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Purification of Shiga toxin by α-D-galactose-(1→4)-β-D-galactose-(1→4)-β-D-glucose-(1→) receptor ligand-based chromatography

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A simple and rapid method for Shiga toxin purification based on specific binding to the Galα1→4Galβ1→4Glc globotrioside trisaccharide covalently linked to polyacryl/polyvinyl (Fractogel) has been developed A cell-free sonicate-filtrate of *Shigella dysenteriae* type 1, strain 114Sd was passed over the globotrioside-Fractogel column, and bound toxin eluted with 6 M guanidine-HCl A yield of 36 mg pure toxin/l sonicate-filtrate was obtained, i e a one step 1224-fold purification The recovery of biologically active toxin was 87% The toxin was purified to homogeneity as shown by native PAGE and SDS-PAGE

Shiga toxin, Purification, Globotriose, Receptor, Chromatography, (*Shigella dysenteriae*)

1. INTRODUCTION

The toxin produced by *Shigella dysenteriae* type 1 bacteria, and a few *Escherichi coli* strains, is named Shiga toxin (also Shiga-like toxin or Vero toxin) [1–4]. The holotoxin has a molecular mass of 70 500 and is composed of one A subunit with a molecular mass of 30 500 [5], and several B subunits with a molecular mass of 7691 [6]. The A subunit is the active component and inactivates the 60-S ribosomal subunits [7] by depurination of a single adenosine residue in 28-S RNA [8]. The B subunit binds to receptors on mammalian surfaces, and the Galα1→4Gal disaccharide (galabiose) in a terminal position in glycolipids has been shown to be a binding domain [9]. The toxin is produced when *S. dysenteriae* type 1 strains are grown in iron-depleted medium [10]. The toxin has been purified in processes involving precipitation with ammonium sulphate, ion exchange, gel chromatography and immuno-affinity chromatography [5,11–14]. These processes are cumbersome and reported yields have been low. We here report on the purification of the Shiga toxin by affinity chromatography using the trisaccharide Galα1→4Galβ1→4Glc, (globotriose) covalently linked to a polyvinyl/polyacryl gel.

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Abbreviations MEM, minimum essential medium; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; ^{125}I, ^{125}iodine; Glycolipid nomenclature is according to ICBN, Eur J Biochem (1977) 79, 11–21 and J Biol Chem (1982) 257, 3347–3351

2. MATERIALS AND METHODS

2 1 *Bacterial strain*

Shigatoxin was prepared from *Shigella dysenteriae* type 1, strain 114Sd, which is an auxotrophic mutant because of a transposon Tn 10 inactivated *aroD* gene (unpublished).

2 2 *Medium and culture conditions*

A 12-l fermentor (LKB, Stockholm, Sweden) containing 10 l iron-depleted modified syncase medium [10] was inoculated with 2 l of an overnight starter culture Bacterial growth was allowed to continue for 20 h at 37°C (pH 8 0), with an aeration of 6 l/min The cells were harvested in a Sorvall RC-5B centrifuge at 4°C and 10 000×*g* for 30 min, washed twice with phosphate-buffered saline, dirupted by sonication (Branson Sonifier Cell Disrupter B 15) The sonicate (50 ml) was centrifugated (4°C, 10 000×*g*, 45 min), the supernatant decanted and then passed through a Millipore filter (pore size, 0 45 μm) The filtrate is referred to as the crude toxin fraction

2.3 *Preparation of glycosylated gels and toxin purification*

Fractogel TSK AF-Amino 32-63 μm (Merck, Darmstadt, FRG) was suction-dried and washed with methanol To one gram, 100 mg of saccharide and 3 ml of methanol were added in a sealed tube The mixture was heated for 6 h at 80°C under shaking After cooling to 22°C, 3 ml acetic anhydride was added and the mixture left for 24 h at 22°C The gel was filtered off on a glass filter, and washed thoroughly with water Ten saccharides (Galβ1→, GlcNAcβ1→4GlcNAcβ1→, Manα1→3Manβ1→, Galβ1→3GlcNAcβ1→3Galβ1→, NeuNAcα1→3Galβ1→; NeuNAcα2→6Galβ1→; Fucα1→2Galβ1→; Fucα1→2Galβ1→3GlcNAcβ1→Galβ1→, GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→(globotetraose), Galα1→4Galβ1→4Glcβ1→(globotriose)), were covalently linked to Fractogel. For globotriose, 100 mg was added and the resulting gel contained, as shown by sugar analysis, 16 μmol Gal/g and 3 μmol Glc/g The crude toxin fraction was applied to a 5-ml glass pipette column containing 1 ml of the gel equilibrated with phosphate-buffered saline After washing the gel with 25 ml phosphate-buffered saline, bound material was eluted with 6 M guanidine-HCl (pH 6 7), 1 ml fractions were collected and immediately transferred to dialysis tubing for dialysis against 500 ml phosphate-buffered saline with 3 changes every 3 h and then overnight

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2 4 *Analytical methods*

Cytotoxic activity was measured according to the method of Gentry and Dalrymple [14] with the following modifications. 100 μl of toxin containing fractions, serially diluted in 25 mM Hepes-buffered MEM (Gibco, Paisley, Scotland), were added to a microtiterwell containing 2 10^4 Verocells in 100 μl Hepes MEM which had been kept at 0°C for 30 min After 60 min incubation at 0°C, the cells were washed 3 times with MEM to remove unbound toxin, and then incubated at 37°C in 5% CO_2 overnight. The amount of toxin inducing cytotoxicity in 50% of the cellmonolayer (CD_{50}) were recorded

Shiga toxin receptor binding activity was quantitated using a solidphase enzyme-linked immunoreceptor assay (ELIRA) globotriose covalently linked to bovine serum albumin was diluted to 10 μg/ml in phosphate-buffered saline Aliquots (50 μl) were added to wells of a 96-well microtiterplate and incubated in 20°C overnight Wells were emptied and allowed to incubate at 37°C for 30 min with 200 μl of 1% bovine serum albumin in phosphate-buffered saline. After washing 3 times with phosphate-buffered saline, 50 μl of toxin-containing samples, serially diluted in phosphate-buffered saline, were applied and incubated at 20°C for 2 h. Unbound toxin was removed by washing 3 times with phosphate-buffered saline containing 0.1% Tween 20. After incubation with 50 μl of polyclonal rabbit anti-shigatoxin antibodies in phosphate-buffered saline for 2 h at 20°C, the wells were washed 3 times and reincubated with 50 μl of (Fab) fragment anti-rabbit IgG-alkaline phosphate conjugate (Sigma, St Louis, USA) in phosphate-buffered saline, 20°C overnight After washing, enzyme substrate (0.1 mg *p*-nitrophenylphosphate) was added. The plates were incubated at 37°C for 50 min and absorbance at 405 nm was measured.

Iodination of toxin was performed with ^{125}I, using Bolton and Hunter reagent, NEX120 (NEN Research Products, Dreieich, FRG)

SDS and native PAGE were run on Phast System (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions

Protein concentrations were determined by the method of Lowry et al [15] with bovine serum albumin as standard

3. RESULTS AND DISCUSSION

3.1. *Testing of glycosylated Fractogels*

Ten different saccharide-coupled gels were tested for binding of already purified and ^{125}I-labelled shiga toxin (63 ng toxin per 250 μl gelbed). Only the globotriose- and globotetraose-coupled gels retained the toxin, 85% and 72%, respectively. Thus the toxin bound only to glycosylated gels with Galα1→4Gal disaccharides, in accordance with earlier results [9]. No binding of toxin to unsubstituted or to any of the other eight glycosylated Fractogels was observed. Bound toxin was eluted with 6 M guanidine-HCl (pH 6.7) and immediately dialyzed against phosphate-buffered saline. Approximately 60% of bound toxin eluted in the main peak (measured as radioactivity). The toxin retained its cytotoxicity, approaching 100%, when applied on Verocells. Attempts to elute the toxin by increasing the ionic strength (up to 1M NaCl) or lowering the pH (acetate buffer to pH 4.6 or glycine-HCl buffer to pH 2.6) were unsuccessful. Not until 6 M ureas was added to the glycine-HCl buffer did the toxin elute. Selective eluents such as D-galactose (in concentrations up to 0.4 M in phosphate-buffered saline) and globotriose (up to 0.3 mM in phosphate-buffered saline) failed to release the toxin from the globotriose-Fractogel.

3.2 *Preparative purification of Shigatoxin*

Results on the purification of the toxin are summarized in table 1. A cell-free sonicate-filtrate of an overnight culture of *S. dysenteriae* type 1, strain 114Sd, had a cytotoxic activity corresponding to $500 \cdot 10^4$ CD_{50} units/mg protein. Fifty ml of the crude toxin was passed through the globotriaosyl-Fractogel column. The bound toxin was mainly recovered after elution in fraction two, with a cytotoxic activity corresponding to $1.3 \cdot 10^8$ CD_{50} units/mg protein. That means a one step 1224-fold purification with a recovery of 87% and a yield of 36 mg toxin/l cell lysate. A comparison with conventional methods, which are a lot more time-consuming, showed a yield of only 6.25 mg/l cell lysate and 3.6% recovery [11]. Kongmuang et al. [12] reported a Shiga toxin purification based on immunoaffinity, with a 3685-fold purification and a 50% recovery, but their method also included DEAE cellulosa chromatography and ammonium sulphate precipitation. The globotriose affinity purification procedure has been done on four occasions, and the recoveries have varied from 55%–95%, respectively.

Table 1

Purification of Shiga toxin from *Shigella dysenteriae* type 1, strain 114Sd

	Volume (ml)	Protein (mg/ml)	Cytotoxicity $\times 10^4$ (CD_{50}/ml)[a]	Spec act. $\times 10^4$ (CD_{50}/mg)	ELIRA end-point titre $\times 10^4$	Yield (%)
Culture broth	12 000					
Culture supernatant	12 000	0 50	2 0	4.0	<0.02	
Wash cellpellet	300	27	5 0	0 2	110	
Crude toxin fraction	50	51	500	9 8	300	100
Fraction 1	0 9	0.04	0 08	2 0	1.3	<0 1
Fraction 2	1 7	1 06	13 000	12 000	800	87
Fraction 3	1 3	0.08	1 5	19	52	<0 1
Fraction 4	1 3	<0 01	<0 001	-	<0.02	-
Fraction 5	1 3	<0 01	<0 001	-	<0 02	-
Fraction 6	0 8	<0 01	<0.001	-	<0 02	-

[a]CD_{50} is the amount of toxin activity required to reduce dye staining to half the control values, as described in the text

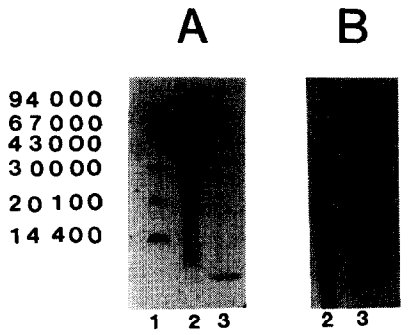

Fig.1. (A) SDS-PAGE, (B) native PAGE. Lane 1 low molecular weight markers (Pharmacia, Uppsala, Sweden), Lane 2 crude toxin fraction and lane 3 fraction two (from Shiga toxin purification from *S. dysenteriae* type 1, strain 114Sd, in this study).

The toxin was also quantified in a Shiga toxin assay based on detection of toxin bound to globotriose-BSA by antibodies (table 1). A good correlation between this assay and cytotoxicity on Vero cells was noted which means that purified toxin was biologically active. A similar correlation was seen with a Shiga toxin preparation (NEZ 3C) purified by conventional methods [11].

The crude Shiga toxin fraction, and eluted affinity-purified fractions, were analysed by polyacrylamide gel electrophoresis under denaturing (fig.1A) and native (fig.1B) conditions (stained with Coomassie blue). After purification, a significant reduction in bands was seen. Most was seen in fraction 2 (one major band corresponding to M_r 70 500, and 3–4 slightly smaller and closely migrating proteins), and faint bands in fractions 1 and 3. When run under denaturing conditions, only 2 bands were seen, corresponding to M_r 30 500 and M_r 7691 of the A and B subunits, respectively. The same banding patterns in native PAGE and SDS-PAGE were seen with the Shiga toxin NEZ 3C preparation (data not shown). The data suggest that the native toxin preparation, purified either by receptor affinity or by conventional methods contains complete holo-toxin but also the A subunit with 4 or fewer B subunits.

The described affinity-based purification of Shiga toxin is both simple and rapid, and the gel can be reused.

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