Production and purification of Clostridium botulinum type C and D neurotoxin

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Abstract

Neurotoxins of Clostridium botulinum are needed in basic neurologic research, but as therapeutic agent for certain neuromuscular disorders like strabism as well. A method for the production and purification of botulinum neurotoxins C and D is reported using a two-step hollow-fiber cross flow filtration and a newly developed chromatographic purification procedure. Hollow-fiber filtration proved to be a rapid and safe concentration and pre-purification step, which can easily be scaled up. The chromatographic purification included hydrophobic interaction, anion exchange and size exclusion chromatography runs. Botulinum neurotoxins C and D could be recovered with an overall yield of 12.6% and 10.6%, respectively. A specific toxicity of $1.86 \times 10^{7}$ minimal lethal dose mg$^{-1}$ (type C) and $5.26 \times 10^{7}$ minimal lethal dose mg$^{-1}$ (type D) was determined in the mouse bioassay.

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1. Introduction

Botulinum neurotoxins (BoNT), which are produced by Clostridium botulinum, an anaerobic spore former, belong to the most toxic biological substances. For more than a century, research focused on the structure and pathogenesis of the seven distinct neurotoxins (A–G) and the associated diseases. Types A, B and E are generally considered as being pathogenic for humans, types C and D are often isolated in cases of animal botulism.

One of the first reports on botulism was written by Justinus Kerner, a German physician and poet. But he did not only increase our basic knowledge on the disease and the causative agent. As early as 1822, he already discussed the possible therapeutic use of BoNT or the ‘fatty acid’ as he called it [1]. However, it lasted 160 years until the benefits of BoNT were discovered and investigated again [2]. Since then, BoNT has become an important agent in treating strabism, blepharospasm, dystonia and other, similar diseases, caused by neuro-muscular disorders [3]. This, on the other hand, increased the demand for purified BoNT, which is also needed in basic neurologic research, e.g. transmitter release studies [4].

Previously published purification procedures for BoNT usually include an initial precipitation [5,6] or at least a centrifugation step [7]. Due to the high toxicity of BoNT, this purification is difficult and/or hazardous to run with larger volumes of cul-
tured broth. Based on previous experiences with hollow-fiber cross flow filtration [8], we have attempted to develop an initial, self-contained two-step filtration, which separates the metabolites including the toxin from the bacteria and the low molecular weight substances, e.g. the media peptides. The concentrate was subject to a newly developed chromatographic purification procedure to obtain highly purified BoNT/C and D, which were intended to be used for antibody production.

2. Material and methods

2.1. Bacterial strains

C. botulinum type C IBT 2300 and type D IBT 2301 (kindly donated by S. Kozaki, Osaka Prefecture University, Japan) were used in this study.

2.2. Protein electrophoresis

Fermentor and chromatography samples were routinely separated electrophoretically by SDS-PAGE using a 7.5% separating and 2% stacking gel. For reducing conditions, the sample was diluted 1:2 with sample buffer, which was supplemented with DTT (30 mg ml⁻¹) and incubated in a water bath (100°C, 3 min). The separation was run at a constant voltage of 200 V.

Gels were silver-stained according to Heukeshoven and Dernick [9], scanned and analyzed with RFLPscan (Scanalytics).

2.3. Mouse bioassay

The biological activity of BoNT/C and BoNT/D was measured in NMRI mice weighing 18–25 g. To reduce the number of animals needed in this study, 0.5 ml of serial 10-fold dilutions of the samples were injected intraperitoneal into two mice per dilution only and the animals were observed for clinical signs or death over a period of 3 days. In addition samples of the fermentor, harvests and the purified toxins were diluted 2-fold in between the positive and negative 10-fold dilutions. The minimal lethal dose (MLD) describes the reciprocal value of the highest dilution at which both mice died.

2.4. Hemagglutinin assay

The hemagglutinating activity of harvest and chromatographic fractions was quantified in a microtiter plate assay. Serial 2-fold dilutions of the samples were made in 20 mM Tris-HCl, pH 7.2, 150 mM NaCl using 96-well microtiter plates (U-shaped). An equal volume of 1% human red blood cell suspension in the dilution buffer was added. Plates were incubated at room temperature overnight. The hemagglutination titer corresponds to the reciprocal value of the highest dilution at which agglutination of the cells could be observed.

2.5. Toxin production

Inoculum of both strains was prepared in 100-ml flasks. The strains were incubated overnight in 50 ml cooked meat medium (Difco) at 37°C. The flasks were placed in an anaerobic jar. A suitable atmosphere, which initially consisted of 90% N₂, 5% H₂ and 5% CO₂, was generated with the gas supplying system Anoxomat (Mart).

For toxin production, the ‘Göttinger Bioreactor’ was used with slight modifications as previously reported [10]. Briefly, a 20 l fermentor vessel containing 10 l medium, which consisted of 1% peptone from casein, pancreatically digested, 1% meat extract, 0.3% yeast extract, 0.1% soluble starch, 0.5% D-glucose, 0.5% sodium chloride, 0.3% sodium acetate and 0.05% l-cysteine-HCl, was inoculated with the overnight culture of type C or D, respectively. Medium for type C was supplemented with 3% cooked meat (Difco). Initially, the pH was adjusted to 6.8. The fermentor culture was incubated for 4 days at 37°C with a continuous N₂ overflow. Every day, samples where taken, checked for contamination and examined in SDS-PAGE.

For the two following batches of each strain, no inoculum was prepared. 10 l of new medium was added to approximately 300 ml culture, left over from the previous cultivation.

2.6. Toxin filtration and chromatographic purification

In general, type C and D toxin were purified according to the same procedure. The filtration and
Further purification steps of the toxins are summarized in Fig. 1.

Within a self-contained system, the cultures were harvested and filtered using a hollow-fiber cartridge, which retains molecules >1 mio D (SPS 9005-8B/4, Fresenius). The filtrate of this step was applied to a cartridge with a 30-kDa cut-off (SPS 6005-8B/4). During filtration, buffer was exchanged to 50 mM Tris-HCl, pH 8.0, 1.0 M ammonium sulfate.

The retained suspension of the second filtration step was subject to chromatographic purification, which was run on an Äkta system (Amersham Pharmacia). Column hardware and resins were supplied by Amersham Pharmacia if nothing else is stated. All fractions collected during the chromatographic runs were analyzed by SDS-PAGE. If a presumptive toxin band could be detected electrophoretically within a peak, the fraction was checked for the presence of toxin in the mouse bioassay, but only the purified toxin was quantified.

For hydrophobic interaction chromatography, the concentrated supernatant was applied to a phenyl-Sepharose HP column, equilibrated with 50 mM Tris-HCl, pH 8.0, 1.0 M ammonium sulfate. The toxin was eluted with a linear gradient from 1.0 to 0.0 M ammonium sulfate in the same buffer. The fractions in which toxin was detected were pooled and dialyzed against 50 mM Tris-HCl, pH 8.0, for 48 h.

The dialyzed fractions were further purified on an anion exchange column, which was packed with Source 30 Q. The column was equilibrated with 50 mM Tris-HCl, pH 8.0, and fractions were eluted with an increasing NaCl gradient from 0.0 to 0.24 M in the same buffer. Again, the fractions were checked for the presence of toxin and the positive fractions pooled and dialyzed against 50 mM Bis-Tris, pH 6.0, for 48 h.

The next step was the application of the pool to a Source 15 Q column, pre-equilibrated with the dialyzing buffer. Elution was run with a linear gradient of 0.0–0.14 M NaCl in the same buffer. For the final polishing, the pooled toxin fractions were applied to
Fig. 3. Chromatographic purification of BoNT/C and D. Chromatograms of the purification steps are shown in chronological order.
a HighLoad 16/60 Superdex 200 column, isocratically eluted in 0.01 M phosphate buffer, pH 8.0, with a continuous flow of 2.0 ml min$^{-1}$.

2.7. Purity control

The purity of the chromatographed toxin was assessed in SDS-PAGE with and without DTT treatment and on an analytical anion exchange column.

For the analytical anion exchange chromatography on a MonoQ column, 1 ml of the toxin solution was diluted 10-fold to decrease the NaCl concentration. The same buffers with pH 8.0 for equilibration and elution were used as in the anion exchange chromatography step with Source 30 Q during toxin purification.

2.8. Protein measurement

Protein concentrations of the fermentor harvest, the filtrated cultures and the purified toxin were measured with a commercially available assay (BCA Protein Assay, Pierce).

3. Results

3.1. Toxin production

During the three consecutive batches, an average amount of $4.0 \times 10^8$ MLD for type C and $1.6 \times 10^9$ MLD for type D could be harvested with each run. This corresponds to $4.0 \times 10^4$ MLD ml$^{-1}$ (type C) and $1.6 \times 10^5$ MLD ml$^{-1}$ (type D).

3.2. Toxin filtration and chromatographic purification

The results of the purification steps of BoNT/C and D are summarized in Table 1. The data given represent the average values of the three consecutive batches run with each strain. During the hollow-fiber filtration, the cultures could be concentrated more than 10-fold. Fig. 2 shows this effect on the high molecular weight metabolites including the neurotoxins in SDS-PAGE. The protein concentration was reduced to at least 10% of the amount measured in the untreated fermentor broth.

Fig. 3 gives representative chromatograms obtained during the four purification steps. In hydrophobic interaction chromatography, no toxin was detected in the fractions containing the unbound proteins. The toxin started to elute at an ammonium sulfate concentration of 190 mM. However, separation of the hemagglutinin could only partially be achieved, although alkaline conditions (pH 8.0) were applied. In the following anion exchange chromatography at pH 8.0, neurotoxin and hemagglutinin eluted in separate peaks. At pH 6.0, the next step, BoNT eluted in one distinct peak. No hemagglutinin activity could be detected within any fraction. During final polishing on Superdex 200, only one homogeneous peak eluted, containing the neurotoxin.

According to the mouse bioassay, an average over-

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<th>Table 1 Purification of BoNT/C and D</th>
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<td>BoNT/C</td>
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<tr>
<td>Fermentor culture</td>
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<td>After filtration</td>
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<td>Chromatographically purified toxin</td>
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<td>BoNT/D</td>
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<td>After filtration</td>
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Average values of three batches for each type
all toxicity yield of 12.8% (BoNT/C) and 10.6% (BoNT/D) could be recovered with a specific toxicity of $1.86 \times 10^7$ MLD mg$^{-1}$ and $5.26 \times 10^7$ MLD mg$^{-1}$, respectively.

A high purity of the toxins was proven with the use of the MonoQ column. All the protein present in the sample bound to the column and eluted in one distinct peak.

The separation of the purified neurotoxins in SDS-PAGE is shown for type D in Fig. 4. Both toxins banded at $\sim 144$ kDa, if no reducing conditions were applied. With DTT treatment, two distinct bands were found at $\sim 98$ kDa and $\sim 49$ kDa. No impurities could be detected electrophoretically.

## 4. Discussion

Several reports on the purification of BoNT/C and BoNT/D have been published. The purification usually starts with an ammonium sulfate [5] or an acid precipitation [6]. This step is time consuming, since precipitating and resuspending takes 1–2 days. De Jongh et al. [7] already used hollow-fiber filtration for the concentration of the culture supernatant of BoNT/D. However, they centrifuged the bacterial culture prior to the filtration to separate bacteria and supernatant. But centrifugation is difficult to perform with larger amounts of fermentor culture, especially with high toxic material like C. botulinum. With an initial two-step hollow-fiber filtration, the culture supernatant can be separated from the cells, concentrated and partially purified. The volume, which can be filtered, depends mainly on the filter cartridges used. With larger cartridges or several cartridges, which are run parallel, this process can easily be scaled up to industrial needs. Since the culture can be transferred to the filtration unit within a self-contained system, the risk of contamination and aerosol formation can be minimized. In addition, buffer can be exchanged to the sample buffer needed in the first chromatographic step. Thus, the filtration offers a convenient, quick and safe procedure to prepare the culture for the following chromatographic purification.

Hydrophobic interaction chromatography was used as the first step in further purification. According to unpublished results, obtained in our lab, this technique was superior to initial ion exchange chromatography, which was used in previous reports on toxin purification [7]. However, total separation of neurotoxin and hemagglutinin could only be achieved in the second step on an anion exchange column. Depending on the intended use of the purified toxins, final polishing in size exclusion chromatography might be omitted. In the last anion exchange step, the toxin already eluted in one distinct peak.

In SDS-PAGE, samples of the purified preparations banded at $\sim 144$ kDa only. With DTT treatment, the toxin molecules were cleaved into the heavy and the light chain, banding at $\sim 98$ kDa and $\sim 49$ kDa. No impurities could be detected electrophoretically.

The purified toxins had a specific toxicity of $1.86 \times 10^7$ MLD mg$^{-1}$ (BoNT/C) and $5.26 \times 10^7$ MLD mg$^{-1}$ (BoNT/D). Moriishi et al. [11] reported that they were able to increase the toxicity of a purified BoNT/D toxin (D-SA) by trypsin treatment. Trypsinization of the purified BoNT/C and D obtained in this study resulted in a total loss of the neurotoxic activity in the mouse bioassay (data not published). This might provide further evidence for the theory of molecular diversity within the tertiary structure of type D strains [11]. However, it cannot be ruled out that the toxins, purified according to the...
presented procedure, are totally separated from any protecting proteins, thus being highly susceptible to proteolytic cleavage.

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References