# RECONSTITUTION OF FULLY ACTIVE DIPHTHERIA TOXIN FROM PURIFIED FRAGMENTS A AND B

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## 1. Introduction

The structure–function relationship between the constituent fragments of diphtheria toxin is now well understood. Fragment A (mol. wt 21 150) catalyses the transfer of the ADP-ribose moiety from NAD to elongation factor 2 (EF2), causing inhibition of protein synthesis [1]. Fragment B (mol. wt 40 850) is itself nontoxic but is responsible for the specific binding of diphtheria toxin to susceptible cells and, by some as yet undetermined mechanism, allows fragment A to traverse the plasma membrane and exert its action in the cytoplasm [2,3].

The highly stable fragment A can be easily purified in an active form. In contrast, fragment B is very unstable and has been reported to be soluble only in the presence of urea or other protein denaturants [4]. Recently, however, several groups have succeeded in purifying stable fragment B possessing some biological activity as judged by its ability to interfere with the binding of intact toxin to cells [5,6].

However, there are no reports to date of the preparation of stable fragment B and its subsequent combination with fragment A to reconstitute fully active diphtheria toxin. Modified fragment B was shown [7] to facilitate the uptake of noncovalently associated fragment A into approx. 60% of HeLa cells on monolayers, depending on the molar ratios used. However, the toxicity achieved represented less than 3% of that of native toxin.

We report for the first time the purification of stable, biologically active diphtheria toxin fragment B by *S*-sulphonation [6] and its combination, by the asymmetric disulphide synthesis [8], with reduced fragment A to form a reconstituted toxin molecule possessing full activity.

## 2. Materials and methods

### 2.1. *Diphtheria toxin*

This was a gift from Dr R. O. Thomson, Wellcome Research Laboratories, Beckenham. The toxin was almost completely nicked.

*S*-Sulphonated fragment B ($BSSO_3$) was prepared from 50 mg diphtheria toxin as in [6], fractionated by Sephadex G-100 gel chromatography and shown to be pure by SDS–polyacrylamide gel electrophoresis [9]. The receptor binding activity of $BSSO_3$ was judged by its ability to bind to receptors on HeLa cells and so inhibit the subsequent binding and toxin effect of diphtheria toxin in the toxicity test system described below.

Reduced fragment A (ASH) was prepared from 50 mg diphtheria toxin in 5 ml 0.4 M Tris–HCl, pH 7.5, buffer by reductive cleavage with dithiothreitol [7]. The mixture was then centrifuged to remove denatured fragment B and ASH purified from the supernatant by Sephadex G-100 gel chromatography. Purified ASH was demonstrably active by its ability to inhibit the synthesis of proteins from tritiated amino acids in a HeLa cell-free system [10].

Whole toxin was reconstituted by mixing 24.6 mg $BSSO_3$ and 14.4 mg ASH in 0.4 M Tris–HCl, pH 6.5, buffer containing 0.15 M $SrCl_2$ as a sulphite trap (molar ratio, 1 $BSSO_3$ : 1.12 ASH, total vol. 10 ml). The reaction mixture was then deaerated and purged

with oxygen-free nitrogen gas several times, and finally incubated at 4°C for 6 days [8]. The mixture was then applied to a Sephadex G-100 superfine column and eluted with 0.4 M Tris–HCl, pH 7.5, buffer. Further purification of reconstituted toxin was achieved by chromatography on a Sephadex G-200 superfine column.

2.2. *Toxicity of reconstituted toxin*

Toxicity testing was carried out on dispersed monolayers of HeLa cells in wells of microtitre plates (NUNC N1480) [7]. All test solutions were dialysed against growth medium (MEM), millipore filtered, then added to cells for 15 min. At the end of this exposure period cells were washed twice with MEM and subsequently incubated for 24 h. Cells were then counted and survival expressed as a percentage of MEM-treated cells.

2.3. *Protein determinations*

Protein concentrations in solutions were determined either by $A_{280}$ or by the Folin method [11]. The relative amount of reconstituted toxin in pooled eluate from gel chromatography was determined by densitometry at 550 nm on stained SDS–polyacrylamide gels.

## 3. Results and discussion

3.1. *Biological activity of $BSSO_3$*

Purified $BSSO_3$ was biologically active in that it bound to HeLa cells and blocked the subsequent binding of native diphtheria toxin in the cytotoxicity test system described above (table 1). The inhibition of protein synthesis observed [6] when HeLa cells were treated for 3 h with diphtheria toxin, however, was unaffected by simultaneous treatment with $BSSO_3$; from this it was deduced [6] that $BSSO_3$ does not bind to HeLa cells.

The apparent discrepancy between our findings and [6] may well reflect the differences in the test systems employed. We feel that pre-incubation of cells with $BSSO_3$ affords less equivocal results than does incubation with a mixture of intact toxin and fragment B [5,6] as it demonstrates that interference with the toxic activity of diphtheria toxin requires the binding of fragment B to the cell surface and is not merely a consequence of some interaction between fragment B and intact toxin in solution.

Table 1
Receptor binding activity of *S*-sulphonated fragment B

| Pre-incubation with $BSSO_3$ (μg/ml) | Challenge with diphtheria toxin (μg/ml) | HeLa cells killed (%) |
|---|---|---|
| – | 1 | 50.1 |
| 100 | 1 | 18.6 |
| 10 | 1 | 28.3 |
| 1 | 1 | 32.5 |

HeLa cell monolayers were treated for 15 min at 37°C with varying concentrations of $BSSO_3$ in MEM, then washed twice with MEM and immediately treated for 15 min at 37°C with native diphtheria toxin in MEM as described in section 2. The diphtheria toxin solution used in this experiment had an $LD_{50}$ higher than that of fresh, native toxin (fig.2) due to prolonged storage at 4°C. $BSSO_3$ alone was nontoxic at the concentrations used

3.2. *Purification and properties of reconstituted toxin*

Toxin was reconstituted as described and Sephadex G-100 chromatography of the reaction mixture yielded three protein peaks, corresponding to fragment A, fragment B and a mixture of high molecular weight reaction products. The latter was shown by SDS–polyacrylamide gel electrophoresis to contain proteins of mol. wt 80 000, 61 000 and 43 000, and corresponded to fragment B dimer, intact toxin and fragment A dimer, respectively (fig.1). Reductive cleavage of these reconstitution products with 2-mercaptoethanol showed them to be composed only of fragments A and B. Densitometry of SDS–polyacrylamide gels revealed that approx. 15% $BSSO_3$ in the reaction mixture had combined with ASH to reconstitute whole toxin.

Fractionation of the high molecular weight material on a Sephadex G-200 superfine column yielded pure reconstituted toxin (fig.1). The purified toxin had an $LD_{50}$ for HeLa cells of 1.25 μg/ml, i.e., approximately the same as that of native toxin (fig.2). Fragments $BSSO_3$ and ASH alone were nontoxic to HeLa cells at the concentrations used in this experiment.

In a second experiment, using freshly prepared $BSSO_3$ and ASH incubated at 4°C for 3 days,

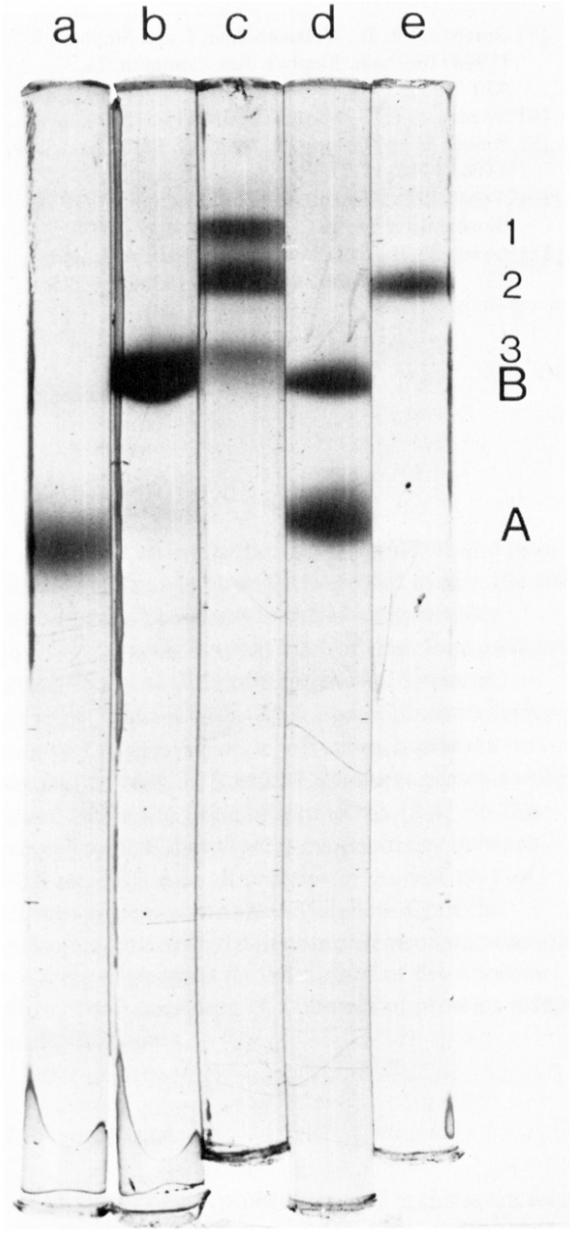


Fig.1. SDS–polyacrylamide gel electrophoresis of (a) ASH; (b) $BSSO_3$; (c) high molecular weight reconstitution reaction products, comprising (1) fragment B dimer, (2) reconstituted toxin, (3) fragment A dimer*; (d) Reconstitution reaction products treated with 2-mercaptoethanol, yielding fragments A and B only; (e) Purified reconstituted toxin.

* This species was considered to be fragment A dimer rather than fragment B by virtue of both its electrophoretic mobility and an examination of the relative amounts of fragments A and B yielded by reductive cleavage of the reconstitution reaction products with 2-mercaptoethanol (see (d))

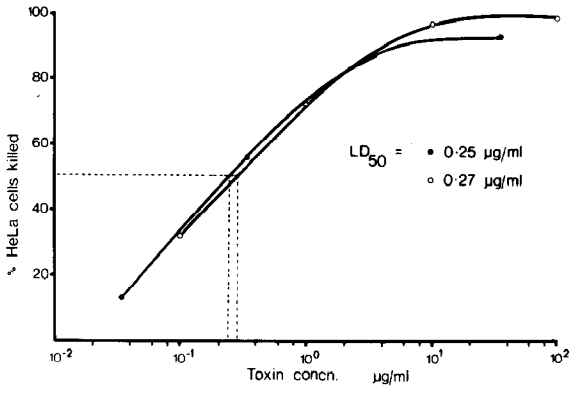


Fig.2. Titration of toxicity of native and reconstituted diphtheria toxin for HeLa cell monolayers. (○) Native diphtheria toxin. (●) Reconstituted toxin.

approx. 45% toxin fragments in the reaction mixture recombined to form fully active toxin.

Thus, we have succeeded in purifying a stable, biologically active preparation of diphtheria toxin fragment B as judged by its ability to combine with reduced fragment A to produce fully active reconstituted toxin.It may therefore prove possible to link fragment B prepared in this manner to other proteins in which there exists or into which has been introduced a functional sulphydryl group, and thereby to effect the uptake of these proteins into animal cells. Such a technique would be of great potential value in the field of cell biology as a means of introducing various macromolecules into cells, and thus of modifying cell activity, without causing gross perturbations in the cell membrane.

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