ISOELECTRIC FOCUSING STUDIES OF STAPHYLOCOCCAL EPIDERMOLYTIC TOXIN

J. P. ARBUTHNOTT, Bronwen BILLCLIFFE and W. D. THOMPSON
Departments of Bacteriology and Pathology, University of Glasgow, Royal Infirmary, 86 Castle Street, Glasgow, G4 OSF, Scotland

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1. Introduction

Strains of *Staphylococcus aureus* belonging to phage group II produce a diffusible toxin (epidermolytic toxin) which causes intra-epidermal splitting in neonatal mice, adult hairless mice and man [1–6]. Epidermolytic toxin (ET) causes the symptoms of staphylococcal toxic epidermal necrolysis (scalded skin syndrome) and apart from enterotoxin, is the only exotoxin known to play a definite role in staphylococcal pathogenicity.

The toxin has been isolated and partly characterised in four laboratories [3–5,7]; it is a protein with a molecular weight between 24 000 and 33 000. However, little is known of its mode of action. Two groups agree that ET has an isoelectric point of approximately 7.0 [4,5], while Kondo et al. [7] report a value of 4.0–4.5. Also, Kondo et al. [7] suggest that ET exists in four stable forms differing in electrophoretic mobility. Previous studies with staphylococcal α-toxin [8] and *Clostridium perfringens* α-toxin [9] have shown that isoelectric focusing is a suitable method of purifying bacterial toxins on a relatively large scale and that it can be used to characterise multiple molecular forms [8,9]. This paper reports the application of isoelectric focusing to the study of ET.

2. Methods

2.1. *Production and purification of ET*

Previously we produced ET by culturing strains of *Staph. aureus* on a semi-solid nutrient agar in an atmosphere containing 20% CO_2 [4]. However, a yeast diffusate medium similar to that used by Bernheimer and Schwartz [10] was found to support ET production in the absence of added CO_2. Using this medium and strain 123A, a non α-toxinogenic strain, ET was prepared and purified by a four stage process similar to that used for staphylococcal α-toxin [8]. Stage I toxin consisted of culture supernatant fluid; in stage II, culture supernatant was concentrated by ammonium sulphate precipitation at 90% saturation. Up to 500 mg of concentrated toxin were applied to an LKB 8102 (440 ml) column. In stage III, peak fractions of ET were pooled and freed from Ampholine by dialysis against 90% saturated ammonium sulphate. Stage III toxin was refocused to yield stage IV toxin.

2.2. *Assessment of purity*

ET preparations were screened by SDS disc gel electrophoresis [8] to determine the number of polypeptide components and to estimate the approximate molecular weight of ET by comparison with a series of standard proteins. The haemolytic toxins (α-, β- and δ-) were tested for by titrating against erythrocytes of different species [8]. Fractions were tested for the presence of staphylococcal enzymes using agar gels containing the appropriate substrates [11].

2.3. *Thin layer gel isolectric focusing*

Gel isoelectric focusing [12] was performed using an LKB Multiphor apparatus. When necessary, increasing concentrations of urea were incorporated as described by Hobart [13] at an angle of 90° to the direction of current flow. After focusing, gels were fixed and stained with an acidic extract of Coomassie Blue [14,15].

2.4. *Preparation of anti-epidermolytic toxin*

Twice focused ET was used to prepare antitoxin in

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rabbits. In a 40-day schedule, injections of antigen in complete Freund's adjuvant were given on day 0 and day 14; a third injection without adjuvant was given on day 35 and animals were bled out on day 40.

2.5. *Assay of ET*

Randomised groups of 3-day old mice were used to assay ET as described previously [4]. Radial immunodiffusion plates [16,17] prepared from 3% agarose, containing 0.02M sodium barbitone 0.17M sodium chloride and 3.5% (v/v) antitoxin were used to determine relative levels of ET in vitro.

3. Results and discussion

From fig. 1 it can be seen that the main component of ET had a pI of 7.05. There was close correlation between the distribution of epidermolytic activity, as detected in 3-day old mice, and the results of radial immunodiffusion assays performed in agarose containing antibody to purified ET. Both assays revealed a second peak having a pI of 6.2 and additional minor peaks and shoulders. A total of 350 mg of Stage III material (fractions between 6.8 and 7.2) were refocused. The protein elution profile and the results of radial immunodiffusion assays again showed a major component having a pI of 7.0 and a second small peak with a pI of 6.0; the main component had a shoulder at pH 6.65. The pI = 7.0 component was designated ET_A and the pI = 6.0 component was designated ET_B.

Table 1 shows the degree of purification obtained at each step. Overall,the procedure results in a 142 fold purification and in the present study, 1.3 g of Stage II toxin yielded 50 mg of Stage IV ET. The degree of purification is of the same order as reported by

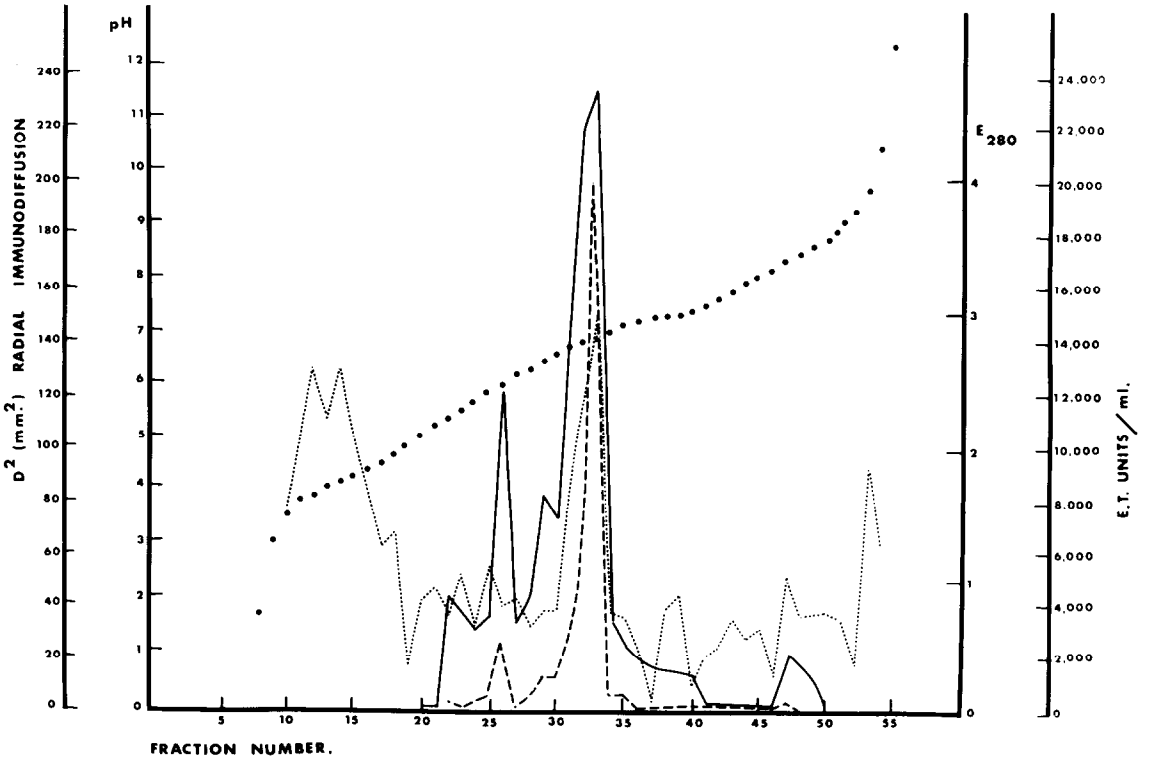

Fig. 1. Preparative density gradient isoelectric focusing of staphylococcal epidermolytic toxin (ET). 460 mg of stage II toxin was applied to an LKB 8102 (440 ml) column using 1% (w/v) Ampholine pH 3.5–10.0. The duration of the run was 70 hr and the final voltage was 800 V. The column was cooled to 4°C and fractions of 8 ml were collected. • • • = pH; - - - - - - - - = E_{280}; – – – – – = epidermolytic activity detected in 3-day old mice; ——— = diameter² of zones detected by radial immunodiffusion.

Table 1
Purification of staphylococcal epidermolytic toxin (ET)

Stage of purification	E.T. units*/ml.	Specific activity (E.T. units/mg)	Purification factor
I	320	20	–
II	5 000	360	18
III	20 000	2 000	100
IV	40 000	2 850	142

* 1 ET unit = smallest amount of toxin which causes epidermal splitting in neonatal (3-day-old) mice 6 hr after injection.

Kondo et al. [7], but the method allows the processing of 100 times more toxic protein.

By SDS polyacrylamide disc gel electrophoresis, refocused ET was found to contain a single component even when gels were overloaded by applying up to 150 μg protein. The molecular weight estimated in SDS disc gels by comparison with 5 proteins having molecular weights ranging from 12 000 to 65 000 was found to be close to 25 000; both ET_A and ET_B had the same molecular weight. This value is close to that obtained by gel filtration [3,7] but is considerably lower than the value of 33 000 obtained for ET purified from extracts of semi-solid nutrient agar cultures grown in the presence of CO_2 [4].

Refocused ET_A was found to be free of the following staphylococcal activities, α-toxin, β-toxin, δ-toxin, lipase, egg yolk factor, gelatinase, DNase, and phosphatase. However, traces of staphylokinase were detected. This was not unexpected since like previous workers [18] we found a peak of staphylokinase activity at pH 6.6. However, the elution profile of staphylokinase followed quite a different outline from that of ET and antibody prepared against ET did not neutralise staphylokinase.

In view of the apparent high purity of ET_A it seems appropriate to quote the minimum effective dose of the toxin. In neonatal mice weighing an average of 2.4 g this was 0.35 μg and tests in three adult human volunteers showed that 0.2 μg produced an area of blistering 15 mm in diameter.

When tested in double diffusion tests against antitoxin to purified ET_A, peak fractions of ET_A gave two distinct immunoprecipitin lines indicating the presence of at least two antigenically distinct components. ET_B was found to contain the same antigenic components but in different proportions. This finding gave the first indication of heterogeneity within individual ET components and it was decided to investigate this aspect further by thin layer gel isoelectric focusing. All ET containing fractions from stage IV of the purification method were analysed in pH 3.5–10.0 gradients. Examples of the complex patterns obtained are shown in fig. 2. Up to seven components having pI's between 6.2 and 7.0 were detected in the peak ET_A fraction while ET_B contained only two components with pI's of 6.0 and 6.3. ET_A was applied to thin layer isoelectric focusing gels containing increasing urea concentrations arranged at 90° to the direction of current flow (fig. 3).

The degree of heterogeneity reduced as the concen-

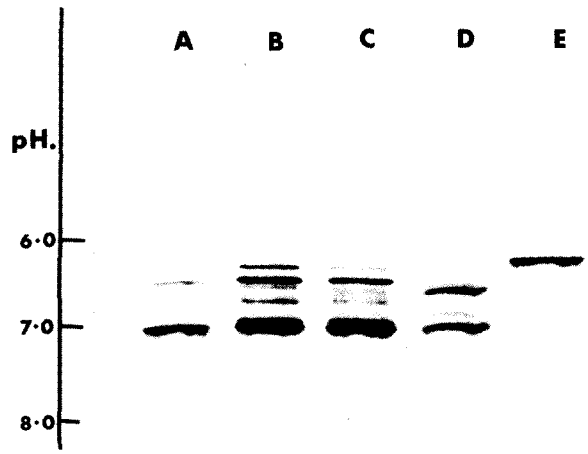

Fig. 2. Thin layer gel isoelectric focusing of fractions of ET obtained from different points in the pH gradient. A = pH 7.2; B = pH 6.95 (ET_A); C = pH 6.75; D = pH 6.55; E = pH 6.0 (ET_B). Between 30 and 45 μg of protein was applied to the gel. The gel was run at 8°C for 70 min at a current of 30 mA and a maximum voltage of 100 V/cm.

tration of urea increased, until at 8M urea most of the protein in ET_A focused as a double band (fig. 3, band i); a second faint band was also detected (fig. 3, band ii). When ET_B was focused alongside ET_A on the same gel in the presence of 6M or 8M urea it focused as a single band corresponding in position to band ii in fig. 3.

It is difficult to give accurate estimates of the pI's of individual components in the presence of urea because of the effect of urea on the pH gradient. For instance, it has been reported [19] that 6M urea causes an increase of approximately 0.4 pH units in the pI of carrier ampholytes under the conditions of preparative density gradient isoelectric focusing. Assuming that the main ET_A band seen in gels containing 8M urea is identical with the most cationic band found in the absence of urea, our experiments would suggest that correction factors of approximately 0.8 pH units and 0.6 pH units should be made in thin layer gels which incorporate 8M or 6M urea respectively.

In general, our findings point to two types of heterogeneity within ET. ET_A and ET_B have closely similar molecular weights but probably differ in covalent structure. If so, the presence of traces of ET_B in ET_A preparations would explain the occurrence of two immunoprecipitin lines in double diffusion tests with antiserum prepared against ET_A..However, ET_A also revealed heterogeneity in thin gel electrofocusing which was markedly reduced in the presence of 6M and 8M urea. This suggests the presence of multiple forms which do not differ in covalent structure.

Further biochemical and physico-chemical characterisation of ET is necessary before it is possible to assess which of the many possible reasons for multiple forms [20] are involved.

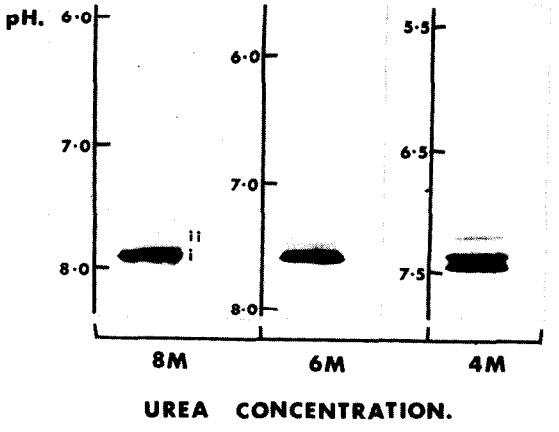

Fig. 3. Thin layer gel isoelectric focusing of ET_A in the presence of different concentrations of urea. Each sample comprised 100 μg of ET_A. The gel was run at 11°C for 115 min. at 30 mA and a final voltage of 100 V/cm.

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References

[1] Melish, M. E. and Glasgow, L. A. (1970) New. Engl. J. Med. 282, 1114–1119.
[2] Arbuthnott, J. P., Kent, J., Lyell, A. and Gemmell, C. G. (1971) Brit. J. Dermatol. 85, 145–149.
[3] Kapral, F. A. and Miller, M. M. (1971) Infect. Immun. 4, 541–545.
[4] Arbuthnott, J. P., Kent, J., Lyell, A. and Gemmell, C. G. (1972) Brit. J. Dermatol., 86, Suppl. 8, 35–39.
[5] Melish, M. E., Glasgow, L. A. and Turner, M. D. (1972). J. Infect. Dis., 125, 129–140.
[6] Wiley, B. B., Allman, S., Rogolsky, M. Norden, C. W. and Glasgow, L. A. (1974). Infect. Immun. 9, 636–640.
[7] Kondo, I., Sakurai, S. and Sarai, Y. (1973). Infect. Immun. 8, 156–164.
[8] McNiven, A. C., Owen, P. and Arbuthnott, J. P. (1972). J. Med. Microbiol. 5, 113.
[9] Smyth, C. J. and Arbuthnott, J. P. (1974). J. Med. Microbiol., 7, 41–66.
[10] Bernheimer, A. W. and Schwartz, L. L. (1963). J. Gen. Microbiol., 30, 455–468.
[11] Arbuthnott, J. P., Gemmell, C. G., Kent, J. and Lyell, A. (1969). J. Med. Microbiol., 2, 479–487.
[12] Karlsson, C., Davies, H., Ohman, J. and Andersson, U-B. (1973). L.K.B. Application Note No. 75.
[13] Hobart, J. J. (1974) in: 'Isoelectric Focusing of Proteins and Related Substances' (Arbuthnott, J. P. and Beeley, J. A. eds.), Butterworth, London, in press.
[14] Malik, N. and Berrie, A. (1972). Anal. Biochem., 49, 173–176.
[15] Billcliffe, B. and Arbuthnott, J. P. (1974). L.K.B. Application Note in press.
[16] Mancini, G., Carbonara, A. O. and Heremans, J. F. (1965). Immunochemistry, 2, 235–254.
[17] Fahey, J. L. and McKelvey, E. M. (1965). J. Immunol., 94, 84–90.
[18] Vesterberg, K. and Vesterberg, O. (1971). J. Med. Microbiol., 5, 441–450.
[19] Ui, N. (1971). Biochem. Biophys. Acta., 229, 567–581.
[20] Arbuthnott, J. P., McNiven, A. C. and Smyth, C. J. (1974) in: 'Isoelectric Focusing of Proteins and Related Substances'. (Arbuthnott, J. P. and Beeley, J. A., eds.) Butterworth, London, in press.