SEPARATION OF *E. COLI* HEAT-LABILE ENTEROTOXIN BY PREPARATIVE ISOTACHOPHORESIS

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1. Introduction

Strains of *Escherichia coli* enteropathogenic for animals and man proliferate in the small intestine and can cause diarrhoea by producing a heat-labile high molecular weight enterotoxin (LT) and a heat-stable low molecular weight enterotoxin (ST) [1]. These toxins were first described in 1967 and 1969 [2,3], but not until very recently have attempts to purify LT and ST been reported [4–6].

Molecular sieve chromatography and ultrafiltration on membranes with defined permeability have been used for purification of cholera enterotoxin (choleragen) [7]. However, these methods have been used with little success when working with the *E. coli* enterotoxin, probably due to heterogeneity in the molecular size of the toxin [4]. Recent attempts to purify LT from the porcine strain 853/67 by separation according to charge, such as CM- and QAE-Sephadex chromatography, gave low recoveries. Furthermore, LT was found to be unstable at its isoelectric point (pI) which made isoelectric focusing less suitable for further purification work [5].

Recent findings indicate that high molecular weight LT (mol. wt $1-40 \times 10^6$) can be split into small subunits possessing full biological activity in the intestinal loop assay and in the skin test [8,9]. Removal of actively growing cells from cultures and treatment with polymyxin B, *n*-butanol or Tris–EDTA buffer in the classical osmotic chock procedure [10] will release an LT with a mol. wt around 100 000 [8] T. Wadström, R. Möllby and O. Söderlind, in preparation).

Isotachophoresis is an electrophoretic method for separating ions of the samenet charge, which all have a common counter-ion. The charged proteins to be separated migrate between a leading and a terminating ion forming a stack of discs. Ampholine® carrier ampholytes are used as 'spacers', i.e. intermediate-mobility compounds between the different proteins. The separation takes place in a polyacrylamide gel and the separated proteins are continuously eluted [11–14].

The high sample capacity and resolving power of preparative isotachophoresis, which is comparable to isoelectric focusing, makes this method superior to most conventional preparative electrophoretic methods [11]. This paper describes separation of the *E. coli* enterotoxin (LT) with the use of this method.

2. Materials and methods

E. coli strain 853/67 (isolated from piglets with diarrhea; serotype 0:149, K:88ac) was grown in Brain Heart Infusion broth (BHI, Difco Labs., Detroit, Mich., USA) in a 10 l fermentor (Biotec FL 110; Biotec AB, Stockholm, Sweden) under control of temperature, aeration, agitation and pH [15]. R. Möllby, O. Söderlind, T. Wadström, in preparation). Cells and culture supernatant were harvested by centrifugation (8000 *g* for 20 min) at the end of the growth phase after 6 hr of cultivation at 37°C. The cells were washed twice in Tris-buffered saline (TBS) and subjected to treatment with polymyxin (2 mg/ml) in 1/16 of the initial culture vol as previously described [16]. The material released from the cells by the poly-

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myxin treatment and the culture supernatant were concentrated six times by ammonium sulphate precipitation (70% saturation, 4°C, pH 6.3). The supernatant was further concentrated 6 times by dialysis against polyethyleneglycol (mol. wt 20 000), dialysed against distilled water and then subjected to chromatography on DEAE-Sephadex (2.5 g dry weight per litre initial culture vol) at pH 8.5. As no enterotoxic activity was adsorbed to the ion exchanger, the effluent was subjected to further purification.

The concentrated polymyxin B released material contained 40 Effective Doses (ED) per mol as determined in the rabbit ileal loop assay [5], 10 ED/ml as determined in the rabbit skin test [17] and 1.3 mg protein per ml as determined by the method of Lowry [18]. The concentrated and prepurified supernatant material contained 300 ED/ml in the rabbit ileal loop test, 100 ED/ml in the skin test and 3.8 mg protein/ml.

The isotachophoresis was performed as described by Winter et al. [11]. 16 ml of the released material and 2 ml of the supernatant material were dialysed against the cathode buffer (terminating electrolyte; 0.23 M ϵ-amino caproic acid (EACA) and 0.012 M Tris, pH 8.9) and supplemented with 0.8 ml Ampholine® carrier ampholytes (pH 6–8, 40%, w/v). Both preparations were layered on top of the polyacrylamide gel (30 ml, T = 3.4 and C = 2.9) in a LKB 7900 Uniphor equipped with the LKB 7960 plastic column and run in separate experiments (LKB-Produkter AB). As leading electrolyte Tris–phosphate, pH 6.25, was used.

Unless otherwise stated all chemicals were of analytical grade and all procedures were performed at 4°C. Acrylamide and bis acrylamide were purchased from Eastman Org. Chemicals, Rochester, N.Y., USA. The gels and all the different solutions were prepared as recently described [11,14].

The sample was separated at 10°C for 18 hr (7.5 mA; 500–1200 V) with an elution flow rate of 20 ml/hr. The transmission (%) at 280 nm was recorded with an LKB Uvicord II as shown in the figure. Deoxyribonuclease (DNase), ribonuclease (RNase), lecithinase, lipase, protease and haemolytic activity were assayed on DNA-, RNA-, egg yolk-, casein- and blood-agar plates [19]. 3′- and 5′-nucleotidase (uridine diphosphate-sugar-hydrolase) were assayed on adenosine-5′-and 3′-monophosphate in a Tris-maleate buffer [20,21].

3. Results

Fig.1A shows separation of LT in the material released from the cells and fig.1B the separation of extracellular LT found in the culture fluid. In the former case, the main peak containing rabbit and ileal loop skin test positive material, i.e. classical LT enterotoxin, was separated followed by a second peak containing material which only gave a positive rabbit skin test. 5′-nucleotidase was eluted in a broad peak before the enterotoxin but the peak of 3′-nucleotidase activity coincided with the peak containing LT (fig.1A). The separation of extracellular LT (fig.1B) resulted in a better coincidence between the two test methods. The peak of activity also coincided with maximal transmission inhibition. Dnase, RNase, lecithinase, lipase, protease or haemolytic activity was not detected in anyone of the two starting materials nor in the separated fractions.

An increase in specific activity (ED in skin test per mg of protein) from three to six times was obtained. As analysed by polyacrylamide gradient gel electrophoresis (Pharmacia, Uppsala, Sweden) the number of protein components diminished from eleven to five upon separation.

The recovery of LT was 5–15% when separating cell released material. This low yield was partly explained by precipitations in the starting material and on top of the gel when the isotachophoresis started. The precipitated material was thus prevented from entering the gel and was also found to contain most of the applied enterotoxic activity. However, when separating the prepurified extracellular toxin, no precipitation was observed and consequently the recovery was considerably better –25% in the ileal loop and 65% in the skin test.

4. Discussion

The concentration of the LT enterotoxin in a crude state caused great difficulties because of precipitation of the active material, although different methods such as ultrafiltration, dialysis against polyethyleneglycol, precipitation with polyethyleneglycol and freeze drying were tried. This resulted in low yields and loading capacity on BioGel and Sephadex chromatography [5]. As the enterotoxin was unstable at its

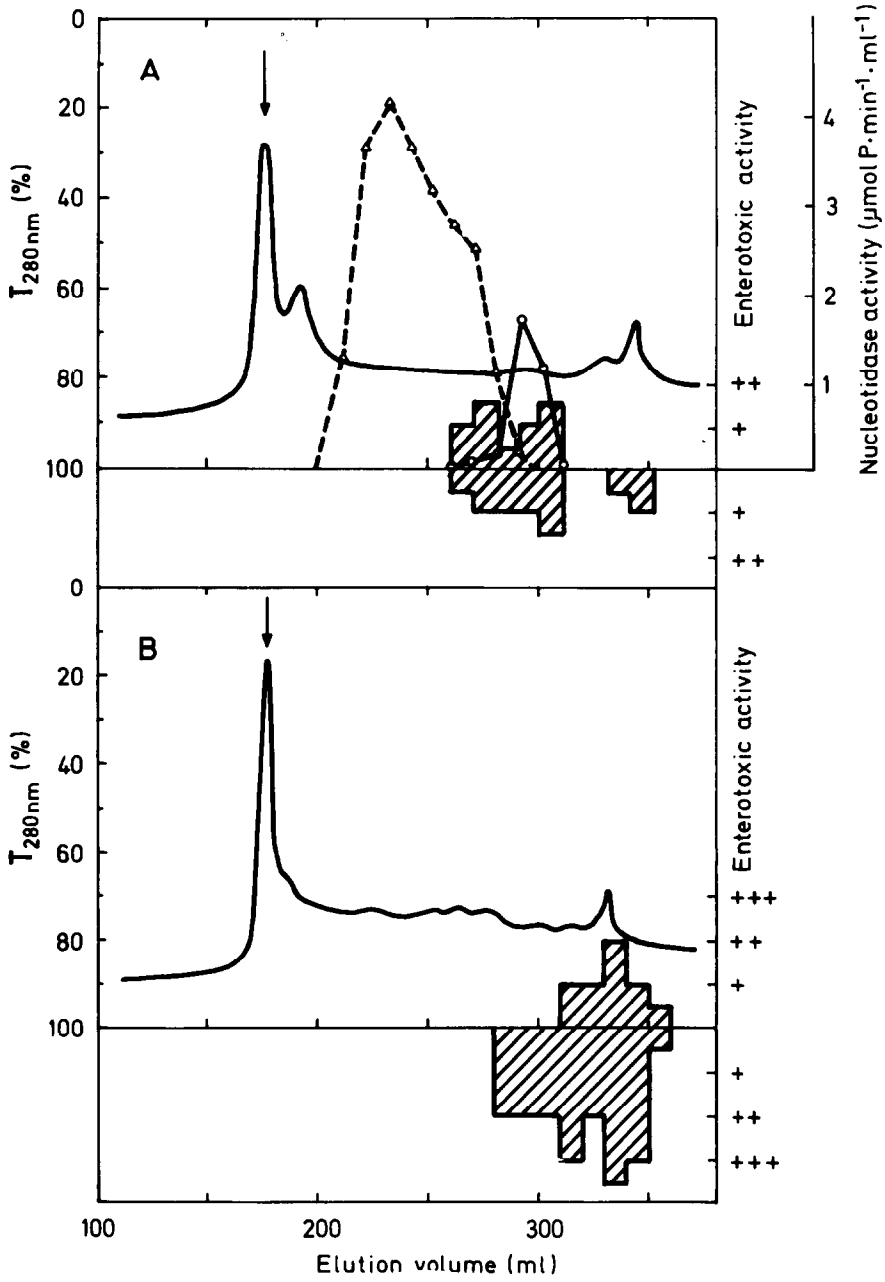

Fig.1. Separation of heat-labile enterotoxin (LT) from *E. coli* 853/67 by preparative isotachophoresis as described in Materials and methods. (A) Polymyxin B released material; (B) Culture fluid material. Both LT preparations were concentrated several times before separation. Fractions of 10 ml were harvested at 4°C and 0.5 ml were assayed directly in the rabbit ileal loop test (upward staples) and 0.1 ml in the rabbit skin test (downward staples). Enterotoxic activity was graded from + to +++ and the values presentec represent a mean of at least three rabbits for each sample. ++ is considered to equal one Effective Dose (ED), i.e. about 1 ml intraluminal fluid per cm of rabbit ileal loop or blue induration of about 7 mm in the rabbit skin. (o---o) Denotes 3'-nucleotidase activity and (△---△) 5'-nucleotidase activity; (——) Transmission (T) at 280 nm. The arrow indicates an artefact peak due to the polyacrylamide gel.

isoelectric point [5] for theoretical reasons the method of isotachophoresis was more attractive than isoelectric focusing for purification on a preparative scale, especially since ion exchange chromatography has given low recoveries and not highly reproducible results (R. Finkelstein, personal communication, R. Möllby and T. Wadström, unpublished data).

Relatively large quantities of crude protein solution (approx. up to 300 mg) can be applied in volumes of 20 to 30 ml in this standard procedure of preparative isotachophoresis. However, by increasing the length of the gel larger samples can be separated in single experiments. Better methods of prepurification should also permit higher loads of the Uniphor column. Further studies changing the separation parameters, such as the choice of leading and terminating ions and spacers, will probably also improve the separation of LT enterotoxin.

Separation of extracellular LT in the culture fluid by isoelectric focusing in a density gradient [22] gave an isoelectric point (pl) of 7.0. This is compatible with the elution profile shown in fig.1B, but not with the pl of 4.5 previously reported for LT from preparations of whole cell lysates [5].

Studies are under way to find a mild and simple purification process to obtain large quantities of LT for biochemical and immunological characterization of this toxin. This would make comparative studies with heat-labile enterotoxins possible, in order to find out the relations between LT produced by *E. coli* strains enteropathogenic for different animal species and for man. It is still unclear, if toxinogenic *E. coli* diarrhoea in different species is caused by one single LT or by a family of enterotoxins, all possibly acting in a similar way as the enterotoxin from *Vibrio cholerae* (choleragen).

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References

[1] Smith, H. W. and Gyles, C. L. (1970) J. Med. Microbiol. 3, 387–401.
[2] Smith, H. W. and Halls, S. (1967) J. Path. Bact. 93, 531–543.
[3] Gyles, C. L. Barnum, D. A. (1969) J. Infect. Dis. 120, 419–426.
[4] Larivière, S., Gyles, C. L. Barnum, D. A. (1973) J. Infect. Dis. 128, 312–320.
[5] Söderlind, O., Möllby, R. and Wadström, T. (1974) Zbl. Bakt. Hyg., I. Abt. Orig. A229, 190–204.
[6] Jacks, T. M. and Wu, B. J. (1974) Infect. Immunity 9, 342–347.
[7] Finkelstein, R. A. and Lospolluto, J. J. (1970) J. Infect. Dis. 121 (Suppl.) S 63–S72.
[8] Wadström, T., Söderlind, O., Möllby, R. and Wretlind, B. (1974) IRCS 2, 1312.
[9] Evans, D. J., Evans, D. G. and Gorbach, S. L. (1974) Infect. Immunity 10, 1010–1017.
[10] Heppel, L. A. (1971) in: Structure and Function of Biological Membranes (Rothfield, L. I., ed.) p. 224, Academic Press, New York and London.
[11] Winter, A., Hjalmarsson, S. -G. and Karlsson, C. (1974) LKB Application Note 146.
[12] Haglund, H. (1970) Sci. Tools 17, 2–13.
[13] Routs, R. J. (1971) Ph. D. Thesis, Univ. of Technology, Eindhoven, The Netherlands.
[14] Svendsen, P. J. and Rose, C. (1970) Sci. Tools 17, 13–17.
[15] Wadström. T. and Möllby, R. (1971) Biochim. Biophys. Acta 242, 288–307.
[16] Cerny, G. and Teuber, M. (1971) Arch. Mikrobiol. 78, 166–179.
[17] Holmgren, J., Söderlind, O. and Wadström, T. (1973) Acta Path. Microbiol. Scand. Sect. B 81, 757–762.
[18] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[19] Wretlind, B., Hedén, L., Sjöberg, L. and Wadström, T. (1973) J. Med. Microbiol. 6, 91–100.
[20] Neu, H. C. (1968) Biochemistry 7, 3766–3773.
[21] Neu, H. C. (1968) Biochemistry 7, 3774–3780.
[22] Vesterberg, O., Wadström, T., Vesterberg, K., Malmgren, B. and Svensson, H. (1967) Biochim. Biophys. Acta 133, 435–445.