Purification of anthrax-toxin components by high-performance anion-exchange, gel-filtration and hydrophobic-interaction chromatography

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A procedure has been developed for purification of the tripartite anthrax-toxin components. This involves sequential high-performance anion-exchange, gel-filtration and hydrophobic-interaction chromatography. From an initial culture volume of 15 litres, typical yields of 8 mg of protective antigen, 13 mg of lethal factor and 7 mg of oedema factor are produced to higher degrees of purity than have previously been achieved by conventional chromatographic techniques.

INTRODUCTION

Bacillus anthracis, the causative agent of anthrax, possesses two known virulence determinants: a tripartite protein toxin and a poly-D-glutamic acid capsule [1,2]. The tripartite toxin comprises protective antigen (PA; M_r 85000, pI 5.5), lethal factor (LF; M_r 87000, pI 5.8) and oedema factor (EF; M_r 86000, pI 5.9).

A recent resurgence of interest in *B. anthracis* [3,4] resulted in improved methods for production, separation and purification of the known toxin components [5–7]. This in turn has led to development of much more sensitive toxin-detection systems [8,9] and studies on the production *in vitro* and toxic effects of these proteins [8,10]. Consequently it is now known that EF is a calmodulin-dependent adenylate cyclase [3], and it has been postulated that PA acts as a receptor for which both EF and LF compete in cell internalization [3,7,8,10]. The mechanism of action of LF, although presumably enzymic, has yet to be elucidated [7].

Further understanding of toxin activity, the role of individual components in protection against the disease and the production of antigens for use in diagnostic systems now require higher degrees of purity than have hitherto been achieved. To this end we report the application of h.p.l.c. for rapid production of active toxin components to a consistently high degree of purity.

MATERIALS AND METHODS

General methods

H.p.l.c. was done on a Pharmacia Fast Protein Liquid Chromatography (f.p.l.c.) system comprising an LCC-500 gradient controller, two P-500 pumps, a single-path UV-1 monitor (280 nm filter), a Frac-100 automated fraction collector and a two-channel REC-482 chart recorder. Chromatographic columns and media used were all products of Pharmacia, Uppsala, Sweden, and

included a 20 ml Mono-Q [QAE (quaternary amino-ethyl)] HR 16/10 anion-exchange column, a 1 ml Mono-Q (QAE) HR 5/5 anion-exchange column, a 25 ml Superose-12 prepacked HR 10/30 gel-filtration column, a 100 ml Prep-Grade Superose-12 HR 16/50 user-packed gel-filtration column and a 1 ml phenyl-Superose HR 5/5 hydrophobic-interaction chromatography column.

Materials

Triethanolamine, Tris/HCl buffer, disodium EDTA, 2-mercaptoethanol and Triton X-100 were all purchased from Sigma Chemical Co., Poole, Dorset, U.K. $(\text{NH}_4)_2\text{SO}_4$ and NaCl were of AnalaR grade purchased from BDH Biochemicals, Poole, Dorset, U.K. Water used in chromatographic procedures was of Millipore or Milli-Q standard. All buffers and solutions were pre-filtered (0.22 μm pore size) and degassed before use.

General procedures

All work with live cultures of *Bacillus anthracis* was done under A.C.D.P. (Advisory Council for Dangerous Pathogens) Category 3 containment conditions [11] by immunized personnel only. F.p.l.c. was also done by immunized personnel, but under A.C.D.P. Category 2 conditions.

Culture conditions and toxin production

Batch production (15 litres) of anthrax toxin was done in 500 ml portions in Thompson bottles with the medium of Thorne & Belton [12]. The carbon source (0.25% glucose) and mineral additives were pipetted separately (50 ml) into each bottle together with 0.1 ml of an overnight (16 h) nutrient-broth (Oxoid) culture of the non-encapsulated, toxigenic, avirulent Sterne strain of *B. anthracis*. Bottles were then incubated without agitation on their sides for 26 h at 37 °C.

At the end of the incubation period cultures were harvested into an enclosed 20-litre bottle, and cell-free

Abbreviations used: PA, protective antigen; LF, lethal factor; EF, oedema factor; PAGE, polyacrylamide-gel electrophoresis; f.p.l.c., fast protein liquid chromatography.

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culture medium was collected by filtration under pressure through a 0.22 μm -pore-size Millipore Durapore hydrophilic disc filter (293 mm diam.). This cell-free filtrate was then diluted up to a total volume of 80 litres with 2.0 mM-EDTA at pH 8. Anion-exchange-resin slurry (500 ml; Whatman DE52 DEAE-cellulose) equilibrated in 20 mM-Tris/HCl buffer (pH 8.0)/1.0 mM-EDTA/2 mM-2-mercaptoethanol was then added, and the resin was kept suspended by stirring with a top-drive IKA-RN18 stirring motor (Sartorius). After 60 min the resin was allowed to settle and the supernatant was aspirated off. The DE52 DEAE-cellulose was then collected in a large-diameter chromatography column and washed with 3 bed vol. of equilibration buffer. Protein was eluted in 3 bed vol. of 1.0 M-NaCl in 20 mM-triethanolamine/NaOH buffer, pH 8.0, and precipitated overnight at 4 °C by addition of $(\text{NH}_4)_2\text{SO}_4$ to 70% saturation. The precipitate was collected by centrifugation at 23000 g (r_{av} 8.0 cm) for 60 min, resuspended in 20 ml of 20 mM-triethanolamine/NaOH buffer, pH 8.0, and dialysed at 4 °C for 5 h against 4×2 -litre hourly changes of the same buffer. The dialysed crude toxin preparation was then centrifuged at 15000 g (r_{av} 4.5 cm) for 10 min to remove insoluble material, and the supernatant was filter-sterilized through a 0.22 μm -pore-size disposable filter (Anderman and Co., East Moseley, Surrey, U.K.) before anion-exchange f.p.l.c. Batches of dialysed crude toxin (approx. 20 ml) contained 2.4–4.0 mg of total protein/ml.

Separation of toxin components by anion-exchange f.p.l.c.

Dialysed crude toxin was chromatographed on a Mono-Q (QAE) HR 16/10 column of 20 ml bed volume (Pharmacia) in 20 mM-triethanolamine/NaOH buffer, pH 8.0, with a two-stage NaCl gradient from 0.1 M to 1.0 M. All predominant peaks were collected and tested against antisera specific to individual toxin components. Fractions identified as containing PA or LF were diluted 2-fold in an equal volume of 20 mM-triethanolamine/NaOH buffer, pH 8.0, and concentrated by re-binding to a 1 ml Mono-Q column, followed by pulse elution in 0.3 M-NaCl in the same buffer. Fractions containing the EF component were also rechromatographed in this manner, but with the inclusion of 0.5 mM-Triton X-100 in the running buffers.

Gel-filtration f.p.l.c. Samples (1.0 ml, 1.0 mg/ml) were loaded in 0.1 M-Tris/HCl buffer (pH 8.0)/1.0 mM-EDTA/50 mM-NaCl with a flow rate of 0.4 ml/min. For purification of EF, 0.5 mM-Triton X-100 was included in running buffers.

Hydrophobic-interaction f.p.l.c. All components were brought to their final stage of purity by hydrophobic-interaction chromatography on a 1 ml phenyl-Superose HR 5/5 column (10 mg total protein capacity). On these columns the hydrophobic matrix consists of phenyl groups covalently bound to a Superose-12 support (Pharmacia). Samples of up to 6 mg of protein were diluted 2-fold in an equal volume of 20 mM-triethanolamine/NaOH buffer, pH 8.0, containing 3.4 M- $(\text{NH}_4)_2\text{SO}_4$, and loaded on to the column from a 10 ml Superloop (Pharmacia). Protein was eluted in a 20 ml linear gradient of decreasing $(\text{NH}_4)_2\text{SO}_4$ concentration. The predominant peak in each elution profile was collected for assessment of activity and purity.

Production of antiserum

Antisera to individual toxin components purified by conventional techniques [3] were raised in rabbits by subcutaneous injection of 200 μg of protein in 0.2 ml of phosphate-buffered saline (0.1 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4) plus 0.15 ml of Freund's complete adjuvant at week 0. Rabbits were boosted intramuscularly at week 1 with the same antigen preparation and at week 2 with Freund's incomplete adjuvant with the same antigen content. At week 3 rabbits were injected subcutaneously with 200 μg of antigen alone in 0.2 ml of phosphate-buffered saline. Animals were bled for antisera from the marginal ear vein at week 6. Initial toxin components were kindly supplied by Dr. S. H. Leppa, U.S. Army Medical Research Institute for Infectious Diseases, Fort Detrick, MD, U.S.A.

Purity assessment

Purity was determined by double immunodiffusion [13] against a 40%-satn- $(\text{NH}_4)_2\text{SO}_4$ -precipitated globulin fraction of hyperimmune horse antiserum raised against the live-spore anthrax vaccine (Anvax; Wellcome). Samples of toxin components were also analysed by polyacrylamide-gel electrophoresis (PAGE).

SDS/PAGE was done under reducing conditions (50 μM -dithiothreitol) on gradient slab gels (4–40% polyacrylamide; Pharmacia) [14]. Gels were diffusion-stained overnight in ethanol/acetic acid (5:1, v/v) containing 0.1% Brilliant Blue R-250 (Sigma Chemical Co.) and destained electrophoretically in methanol/acetic acid (5:2, v/v). M_r values of proteins were estimated by using Pharmacia low- M_r and high- M_r standards. Protein was measured spectrophotometrically by the method of Warburg & Christian [15] on crude material, and by the method of Lowry *et al.* [16] on purified material with bovine serum albumin (Sigma Chemical Co.) as a standard.

Toxic activity

The adenylate cyclase activity of EF [3] was assayed by incubating a range of EF concentrations (0.2–3 ng/ml) in 50 mM-Tris/HCl buffer, pH 7.5, containing 1 μM - MgCl_2 , 1 μM - CaCl_2 , 1 μM - MnSO_4 , 5 mM-5'-ATP and 20 units of calmodulin/ml for 60 min. Samples (50 μl) of each dilution were then assayed for cyclic AMP content by a competitive assay system kit containing tritiated cyclic AMP (Amersham International, Amersham, Bucks., U.K.).

Activities of PA and LF were shown by a mouse lethality assay. Groups of eight mice were injected intravenously via the tail vein with 120 μg of PA, 25 μg of LF or 200 μg of a PA/LF mixture (5:1, w/w) [8] in 0.5 ml of phosphate-buffered saline. Deaths occurring within 5 days were recorded.

RESULTS

Separation of toxin components by anion-exchange f.p.l.c.

The anion-exchange procedure on the Mono-Q HR 16/10 column readily separated the three toxin components with a high degree of reproducibility. This allowed direct identification of PA, EF and LF elution peaks (Fig. 1). SDS/PAGE showed each peak to contain

Fig. 1. Mono-Q (QAE) HR 16/10 f.p.l.c. chromatogram of crude anthrax toxin with SDS/PAGE gel of pooled fractions

For chromatography details see the Materials and methods section. Photo inset: lane 1, low- M_r markers (Pharmacia); lane 2, crude PA; lane 3, crude LF; lane 4, crude EF. —, A_{280} ; ----, NaCl gradient.

Fig. 2. Purification of PA by f.p.l.c. Superose-12 gel filtration (a) and phenyl-Superose HR 5/5 hydrophobic-interaction chromatography (b) with SDS/PAGE gel of pooled fractions

For chromatography details see the Materials and methods section. Photo inset: lane 1, low- M_r markers (Pharmacia); lane 2, crude toxin; lane 3, crude PA (anion exchange); lane 4, crude PA (gel filtration); lane 5, purified PA (hydrophobic-interaction chromatography). —, A_{280} ; ----, $(\text{NH}_4)_2\text{SO}_4$ gradient.

the appropriate toxin-component band, but with considerable amounts of high- M_r and low- M_r contamination. At this stage the EF fraction tended to exhibit cross-reaction with anti-PA serum.

Purification of PA

PA was further purified by sequential gel-filtration and hydrophobic-interaction chromatography. The gel-filtration step was effective in removing most of the low- M_r contaminants, the major elution peak being recovered with a column retention of 118 ml (295 min) (Fig. 2*a*). However, high- M_r proteins were observed running close to the main toxin band (Fig. 2). The final stage in the purification was hydrophobic-interaction chromatography on a 1 ml phenyl-Superose HR 5/5 column (10 mg total protein capacity). The predominant protein peak was eluted at 0.43 M-(NH₄)₂SO₄, and this was collected for assessment of purity (Fig. 2) and activity (Table 1). Typical yields of PA (M_r 85000) are 6–8 mg from a 15-litre batch culture.

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Purification of LF

The same protocol as described for PA above resulted in purification of LF to the same high degree. The LF was eluted from Superose-12 gel filtration with a retention of 123 ml (307.5 min) (Fig. 3*a*). As with PA, this was only successful in eliminating low- M_r contamination (Fig. 3). Hydrophobic-interaction chromatography resulted in elution of a broad peak at 0.3 M-(NH₄)₂SO₄ (Fig. 3*b*), the leading edge of which was collected for assessment of purity (Fig. 3) and activity (Table 1). Typical yields for this toxin component (LF; M_r 87000) are 10–13 mg from a 15-litre culture.

Purification of EF

Although PA and LF could be readily purified by sequential gel-filtration and hydrophobic-interaction chromatography, certain problems were encountered with EF. After the initial separation of the toxin components by anion-exchange chromatography, it was occasionally found that the EF fraction contained PA-immunoreactive activity. Under a variety of elution

Table 1. Mouse lethality assay for detection of PA and LF activity

Group	Toxin component injected (μ g)		Survivors per group
	PA	LF	
1	125	—	8/8
2	—	25	8/8
3	125	25	0/8

Fig. 3. Purification of LF by f.p.l.c. Superose-12 gel filtration (a) and phenyl-Superose HR 5/5 hydrophobic-interaction chromatography (b) with SDS/PAGE gel of pooled fractions

For chromatography details see the Materials and methods section. Photo inset: lane 1, low- M_r markers (Pharmacia); lane 2, crude toxin; lane 3, crude LF (anion exchange); lane 4, crude LF (gel filtration); lane 5, purified LF (hydrophobic-interaction chromatography). —, A_{280} ; ----, (NH₄)₂SO₄ gradient.

Fig. 4. Purification of EF by f.p.l.c. Superose-12 gel filtration (a) and phenyl-Superose HR 5/5 hydrophobic-interaction chromatography (b) with SDS/PAGE gel of pooled fractions

For chromatography details see the Materials and methods section. Photo inset: lane 1, low- M_r markers (Pharmacia); lane 2, crude toxin; lane 3, crude EF (anion exchange); lane 4, crude EF (gel filtration); lane 5, purified EF (hydrophobic interaction chromatography). —, A_{280} ; ----, $(\text{NH}_4)_2\text{SO}_4$ gradient.

conditions gel filtration resulted in the elution of a broad diffuse protein peak in the void volume of the column, with no apparent peak separation. As EF is a calmodulin-dependent adenylate cyclase [3], affinity and dye-ligand chromatography were attempted, based on specific interaction of the enzyme with its substrate or chemical analogues [17]. A series of possible 5'-ATP affinity ligands (C-8-bound, N^6 -amino-group-bound and ribosyl-hydroxy-group-bound) and a range of operating conditions (varied pH, Mn^{2+} ions, Mg^{2+} ions, different buffer species and concentrations) were investigated, but no significant binding of EF was obtained, over 90% of the protein being eluted in the wash fractions. Similar results were obtained by using calmodulin-agarose under a variety of experimental conditions.

The textile dye Cibacron Blue F-3GA (I.C.I.) bound to Sepharose 4B (Pharmacia) has been used successfully in the purification of several nucleotide-dependent enzymes [18–20] and of calf brain adenylate cyclase [17]. Although it was found that EF could be reproducibly bound at high protein/dye ratios with salt concentrations of up to 0.5 M- Cl^- in the equilibration buffer and in the presence of 10 mM- Mg^{2+} ions, no effective method of elution could be found with the mild conditions preferred for recovery of an active enzyme.

Small amounts of EF were eluted by mild chaotroph treatment (0.1 M-KSCN) or with 20 mM-EDTA and 10 mM-5'-ATP if the column had initially been over-

loaded and EF had been detected in the wash fractions; increasing salt concentrations up to 2.0 M- Cl^- were ineffective. Organic solvents and denaturing agents such as 8 M-urea or 40% (w/w) ethylene glycol were avoided.

It was suspected that hydrophobic interactions were the cause of elution problems, and hence 0.5 mM-Triton X-100 was included in the running buffers. Re-chromatography of EF on Mono-Q then resulted in elimination of cross-reaction with anti-PA serum. Consequently crude EF was treated by Triton X-100 anion exchange and gel filtration, from which it was eluted at 90 ml (225 min) (Fig. 4a). Detergent was removed by re-binding the protein to the Mono-Q HR5/5 column, washing in 20 mM-triethanolamine/NaOH buffer, pH 8.0, and eluting in 0.3 M-NaCl in the same buffer. EF was then further purified by phenyl-Superose hydrophobic-interaction chromatography (Fig. 4b), as used for purification of PA and LF. Purification of the enzyme (M_r 86000) by this method typically yields 4–7 mg from an initial culture of 15 litres.

Purity assessment and toxic activity

By using the protocols described, all three known toxin components can be purified to at least 90% homogeneity in an active form (Fig. 5 and Table 1), with EF having a specific activity of 27.5 $\mu\text{kat}/\text{mg}$ of protein. The proteins have also been shown to be free of immunological cross-reaction by double immuno-

Fig. 5. SDS/PAGE gradient gel of f.p.i.c.-purified anthrax-toxin components

Lane 1, low- M_r standards (Pharmacia); lane 2, PA; lane 3, LF; lane 4, EF.

Fig. 6. Ouchterlony double-immunodiffusion gel of f.p.i.c.-purified toxin components

Wells 1 and 4, PA; wells 2 and 5, EF; wells 3 and 6, LF; central well contains 40% satn.-(NH₄)₂SO₄ globulin fraction of horse anti-(live-spore vaccine) (Anvax; Wellcome) serum.

diffusion (Fig. 6), and this has been confirmed by enzyme-linked immunosorbent assay techniques.

DISCUSSION

We have detailed here a procedure for production of the three separate anthrax-toxin components that, we believe, provides the highest degree of purity yet achieved by laboratories working in this field. Furthermore, the speed and resolution of the system make it extremely simple to use on a routine basis for recovery of active biomolecules, and considerably decrease the labour intensity of conventional chromatographic techniques, with minimal losses of labile components. This is largely facilitated by employing the same chromatographic parameters for each of the proteins in question. Only the

anion-exchange chromatography of EF requires column re-equilibration. The effects of doing the entire anion-exchange steps in 0.5 mM-Triton X-100 have not yet been investigated and may serve to decrease production time even further. The high reproducibility allows direct selection of relevant protein fractions without the need for activity assay or immunological detection during the earlier stages of preparation.

Yields from a 15-litre batch growth are currently of the order of 8 mg of PA, 13 mg of LF and 7 mg of EF, which are adequate for use in sensitive enzyme-linked immunosorbent assay detection of anti-toxin antibodies, as standards for the detection of specific serum antigens in suspected cases of infection and for use in vaccine trials.

We gratefully acknowledge the support of the British Ministry of Defence Procurement Executive for the funding of this work. We thank Dr. S. H. Leppla, of the U.S. Army Medical Institute for Infectious Diseases, for kindly providing an initial supply of anthrax-toxin components, and Mr. J. A. Carman for advice and technical assistance in toxin production.

REFERENCES

- Smith, H., Keppie, J. & Stanley, J. (1955) *Br. J. Exp. Pathol.* **36**, 460–472
- Zwartouw, H. & Smith, H. (1956) *Biochem. J.* **63**, 437–442
- Leppla, S. (1984) *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **17**, 189–198
- Turnbull, P. C. B. (1986) *Abstr. Hyg. Trop. Dis.* **61**, R1–R9
- Thorne, C. B., Molnar, D. M. & Strange, R. E. (1959) *J. Bacteriol.* **79**, 450–455
- Ristroph, J. & Ivins, B. (1983) *Infect. Immun.* **39**, 483–486
- Leppla, S., Ivins, B. & Ezzell, J. (1985) in *Microbiology—1985* (Lewe, L., ed.), pp. 63–66, American Society for Microbiology, Washington
- Ezzell, J., Ivins, B. & Leppla, S. (1984) *Infect. Immun.* **45**, 761–767
- Johnson-Winegar, A. (1984) *J. Clin. Microbiol.* **20**, 357–361
- O'Brien, J., Friedlander, A., Dreier, T., Ezzell, J. & Leppla, S. (1985) *Infect. Immun.* **47**, 306–310
- Advisory Committee on Dangerous Pathogens (1984) *Categorisation of Pathogens according to Hazard and Categories of Containment*, pp. 11–13, HSE, Bootle
- Thorn, C. B. & Belton, F. C. (1957) *J. Gen. Microbiol.* **17**, 505–509
- Ouchterlony, Ö. (1964) in *Immunological Methods* (Ackroyd, J. F., ed.), pp. 168–169, Blackwell Scientific Publications, Oxford
- Tse, C. K., Dolly, J. O., Hambleton, P., Wray, D. & Melling, J. (1982) *Eur. J. Biochem.* **122**, 493–500
- Warburg, J. K. & Christian, C. W. (1941) *Biochem. Z.* **310**, 384–386
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Stellwagen, E. & Baker, B. (1976) *Nature (London)* **261**, 719–720
- Kopperschlager, G., Bohme, H.-J. & Hofman, E. (1982) *Adv. Biochem. Eng.* **25**, 101–138
- Subramian, S. (1983) *Crit. Rev. Biochem.* **16**, 169–205
- Lowe, C. R. & Pearson, J. C. (1984) *Methods Enzymol.* **104**, 97–113