein.

** One unit is defined as the minimum amount of protein in the extract capable of producing haemagglutination of sheep red blood cells.

Fig. 2. *Diagram of the immunoprecipitation patterns of the three peaks obtained in the QAE-Sephadex A-50 column against anti-*E coli *637 serum (t) and anti-K99 serum (k).*
a) peak 1; *b*) peak 2; *c*) peak 3.

lyzed in agarose gels, where a single band is also obtained. Peak 3 also shows one precipitation line with anti *E. coli* 637 serum (fig. 2). This peak is also homogeneous as demonstrated by isoelectrofocusing and analytical gel filtration (not shown). The pI calculated for the adhesin was 9.45, and its Mr at about 2×10^6.

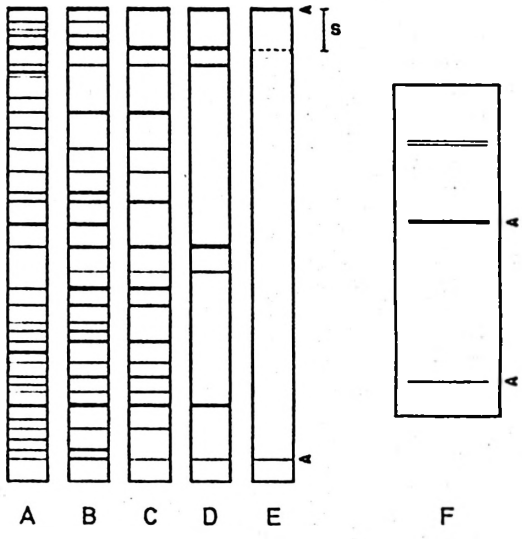

Fig. 3. *Diagram of the electrophoretic patterns of the active fractions obtained in each purification step.*
A) PBS extract; *B*) Ammonium sulphate fraction; *C*) DOC fraction; *D*) Sepharose CL-4B fraction; *E*) and *F*) peak 3 of the QAE-Sephadex A-50 column. *A*, *B*, *C*, *D* and *E* are polyacrylamide gels. *F* is an agarose gel. *S* indicate the stacking gel. (◀) Fimbriae. (◁) Bromphenol blue marker.

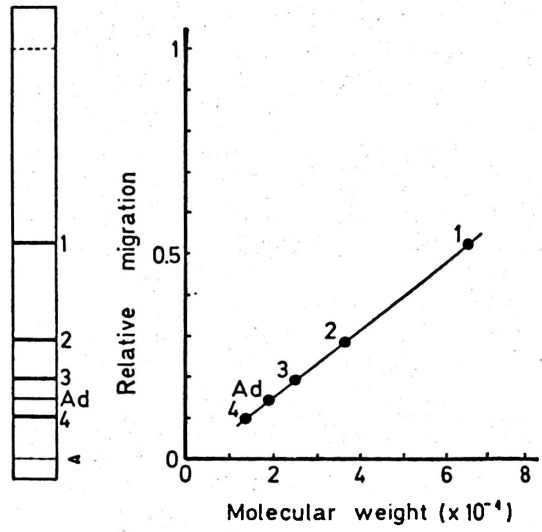

Fig. 4. *Molecular weight determination of the fimbrial sub-units of the K99-antigen associated adhesin.*
(A) SDS-polyacrylamide gel electrophoresis. (B) Plot of molecular weight versus relative migration distance in the gels. (1) Bovine serum albumin; (2) papain; (3) trypsin; (4) lysozyme; (Ad) adhesin (◁) brophenol blue marker.

Protein and carbohydrate analysis of the purified adhesin showed the absence of sugars. SDS-polyacrylamide analysis showed that the adhesin is composed of sub-units with an Mr of 18,900 ± 950 (fig. 4).

Discussion

The fimbriae purification procedure used is based on those of other authors (10, 15). The use of sodium deoxycholate prevents the hydrophobic interactions of the fimbriae with other membrane components such as the outer membrane proteins (OMP) or lipopolysaccharides (LPS) and was introduced for this purpose by DATA *et al.* (5). It was used recently for the purification of type 1 fimbriae (12) and of the K99 antigen (6).

From the electrophoretic, immunoprecipitation, analytical filtration, isoelectro-

focusing and SDS-electrophoretic analyses, it can be assumed that the K99-associated adhesin has been purified to homogenity. From the QAE-Sephadex column results it can be seen that the adhesin and the K99 antigen are eluted in different peaks. With the same type of colums, MORRIS *et al.* (15) obtained both activities in the same peak. The differences between the two purification procedures are in the extraction and precipitation methods, which, in the procedure of MORRIS *et al.* (15) do not allow the annulation of the hydrophobic forces. This can make it impossible to separate the fimbriae from other membrane components during purification.

The different behaviour found by ISAACSON (10) for the K99 antigen (it is eluted in the void volume of a DEAE-Sephadex column) can be explained by the same reasons indicated above. Furthermore, DEAE-Sephadex is an anionic exchanger, whereas QAE is cationic. This can also explain for the differences found (4).

ISAACSON (10) found that the agglutinating activity eluted later with the application of the NaCl gradient, and that the K99 antigen was devoid of agglutinating activity.

In this sense, the results presented in this paper are similar, because the K99 antigen and the adhesin are obtained as different molecules, at different peaks.

Recently, DE GRAAF *et al.* (6) have purified the K99 antigen by a method that includes the use of DOC, but they have not investigated the possible association of the antigen with the adhesin. The K99 antigen was detected by these authors by immunoenzymatic techniques.

The isoelectric point found for the K99 antigen was 9.45, and agrees with those found by ISAACSON (10) and DE GRAAF *et al.* (6). which were over 10 and 9.5 respectively. The isoelectric point found by MORRIS *et al.* (16, 17), 4.2, is completely different. The later authors postulated the existence of different K99 antigens in different *E. coli* strains, but this hypothesis was questioned by ISAACSON (11) and DE GRAAF *et al.* (6).

Resumen

Se purifica hasta la homogeneidad, por métodos cromatográficos habituales, una adhesina asociada al antígeno K99 en la cepa 637 de *Escherichia coli*. El uso de deoxicolato sódico en las etapas de precipitación tiene como finalidad el evitar las interacciones hidrofóbicas entre las fimbrias y otros componentes asociados a la membrana.

Se ensaya la homogeneidad de la adhesina purificada por electroforesis, isoelectroenfoque, filtración analítica en geles e inmunoprecipitación frente a antisuero específico anti-K99, siendo homogénea en todos los casos. La adhesina purificada está compuesta de subunidades de proteína con un peso molecular de 18.900 ± 950 daltones. No se detectan azúcares en la molécula. El peso molecular de la adhesina es superior a 2×10^6 daltones, y su punto isoeléctrico se estima alrededor de 9,45.

References

1. BEACHEY, E. H.: *J. Infect. Dis.*, **143**, 325-345, 1981.
2. BRADFORD, M.: *Anal. Biochem.*, **72**, 248-254, 1976.
3. BURNETT, D., BRADWELL, A. R. and RAMSDEN, D. B.: *Science Tools*, **27**, 19-20, 1980.
4. COOPER, C.: In «The tools of biochemistry». John Wiley and Sons Inc. New York, 1977, pp. 136-168.
5. DATA, T., INUZUKA, M. and TOMOEDA, A.: *Biochemistry*, **16**, 5579-5585, 1977.
6. DE GRAAF, F. K., KLEMM, P. and GAASTRA, W.: *Infect. Immun.*, **33**, 877-883, 1981.
7. DUBOIS, M., GILLES, K. A., HAMILTON, J. K. REVERS, P. A. and SMITH, F.: *Anal. Chem.*, **28**, 350-356, 1956.
8. GUINEE, P. A. M., JANSEN, W. H. and AGTERBERG, C. M.: *Infect. Immun.*, **13**, 1369-1377, 1976.
9. GUINEE, P. A. M., VELKAMP, J. and JANSEN, W. H.: *Infect. Immun.*, **15**, 676-678, 1977.
10. ISAACSON, R. E.: *Infect. Immun.*, **15**, 272-279, 1977.

11. ISAACSON, R. E.: *Infect. Immun.*, 22, 555-559, 1978.
12. KORHONEN, T. K., NURMIAKO, E., RANTA, H. and SVANBORG-EDEN, C.: *Infect. Immun.*, 27, 569-575, 1980.
13. LAEMLI, U. K.: *Nature*, 227, 680-685, 1970.
14. MORGAN, R. L., ISAACSON, R. E., MOON, H. W., BRINTON, C. C. and TO, C. C.: *Infect. Immun.*, 22, 771-777, 1978.
15. MORRIS, J. A., STEVENS, A. E. and SOJKA, W. J.: *J. Gen. Microbiol.*, 99, 353-357, 1977.
16. MORRIS, J. A., STEVENS, A. E. and SOJKA, W. J.: *J. Gen. Microbiol.*, 107, 173-175, 1978.
17. MORRIS, J. A., THORNS, C. J. and SOJKA, W. J.: *J. Gen. Microbiol.*, 118, 107-113, 1980.
18. ORSKOV, I., ORSKOV, F., SMITH, H. W. and SOJKA, W. J.: *Acta Pathol. Microbiol. Scand.*, 88, 31-36, 1975.
19. OTTOW, J. C.: *Ann. Rev. Microbiol.*, 29, 79-108, 1975.
20. SIMPSON, D. L., ROSEN, S. D. and BARONDES, S. H.: *Biochim. Biophys. Acta*, 412, 109-119, 1975.
21. VAN ARKEL, C., BALLIEUX, R. E. and JORDAN, F. L. J.: *J. Chromatog.*, 11, 421-423, 1963.