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Note

Rapid purification of diphtheria toxin by Phenyl Sepharose and DEAE-cellulose chromatography

R. RAPPUOLI*, M. PERUGINI, I. MARSILI and S. FABBIANI
Sclavo Research Center, Via Fiorentina 1, 53100 Siena (Italy)
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Diphtheria toxin is a protein of molecular weight 62,000 daltons which is produced by strains of *Corynebacterium diphtheriae* lysogenic for phages β (ref. 1) or ω (ref. 2) which carry the tox structural gene. These lysogens secrete the protein into the culture medium where it can reach up to 70% of the total protein. Owing to its abundance in the culture supernatants, purification of diphtheria toxin is not a great problem and for most purposes (including production of antidiphtheria vaccines) a rough purification procedure such as ammonium sulphate fractionation[3,4] or trichloroacetic acid precipitation[5] is sufficient. Highly purified diphtheria toxin can be obtained by ion-exchange chromatography on a DEAE column[6]. However, the DEAE-cellulose binds proteins at low ionic strength and is not able to absorb the toxin from the supernatant, making an ammonium sulphate precipitation and an extensive dialysis of the resuspended pellet necessary before the DEAE step. This is time-consuming and difficult to perform on large volumes. The method we propose takes advantage of the property of the hydrophobic resin "Phenyl Sepharose" which, being able to bind proteins at high ionic strength, absorbs the toxin directly from the culture supernatant. The toxin is then eluted in a small volume which, diluted 1:3, has an ionic strength low enough to be loaded on to the DEAE column. The entire purification procedure can be carried out in a few hours, can handle small or large volumes and gives highly purified diphtheria toxin (97%).

EXPERIMENTAL

Diphtheria toxin production

Cultures of C7(ω^{tox+})16 (ref. 7) were grown in 2-l flasks containing 100 ml of CY medium[8] with shaking for 24 h. Larger preparations were carried out in a 5-l Biolafitte fermentor. Bacteria were removed by centrifugation and the diphtheria toxin released in the supernatant was determined by rocket immunoelectrophoresis[9] and/or by flocculation[10]. Routinely the supernatants contained 70–100 Lf/ml (1 Lf = 2.5 μg of diphtheria toxin antigen)[8]. Protein nitrogen was determined by the Kjeldahl semimicro method, according to the U.S. Pharmacopeia[11].

Phenyl Sepharose

Phenyl Sepharose (Pharmacia, Uppsala, Sweden) was washed extensively with

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distilled water and then poured into a column with a ratio of diameter to height of about 3, to allow high flow-rates. To increase the gel-binding capacity, 13 g per 100 ml of ammonium sulphate was added to the culture supernatant which was then passed through the column. Under the above conditions, the resin was able to bind 10 mg (4000 Lf) of toxin per ml of gel. However, in our experiments we did not usually use more than half of the gel capacity. When all the toxin had been adsorbed on to the gel, the column was washed with two volumes of 0.6 *M* ammonium sulphate, 6 m*M* phosphate buffer (pH 7.5) (obtained by diluting with distilled water a stock solution of 1 *M* ammonium sulphate and 10 m*M* phosphate, pH 7.5) and then eluted with 10 m*M* sodium chloride, 10 m*M* phosphate buffer. After extensive washing with distilled water the column was ready to be reused.

DEAE-cellulose

DEAE-cellulose (preswollen ion-exchange DE-52, Whatman, U.K.) was washed with 1.5 *M* sodium chloride, 0.5 *M* Tris (pH 7.5) until the pH of the eluate was 7.5. It was then suspended in 10 m*M* phosphate (pH 7.5), 10 m*M* sodium chloride, poured into a column (of high diameter-to-height ratio) and washed with the same buffer. The toxin eluted from Phenyl Sepharose was diluted 1:3 with distilled water and loaded on to the DEAE column which was then washed with two volumes of 50 m*M* sodium chloride, 0.5 m*M* phosphate (pH 7.5) and eluted with 110 m*M* sodium chloride, 1 m*M* phosphate. The column was then extensively washed with 1 *M* sodium chloride, 10 m*M* phosphate (pH 7.5), equilibrated with 10 m*M* phosphate (pH 7.5) and was again ready for use. All the above solutions at different ionic strength were obtained by diluting with distilled water a stock solution of 1 *M* sodium chloride, 10 m*M* phosphate (pH 7.5). (Some dark brown pigments stick to the top of the column and do not come off with the above washings, but they do not interfere with subsequent purification procedures.)

SDS polyacrylamide gel electrophoresis

The method of Laemmli[12] was used to prepare 12.5% polyacrylamide gels. To detect low levels of contaminants, each well was loaded with large amounts of proteins (up to 100 μg per well). Gels were then stained with Coomassie Blue G 250 and the intensity of each band was determined by the LKB 2202 ultrascan laser densitometer equipped with the LKB 2220 recording integrator.

RESULTS

Phenyl Sepharose

Fig. 1 shows the toxin adsorption and elution from the gel as monitored by rocket immunoelectrophoresis: after addition of ammonium sulphate (13 g per 100 ml) the culture supernatant (well 1) was loaded on to the Phenyl Sepharose column. No toxin was found in the culture supernatant eluting from the column or in the 0.6 *M* ammonium sulphate washing buffer (wells 2 and 3, respectively). The column was then eluted with 10 m*M* sodium chloride, 10 m*M* phosphate buffer (pH 7.5) (wells 4–10) and the fractions containing the toxin (4–7) were pooled.

Although the toxin eluted from Phenyl Sepharose does not seem to be purer than the toxin in the culture supernatant when analysed by SDS polyacrylamide gel

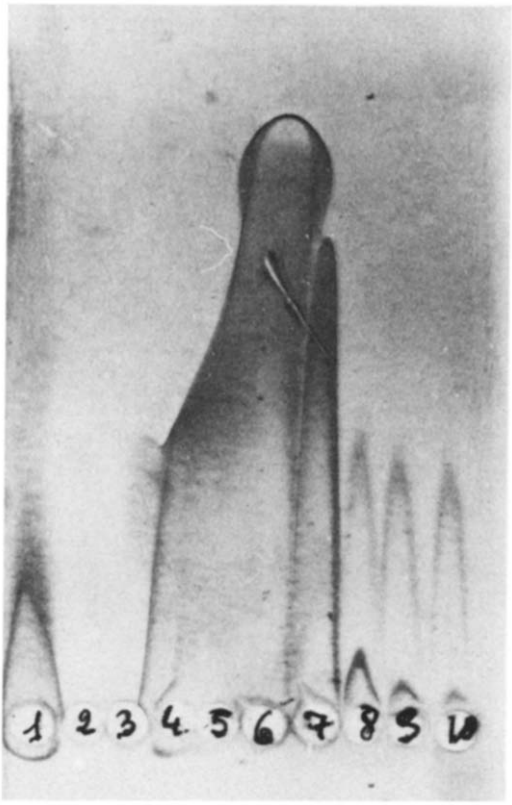

Fig. 1. Rocket immunoelectrophoresis showing the binding and elution of diphtheria toxin from Phenyl Sepharose. 1 = Culture supernatant with the addition of 13 g per 100 ml of ammonium sulphate; 2 = Culture supernatant eluting from the column; 3 = washing with 0.6 *M* ammonium sulphate. 4–10 = elution of diphtheria toxin with 10 m*M* sodium chloride, 10 m*M* phosphate buffer.

electrophoresis (Fig. 2, lanes B and A, respectively), during this process most of the small polypeptides (peptones) present in the culture medium are removed: the toxin concentration (150 Lf per mg of protein nitrogen in the culture supernatant) was increased to 1600 Lf per mg of protein nitrogen by passage through the Phenyl Sepharose column. Furthermore, most of the pigments were removed and the volume was reduced about twenty times. Free of the peptides, pigments and salts which would compete with the toxin for binding to the ion-exchange resin, this toxin was suitable for the DEAE step.

DAEA-cellulose

The toxin eluted from Phenyl Sepharose diluted three times with distilled water (Fig. 3, well 1) was adsorbed on to the DEAE-cellulose column (wells 2–4) and washed with 50 m*M* sodium chloride (wells 5–8). Pure toxin was then eluted with 110 m*M* sodium chloride (wells 9–16) and the remaining proteins were eluted from the column with 1 *M* sodium chloride, 10 m*M* phosphate buffer (wells 17–20). Fractions 9–16 were pooled. The purity of the toxin obtained was analysed by gel electropho-

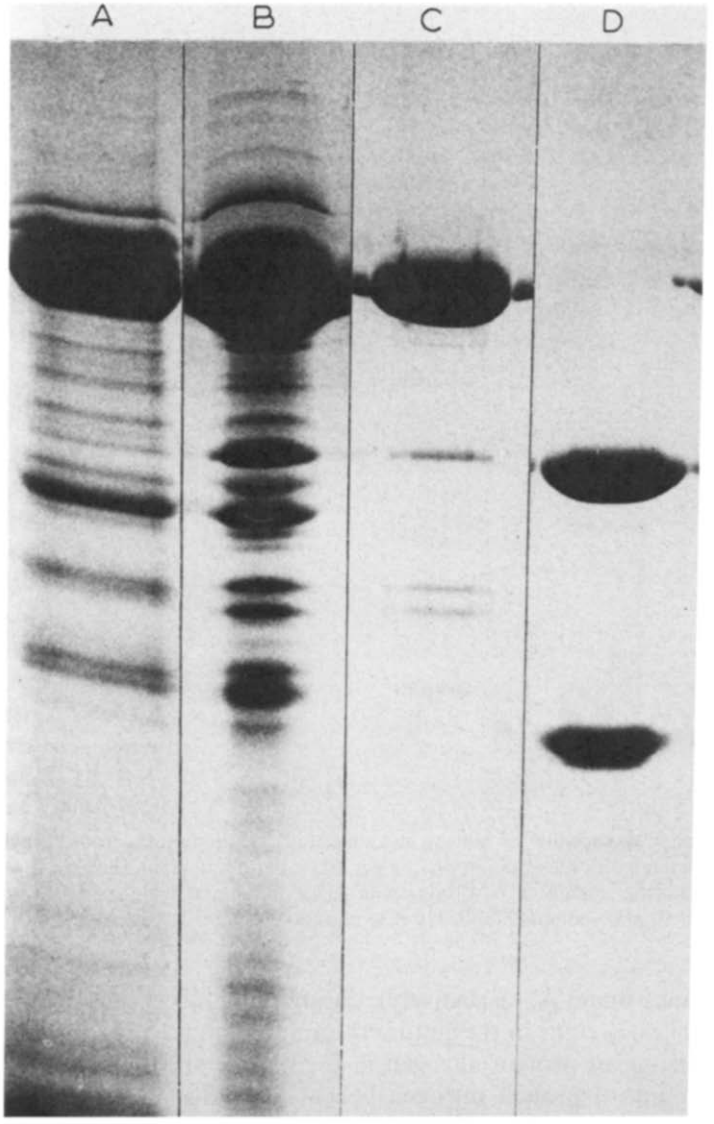

Fig. 2. SDS polyacrylamide gel electrophoresis showing diphtheria toxin from the culture supernatant (A), after elution from Phenyl Sepharose (B), after purification with DEAE-cellulose (C) and divided into fragment A and fragment B by mild trypsin treatment (D).

resis (Fig. 2, lane C). As shown, the toxin is pure: as determined by gel scanning, 94% of the total proteins were intact toxin, 3% nicked toxin and *ca.* 3% contaminating proteins. The above purity was confirmed by the determination of flocculation units which were 2400 Lf per mg of protein nitrogen (96%). After mild treatment with trypsin[6], the toxin could be cleaved into the characteristic fragments A and B (Fig. 2, lane D).

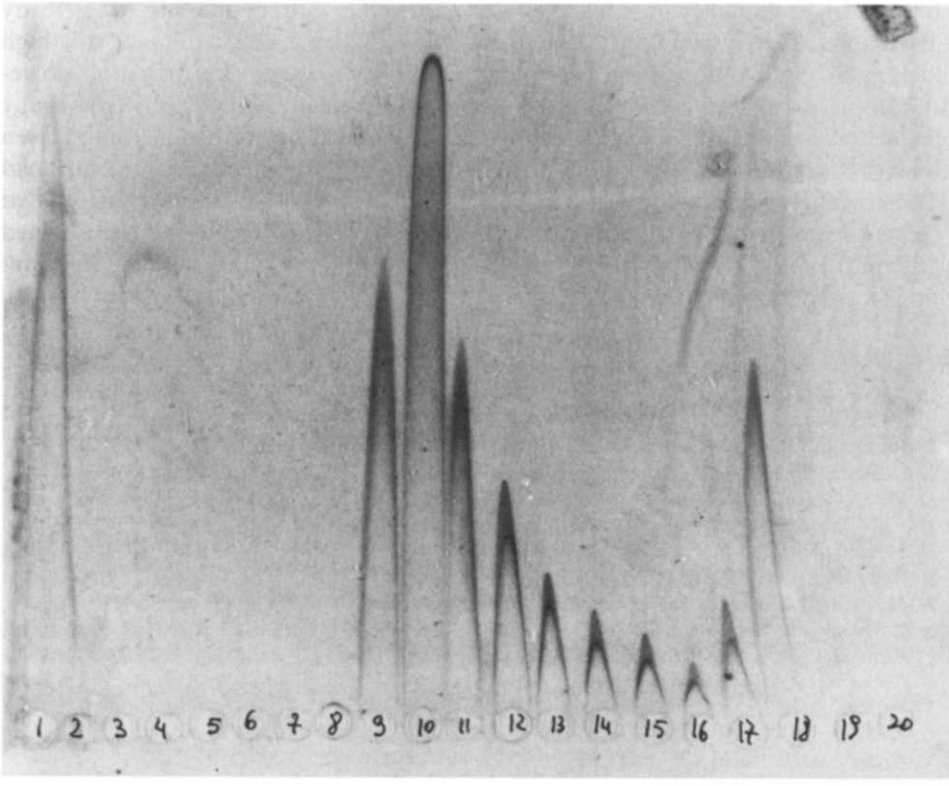

Fig. 3. Rocket immunoelectrophoresis showing the purification of diphtheria toxin on DEAE-cellulose. 1 = Toxin eluted from Phenyl Sepharose diluted three times with distilled water; 2–4 = eluate from the column; 5–8: washing with 50 m*M* sodium chloride; 9–16 = toxin elution with 110 m*M* sodium chloride; 17–20 = column regeneration with 1 *M* sodium chloride.

DISCUSSION

Hydrophobic chromatography has already been used by Nitzan and Michalsky[13] for purification of diphtheria toxin; however, the gel they used [Sepharose–NH–$(CH_2)_5$–CH_3] is not available on the market and is not completely hydrophobic since it contains the –NH– group. Phenyl Sepharose is a commercially available CL-4B Sepharose containing hydrophobic phenyl groups which is able to bind most of the proteins at high ionic strength and release them at low ionic strength. Although it has been used for fine purification of proteins, we find it very useful for a less sophisticated purpose, substitution of ammonium sulphate fractionation. This step, used in almost all protein purification procedures is particularly tedious and time-consuming when dealing with large volumes: in fact, it involves centrifugation of many litres of culture and, furthermore, extensive dialysis of the resuspended pellet is needed to remove all the salts which would otherwise inhibit the binding of the proteins to DEAE-cellulose.

In the method we propose, all this has been substituted by a passage of the culture supernatant through a Phenyl Sepharose column. This concentrates down the

toxin to a small volume which, once diluted three times with distilled water, is ready to be loaded on to the DEAE-cellulose column. When using columns with high diameter-to-height ratios which allow high flow-rates, the entire purification procedure can be carried out in a few hours. The toxin obtained is completely free of any pigment and its purity is comparable with that obtained by other methods. Even cleaner preparations (up to 99%) can be obtained if DEAE-Sepharose is used instead of DEAE-cellulose. The above method was succesfully used to purify other related proteins such as cross-reacting material 197[14], and we feel that it may be adapted for the purification of many other proteins where the ammonium sulphate fractionation is a limiting step.

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