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# Improved Purification Process for Cholera Toxin and its Application to the Quantification of Residual Toxin in Cholera Vaccines

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**A simplified method for the purification of cholera toxin was developed. The 569B strain of *Vibrio cholerae*, a recognized hyper-producer of cholera toxin, was propagated in a bioreactor under conditions that promote the production of the toxin. The toxin was separated from the bacterial cells using 0.2-μm crossflow microfiltration, the clarified toxin was passed through the membrane into the permeate, and the bacterial cells were retained in the retentate. The 0.2-μm permeate was then concentrated 3-fold and diafiltered against 10 mM phosphate buffer, pH 7.6, using 30-kDa crossflow ultrafiltration. The concentrated toxin was loaded onto a cation exchange column, the toxin was bound to the column, and most of the impurities were passed unimpeded through the column. The toxin was eluted with a salt gradient of phosphate buffer, pH 7.0, containing 1.0 M NaCl. The peak containing the toxin was assayed for cholera toxin and protein and the purity was determined to be 92%. The toxin peak had a low endotoxin level of 3.1 EU/μg of toxin. The purified toxin was used to prepare antiserum against whole toxin, which was used in a $G_{M1}$ ganglioside-binding ELISA to determine residual levels of toxin in an oral inactivated whole-cell cholera vaccine. The $G_{M1}$ ganglioside-binding ELISA was shown to be very sensitive and capable of detecting as little as 1 ng/ml of cholera toxin.**

**Keywords:** Cholera toxin, purification, $G_{M1}$ ganglioside, ELISA, vaccine

Following colonization of the small intestine, pathogenic strains of *Vibrio cholera* secrete a powerful exotoxin that triggers cyclic AMP formation resulting in electrolyte and fluid secretion by enteric cells. This toxin-induced fluid loss may lead to severe diarrhea and rapid dehydration, and if not treated with rehydration therapy may lead to death [7]. Cholera toxin consists of five identical B subunits (each B subunit 11.6 kDa) arranged in a ring, which are responsible for binding of the toxin to the $G_{M1}$ ganglioside receptors on intestinal cells [3, 8]. The B subunit pentamer is linked to a single enzymatically active A subunit (27 kDa) that induces adenylate cyclase activity followed by build up of cyclic AMP, resulting in fluid secretion. [5, 10]. Cholera toxin is immunogenic and the antibody response, primarily directed against the nontoxic B subunit, has been shown to neutralize the toxicity caused by the intact halotoxin. An oral cholera vaccine consisting of purified cholera toxin B subunit and inactivated whole bacterial cells has proven to be safe and to confer protection for at least three years [2].

During development of an oral inactivated whole-cell cholera vaccine [1], it was observed that detectable amounts of cholera toxin were present in the culture broth harvested from the bioreactor. Owing to the risk of severe adverse events caused by residual toxin, it is necessary to ensure that the amount of toxin in the final vaccine is very low. Thus, a sensitive assay for detection of minute quantities of residual toxin is required for lot release of finished product [22].

In order to provide reagents for, and to standardize, the toxin assay with multiple vaccine manufacturers, we have developed a new simplified method for toxin production and consequent antitoxin serum preparation. The antiserum and toxin were used in a $G_{M1}$ ganglioside-binding-dependent ELISA [4, 9, 15] to measure quantities of toxin in process samples and final bulk vaccine. We now describe the simplified method for toxin production and purification, and present the results of the toxin quantitation assay.

## Materials and Methods

### Bacterial Strain and Media used for Cultivation

*Vibrio cholerae* serotype Inaba strain 569B was obtained from Prof. Jan Holmgren, University of Goteborg, and this strain is a recognized hyper-producer of cholera toxin. The initial culture was grown on solid LB agar media. Bacterial cell numbers were expanded in flask

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culture prior to seeding the bioreactor. The media composition for flask and fermentation cultures was 10 g/l yeast extract (Oxoid Ltd.), 30 g/l casamino acid (Becton Dickinson and Co.), 0.05 g/l Tryptophan (Gibco BRL), 0.02 g/l $MgSO_4 \cdot 7H_2O$ (Junsei Chemical Co.), 1.31 g/l $Na_2HPO_4 \cdot 7H_2O$ (USB Corporation), and 0.13 g/l $KH_2PO_4$ (USB Corporation). All media components were sterilized by filtration through a Sartorius Sartopore 300, 0.2 μm filter that was placed in line on the bioreactor and sterilized *in situ* during sterilization of the bioreactor.

**Fermentation and Culture Conditions**

Initial culture was performed in a baffled shake flask containing 150 ml of media. Flasks were incubated in a Sartorius AG CERTOMAT BS-T shaker cabinet at 150 rpm, 32°C, for 12 h. The flask culture harvest was aseptically added to a 2.0-l Biostat B-DCU (Sartorius AG) bioreactor containing 1.5 l of sterilized media. The pH was controlled at 7.8 by automatic addition of 10% $NH_4OH$ or 10% HCl. The temperature was controlled at 32°C as it has been demonstrated that lower temperature induces more toxin production by the 569B strain [16]. Dissolved oxygen (DO) was controlled at 35% air saturation by automatic adjustment of the impeller speed, and the air sparging flow rate was set at 1.5 l/min (1.0 VVM).

**Purification of the Cholera Toxin**

The toxin was separated from the cells using a 0.2-μm Hydrosart cassette (0.1 $m^2$; Sartorius AG), fitted into a Sartoflow Alpha cross-flow filtration apparatus (Sartorius AG). The cells were concentrated from 1.5 l to 200 ml, thus collecting 1.3 l of clarified culture broth containing toxin in the permeate. The permeate was then concentrated to 500 ml using a 30-kDa nominal molecular weight cut-off (NMWCO) Hydrosart ultrafiltration cassette (0.1 $m^2$; Sartorius AG) fitted into the Sartoflow Alpha. Using constant volume diafiltration (*i.e.*, permeate flow equals rate of buffer addition to the retentate maintaining retentate at constant volume), the concentrate was then diafiltered against 20 volume changes of 10 mM potassium phosphate buffer, pH 7.6. A pre-packed mono S 5/50 GL cation exchange column (GE Healthcare) installed in an AKTA Explorer 100 (GE Healthcare) was equilibrated with 10 column volumes of 10 mM potassium phosphate buffer, pH 7.6, and then 10 ml of diafiltered crude toxin concentrate was injected onto the column. pH 7.6 was found to be optimal for binding cholera toxin while allowing other contaminants to pass unimpeded through the column (results not shown). The cholera toxin was eluted with a gradient of 10 mM potassium phosphate buffer, pH 7.0, containing from zero to 1.0 M NaCl at a linear flow rate of 300 cm/h. The elution was monitored by UV absorption at 280 nm and 1.0-ml fractions were collected.

**Analyses**

Rocket immunoelectrophoresis (RIE) was carried using the method described by Laurell [11] and described briefly as follows. Gels were made up of 1.1% (w/v) agarose (7.5×5.0 cm×1.5 mm thick) containing 0.3% antiserum (Sigma c3062 or antiserum prepared in our laboratory) and the electrophoresis was run in 0.1 M Tris buffer, pH 8.6. Samples were prepared by centrifugation at 4,500 ×*g* for 20 min at 4°C and collecting the supernatant for analysis. Samples 5 μl were inoculated into 3-mm diameter wells cut into the agarose gel. Commercially available cholera toxin (Sigma c8052) was compared with toxin prepared in our laboratories. Dilutions of cholera toxin to 5, 10, and 20 μg/ml were used as the standard. Electrophoresis was carried out at 4.0 mA for 17 h at 4.0°C. The gel was washed in 0.2 M NaCl for 2 h, repeated 4 times, and then running water for 15 min, and the gel was then pressed, dried, and stained with Coomassie blue (0.25%) in ethanol/acetic acid/water (45:10:45, v/v/v) for 1 h, and destained in ethanol/acetic acid/water (45:10:45, v/v/v). Peak heights were measured and the cholera toxin concentration was calculated by comparison to the standard curve (toxin concentration versus peak height) generated by the Sigma standard preparation.

Quantitation of the purified cholera toxin, in process samples and final vaccine bulk samples, was performed using the $G_{M1}$ ganglioside-binding ELISA. The method used was as described by Dawson [4] and briefly was performed as follows. Microtiter plates (Cat. No. 3590; Corning Costar) were coated by adding 100 μl per well of 0.1 μg/ml $G_{M1}$ ganglioside (Monosialoganglioside, $G_{M1}$, Sigma Cat. No. G7641) and incubated at 4°C overnight. Plates were washed with washing buffer (PBS, pH 7.4, containing 0.01% Tween 20) and then blocked with PBS, pH 7.4, containing 0.01% Tween 20 and 1.0% BSA for 1 h at room temperature. Residual BSA was washed out using washing buffer. Aliquots 200 μl of standard and sample containing approximately 0.1 μg/ml of toxin diluted in PBS, pH 7.4, containing 0.01% Tween 20 and 0.1 % BSA were added to the first well and doubling dilutions performed across the plate (100 μl of diluting buffer in wells other than in first row); 100 μl was removed from the last row. Plates were incubated for 1.5 h at room temperature and then washed with washing buffer. Then, 100 μl of antiserum (rabbit anti-cholera toxin serum prepared in our laboratory diluted 1 in 5,000) was added to each well and the plates were incubated for 1 h at room temperature. Plates were then washed with washing buffer followed by addition of 100 μl of peroxidase-labeled goat anti-rabbit IgG (diluted 1 in 2,000) to each well and then incubated for 1 h at room temperature. Plates were again washed with washing buffer and then 50 μl of color development solution (Sureblue,TMB microwell peroxidase substrate; KPL) was added to each well and the plates were incubated at room temperature. After 15 min, 50 μl of 0.5 M HCl was added to each well and the absorbance read at 450 nm using an Ultramark (Bio-Rad) microplate reader. The toxin concentration in the sample is calculated by interpolation from a standard curve and adjusted for the sample dilution factor.

Endotoxin (LPS) content was measured by *Limulus* amebocyte lysate (LAL) test using a kinetic turbidimetric assay kit (Cambrex Bio Science). Turbidity was measured at 340 nm using a microplate reader (ELX808 ultra microplate reader; Bio-TEK Instruments Inc.), and the result obtained was compared with that of standard endotoxin (*E. coli* O55:B5 endotoxin; Cambrex Bio Science). The endotoxin concentration was calculated using the WinKQCL Version 3.00 software (Cambrex Bio Science). The protein concentration was determined by the Lowry method using bovine serum albumin as a standard [13].

Acrylamide gel electrophoresis was performed in Tris-glycine buffer (25 mM Tris, 192 mM glycine, and 0.1% [w/v] SDS, pH 8.3) using a Mini-protean 3 cell system (Bio-Rad). The acrylamide gel concentration was 12% (w/v). Sample preparation was performed by adding to 20 μl of sample, 5 μl of 5× sample buffer (250 mM Tris-HCl, 500 mM dithiothreitol, 10% [w/v], SDS, 0.5% [w/v] bromophenol blue, and 50% [v/v] glycerol, pH 6.8) and then boiling for 5 min. Then, 20 μl of the prepared samples was loaded onto each sample lane and electrophoresis carried out with a current of 5 mA and a running time of 4 h. Gels were stained with Coomassie blue and then dried.

Size-exclusion chromatography was performed by loading a 0.5-ml sample of the peak fraction from the mono S column onto a pre-packed Superdex 200 10 300 GL column (GE Healthcare) equilibrated with 10 mM potassium phosphate buffer, pH 7.4. The elution with the same buffer, at a flow rate of 32 cm/h, monitored by UV absorption at 280 nm.

**Table 1.** Analysis of in-process samples during purification of cholera toxin.

| Sample | Protein (mg/ml) | Toxin (μg/ml) | Yield (%) | Purity (%) | Endotoxin (EU/ml) |
|---|---|---|---|---|---|
| Fermentation broth | 8.8 | 45 | | 0.51 | $8.36\times10^7$ |
| Clarified fermentation broth | 7.0 | 40 | 77 | 0.57 | $8.39\times10^6$ |
| 30-kDa Concentrate | 3.9 | 53 | 67 (51) | 1.37 | $2.98\times10^7$ |
| Mono S peak fraction | 0.47 | 434 | 81 (42) | 92.3 | $1.32\times10^4$ |

Clarified fermentation broth was 0.2 μm permeate of fermentation broth; 30 kDa concentrate was the clarified fermentation broth concentrated on a 30 kDa Hydrosart membrane and diafiltered against 10 mM potassium phosphate buffer pH 7.6; the mono S peak fraction was the peak fraction eluted with a NaCl gradient, pH 7.0. The yield figures are for the single step process (*i.e.* from the previous step,) and figures in parenthesis are recovery from the starting fermentation broth. The purity is the percentage of specific toxin in the total protein.

## Results

### Toxin Production in the Bioreactor

Toxin production was followed during fermentation; samples were taken at the end of the 12 h growth phase and during the stationary phase at 24 h and quantified by RIE (Table 1). The toxin concentration at the end of the growth phase was 45 μg/ml, which is consistent with previously published yields for the 569B strain [14, 19]. No further increase in toxin concentration was observed during the stationary phase.

### Purification of Cholera Toxin

The toxin was clarified using 0.2-μm cross-flow filtration, which separated the cells from the crude toxin and resulted in 77% recovery of the toxin in the permeate. The permeate was concentrated and diafiltered against 10 mM potassium phosphate buffer, pH 7.6, using a 30 kDa ultrafiltration membrane with a recovery for the process step of 67% (Table 1). The clarified concentrate was loaded onto the Mono S column, which had been equilibrated with 10 mM potassium phosphate buffer, pH 7.6. Fig. 1 shows the elution

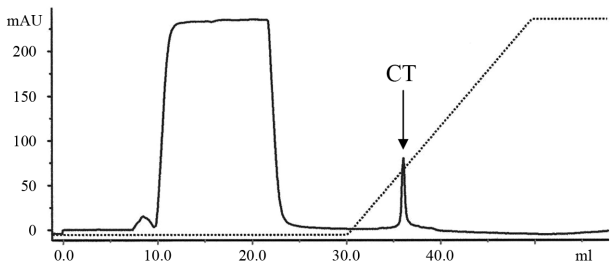


**Fig. 1.** Cation exchange chromatography elution profile.
Clarified concentrated toxin was loaded onto the column as described in the Materials and Methods. The bound toxin was eluted with a NaCl gradient from 0 to 1.0 M NaCl in 10 mM potassium phosphate, pH 7.0, and elution was started at 30 ml. The solid line shows the elution profile measured by UV absorption at $OD_{280}$ and the dotted line shows the NaCl concentration.

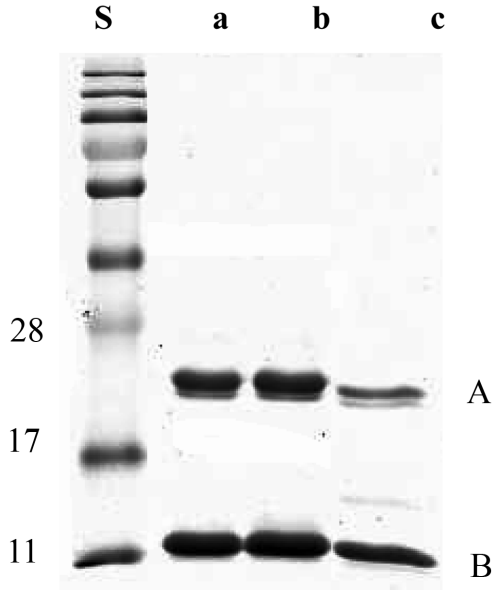


**Fig. 2.** SDS-polyacrylamide gel analysis of purified cholera toxin.
Lane S, molecular weight standards (MW $\times10^{-3}$). Lane a, peak cholera toxin eluted from cation exchange chromatography. Lane b, cholera toxin peak from size exclusion chromatography. Lane c, standard cholera toxin (0.5 mg/ml from Sigma).

profile of the cholera toxin and elution with a NaCl gradient (0 to 1 M) in 10 mM potassium phosphate buffer, pH 7.0. Most of the UV absorbing material ($OD_{280}$) passed unimpeded through the column and appeared in the peak at the beginning of the elution profile. The cholera toxin bound to the column was eluted at elevated ionic strength, with the peak appearing at a concentration of 0.32 M NaCl. The results presented in Table 1 showed that the cation exchange step increased the purity from 1.4% to 92.3%, with 81% of the toxin loaded onto the column recovered in the peak fraction. The toxin peak was assessed for purity by polyacrylamide electrophoresis (PAGE) (Fig. 2) and size exclusion chromatography (Fig. 3). The PAGE showed no obvious contamination from other proteins, with the two major bands present being consistent with the cholera toxin A and B subunits and running in the same position as the cholera toxin from Sigma. The size exclusion chromatography (Fig. 3) also confirmed the absence of any major contaminants and the presence of a single peak, indicating that the toxin was present as predominantly intact toxin. The process efficiently removed endotoxin with an almost 4 log reduction (Table 1) and a final endotoxin content of 3.0 EU/mg cholera toxin.

### Assay of Cholera Toxin Using $G_{M1}$ Ganglioside-binding ELISA

The results presented in Fig. 4 show that under the conditions described in Materials and Methods for the $G_{M1}$ ganglioside-binding ELISA, the log of the toxin concentration versus absorbance is linear in the range of 1 to 50 ng/ml of cholera

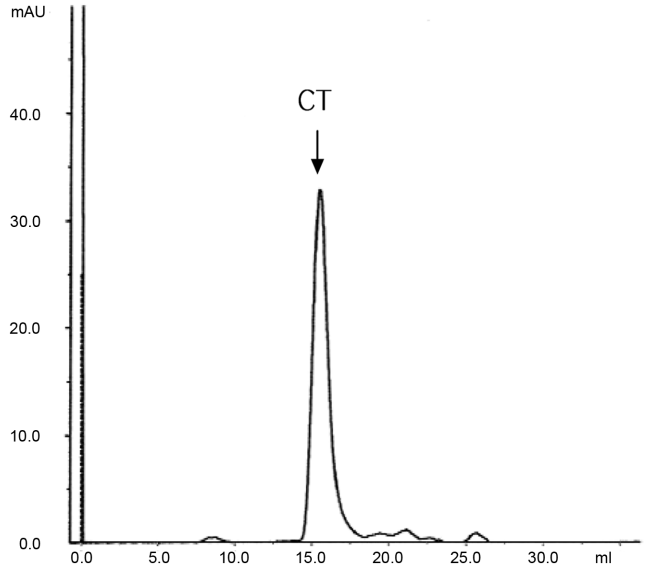


**Fig. 3.** Size exclusion chromatography of purified cholera toxin. A 0.5 ml sample of the peak fraction from the mono S column was loaded onto a pre-packed Superdex 200 column equilibrated with 10 mM potassium phosphate buffer, pH 7.4. The elution was monitored by UV absorption at 280 nm.

toxin. This demonstrates that the $G_{M1}$ ganglioside-binding ELISA can detect as little as 1 ng/ml of intact cholera toxin. The ELISA was used to quantify the toxin content of peak fraction eluted from the cation exchange column. The correlation between the ELISA and RIA was very good, with the cation exchange purified toxin peak being quantified as having 434 µg/ml by RIE and 428 µg/ml by ELISA. The RIE assay (limit of detection 5 mg/ml) was however 5,000 times less sensitive than the ELISA and so not suitable for detection of low levels of residual toxin found in cholera vaccine. The ELISA was used to measure the toxin content in

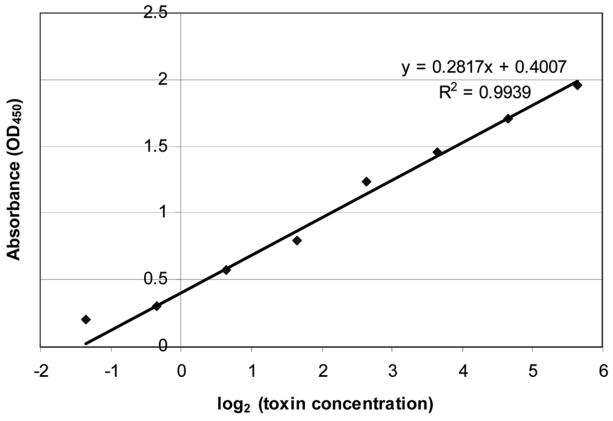


**Fig. 4.** $G_{M1}$ ganglioside-binding ELISA. Graph shows standard curve for Sigma toxin.
The x-axis is the log to the base 2 (doubling dilutions) of the toxin concentration. The solid line is the trend line plotted from 50 ng/ml (log2=5.644) to 0.78 ng/ml (log2=−0.356), demonstrating the linear nature of the plot. Below 0.78 ng/ml, the line is no longer linear.

batches of bulk and final vaccines that had been used in a clinical trial [2], neither the five bulk concentrates nor the final vaccine contained detectable toxin.

## DISCUSSION

Vaccines against cholera are an effective control measure in preventing disease, and because cholera affects mainly poorer developing countries, there is a need for a safe, affordable vaccine [6, 21]. (IVI) has been involved in the development and technology transfer of an oral inactivated cholera vaccine from Vietnam to India [1], and as part of that process to assure safety of this vaccine, it was necessary to introduce a sensitive assay to detect residual cholera toxin. In the development of the assay for residual toxin, it was necessary to prepare reagents including purified toxin and an antitoxin serum. Several methods for the purification of cholera toxin [14, 15, 17, 19], and recombinant B subunit [18] have been reported. The method described here provides a simple procedure, which as the final purification step utilizes binding of the cholera toxin to a cation exchange mono S column. The likely mechanism for this binding is that the toxin has an exposed cationic charge on its surface that resides in the B subunits [14, 18], and the exposed B subunit surface of the whole toxin (or B subunits) binds to the column under the conditions specified in the Materials and Methods. The toxin produced using this simplified method was shown to have a purity of 92% and a yield of 19 mg/l of fermentation broth, which was consistent with that achieved with other purification methods, 15–30 mg/l [14] and 20 mg/l [19]. The method developed does not require use of low pH 2.8 buffers for elution of the toxin from the column as is necessary in the affinity chromatography method [19]. It is recognized that, at pH 2.8, there is complete dissociation of toxin subunits, which renature and recombine after neutralization [12]; avoiding low pH conditions therefore gives much better control of the purification process and increases the probability of selectively purifying intact toxin.

Toxigenic strains of *Vibrio cholerae* characteristically secrete cholera toxin, and the amount of toxin secreted varies from isolate to isolate [20] but is generally significantly less than that seen with the 569B strain. The strains used for vaccine production are wild-type strains that typically secrete toxin. To reduce the risk of residual toxin in the vaccine, strains are grown at higher temperatures to suppress toxin production [16], and processing steps introduced to remove or inactivate the toxin. However, to assure safety of the vaccine, a sensitive assay must be used to detect residual toxin in the final vaccine and this assay should be incorporated into the lot release testing requirements. The results presented here indicate that the antiserum prepared in rabbits from toxin prepared in the IVI laboratories is of good quality and can be used in a toxin-specific $G_{M1}$ ganglioside-binding ELISA

for detecting very low levels of toxin, down to 1 ng/ml. The assay described here will form part of the package of assays required for the technology transfer and internationalization of the whole-cell cholera vaccine used in Vietnam [1] and currently in phase III clinical trials in Kolkata in compliance with the WHO recommendations [22].

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