A new scaleable method for the purification of botulinum neurotoxin type E

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Received 13 January 2005; received in revised form 17 March 2005; accepted 29 March 2005

Abstract

Botulinum neurotoxins belong to the most toxic substances in nature. Well-known as a food poisoning agent for almost two centuries, the beneficial aspects of this bacterial metabolite were rediscovered about 30 years ago. These toxins, which are produced by the anaerobic bacterium Clostridium botulinum are nowadays used to treat a variety of neuro-muscular disorders. The increased demand requires techniques for the production and purification of these toxins on an industrial scale. The method described herein is based on filtration and chromatography procedures only. Precipitation, centrifugation and dialysis steps were consequently excluded to develop a protocol, which can easily be scaled up from the laboratory purification to industrial needs. About 4 mg of BoNT/E were purified from a 10-L batch culture corresponding to an overall recovery of ∼14%.

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Keywords: Botulinum neurotoxins; Clostridium botulinum; Purification; Microfiltration; Ultrafiltration; Chromatography

1. Introduction

Clostridium botulinum, an anaerobic spore-former, is known to produce highly potent neurotoxins (BoNT). Currently seven distinct neurotoxins are known (A–G). These neurotoxins are di-chain molecules of ∼150 kD with a 100-kD heavy and 50-kD light chain, linked through a disulfid bond. In its natural habitat or in liquid culture media complexes are formed, which consist of the neurotoxin, hemagglutinin and non-hemagglutinating proteins (Sakaguchii, 1986).

Composition and size of these pH-dependent complexes or progenitor toxins varies throughout the toxin types. Whereas 12S (300 kD), 16S (500 kD) and 19S (900 kD) can be found in type A (Sugii and Sakaguchii, 1977), only 12S can be detected in type E (Kitamura et al., 1968). BoNTs are well-known as food or feed poisons, leading to flaccid muscular paralysis in man and animal (Smith and Sugiyama, 1988), with BoNT/E being the predominant type in botulism caused by contaminated fish or seafood (Hielm et al., 1998). However, in some diseases C. botulinum colonizes the host, leading to toxico-infectious botulism, e.g. infant botulism (Midura and Arnon, 1976), wound botulism (Mechem...
and Walter, 1994), shaker foal syndrome (Rooney and Prickett, 1976) and visceral botulism in cattle (Böhnel et al., 2001). Grass sickness, a dysautonomia in horses, is likely to be caused by C. botulinum as well (Collier et al., 2001). Grass sickness, a dysautonomia in horses, is likely to be caused by C. botulinum as well (Collier et al., 2001). However, the views on BoNTs have changed. They are no longer looked upon as the most poisonous poison, causing severe, often fatal diseases in man and animal only. For a continuously growing variety of disorders, BoNT has become the treatment of choice (Kessler and Benecke, 1997; Kreyden, 2002).

Therefore, purified BoNT is needed not only as a research tool, but as a vaccine and licensed drug as well. The purity of the BoNT solution mainly depends on the intended use. In many applications highly purified neurotoxin is needed. Recently we have published a report on the purification of BoNT/C and BoNT/D by filtration and chromatography, avoiding any precipitation or centrifugation steps (Gessler and Böhnel, 1999). However, this protocol did not result in pure toxin for type E. Based on the experiences with BoNT/C and D, a production and rapid purification method for type E has been developed.

2. Material and methods

All chemicals and reagents were obtained from Merck, Darmstadt, Germany, if nothing else is stated.

2.1. Bacterial strain

In preceding tests, C. botulinum type E CB-S 21E (kindly provided by Korkeala’s lab, University of Helsinki) proved to produce comparatively high amounts of BoNT/E (data not shown) and was therefore used in this study.

2.2. SDS-PAGE

Samples were separated by conventional SDS-PAGE. A 2% stacking and 7.5% separating vertical gel was used in the MiniProteanII-system (Bio-Rad, Munich, Germany) with High Molecular Weight Calibration Kit (Amersham Pharmacia, Freiburg, Germany) as standard. When reducing conditions were appropriate, the sample was diluted 1:2 with a DTT sample buffer (30 mg DTT mL⁻¹) and incubated in a water bath (100 °C, 3 min). The separation was run at a constant voltage of 200 V for approximately 40 min. Gels were removed and silver-stained according to the procedure of Heukeshoven and Dernick (1986). The gels were photographed with the Gel Documentation System 2000i (MWG Biotech, Ebersberg, Germany). The digital image was analysed using RFLPScan software (Scanalytics, Fairfax, USA).

2.3. Quantitative protein assay

A commercial microtiter plate assay was used to measure the protein content following the manufacturer’s instructions (BCA protein assay, Pierce Biotechnology, Rockford, IL, USA).

2.4. Mouse bioassay

The biological activity of the neurotoxin solutions was determined in NMRI mice weighing 18–25 g. However, the number of animals used in this study was kept at a minimum. The sample was diluted 1:10 in gelatine-phosphate-buffer (GPB: 50 mM PBS, 0.2% gelatine, pH 6.2). For nicking of the neurotoxin, trypsin was added to a final concentration of 20 μg mL⁻¹, mixed and incubated at 37 °C for 30 min. Then serial 10-fold dilutions were made in GPB. 0.5 mL of each dilution was injected intraperitoneally into two mice. To calculate the overall recovery of the toxin, an approximate quantification of the amount of biologically active toxin in the fermentor broth and after each purification step was inevitable. Therefore additional two-fold dilutions were made between the positive and the negative 10-fold dilutions for these samples. The animals were observed for clinical signs or death over a period of 4 days. The toxicity is given in minimal lethal doses (MLD), which corresponds to the reciprocal value of the highest dilution at which both mice showed typical signs and died.

2.5. Fermentor cultivation

The inoculum was prepared and the fermentor cultivation was run as previously reported (Gessler and Böhnel, 1999), but slight modifications applied. Briefly, the inoculum was grown in 100-mL flasks with 50 mL clostridial medium (CM) which consisted of 0.3% yeast extract, 0.75% meat extract, 0.75% peptone...
from casein, pancreatically digested, 0.75% peptone from meat, tryptically digested, 0.1% soluble starch, 0.5% d-glucose, 0.5% sodium chloride, 0.3% sodium acetate, 0.05% l-cysteine-HCl. The pH was adjusted to 7.0. The inoculum was incubated overnight at 26 °C in an anaerobic atmosphere (80% N₂, 5% H₂, 15% CO₂). A 10-L fermentor vessel with 1 L CM was inoculated and the pH initially adjusted to 6.8. To ensure an anaerobic atmosphere, the fermentor was supplied with a continuous N₂ overflow. If the bacteria grew well, 9 L of CM were added after ∼24 h to give a final culture volume of 10 L and incubated for another 4 days at 26 °C. Samples were taken daily and checked for contamination, examined in SDS-PAGE, and for toxicity in the mouse bioassay.

2.6. Filtration and chromatographic purification

All purification steps are summarized in a flow chart (Fig. 1). After the incubation, the whole fermentor was pumped dry to a self contained filtration system, which started with a microfiltration step. A hollow fibre cartridge with 0.1 µm pore size (MD 020 FP 1N, Microdyn-Nadir, Wuppertal, Germany) was used to separate the cells from the culture supernatant. The fermentor culture was concentrated down to ∼400 mL. The microfilter was rinsed with 2.5 L of 50 mM Bis-Tris, 0.05 mM EDTA, pH 6.0 and the retentate was again concentrated to a volume of 400 mL. The retentate was discarded and the total filtrate (∼12 L) was subject to the ultrafiltration in a spiral cartridge with a nominal cut-off of 30 kDa (S1Y30, Millipore, Schwalbach, Germany). The volume was reduced to ∼200 mL and the filter rinsed with ∼3 L of the equilibration buffer of the first chromatographic purification step (50 mM Bis-Tris, 0.05 mM EDTA, pH 6.0, 1 M ammonium sulfate). The retentate was again concentrated down to ∼1 L, which corresponds to 1/10 of the original volume. The filtrate of the ultrafiltration was discarded. System hardware (ÄktaExplorer) and resins for chromatographic purification were supplied by Amersham Pharmacia (Freiburg, Germany), if nothing else is stated. All chromatography steps were performed at room temperature (21 °C ± 2 K).

The concentrated and prepurified supernatant obtained in the filtration step was subject to hydrophobic interaction chromatography (HIC). It was applied to a phenylsepharose HP XK 50/110 column, equilibrated with 50 mM Bis-Tris, 0.05 mM EDTA, pH 6.0, 1 M ammonium sulfate. Fractions of 10 mL each were eluted with a discontinuous decreasing ammonium sulfate gradient in the same buffer (1–0.6 M over three column volumes [CV], 0.6–0.35 M over 5 CV’s). The fractions containing the neurotoxin (as determined in SDS-PAGE and the mouse bioassay) were pooled and passed through a HiPrep Desalt 26/10 column with isocratic elution in 10 mM Tris, 0.05 mM EDTA, pH 8.0 (flowrate 20 mL min⁻¹) for desalting.
Fig. 2. *C. botulinum* type E fermentor culture record. After 24 h (indicated by an arrow) the medium is added. The redox starts to rise at about 40 h after inoculation and reaches almost 0 at the time of the harvest (120 h). After 35 h the pH reaches its minimum value of approximately 5.0, where it remains.

The protein containing fractions were loaded on a MonoQ column (1 mL). The buffer of the desalting step was used for binding, the same buffer + 2 M NaCl for elution. Elution was run with a linear gradient (0–0.2 M NaCl over 35 CV’s).

Again, the toxin peak fractions were pooled and the buffer was exchanged on a HiPrep Desalt 26/10. This time, however, 10 mM Bis–Tris, 0.05 mM EDTA pH 6.0 was used for elution. Last step was cation exchange chromatography on a Mono S column (1 mL). The pooled fractions were applied to the column equilibrated with 10 mM Bis–Tris, 0.05 mM EDTA pH 6.0. 2 M NaCl was added to the same buffer for elution. The toxin was eluted with a linear gradient (0–0.6 M over 40 CV’s).

### 3. Results

The bacteria grew rapidly and the redox dropped to a value between $-350$ and $-400$ mV usually 12–24 h after the medium had been added. However, the redox went up again and reached almost 0 at the time of the harvest. Since the pH was not controlled, it remained comparatively low at 5.0–5.5. The fermentor batches yielded an average overall toxicity of $8.0 \times 10^8$ MLD, corresponding to $8 \times 10^4$ MLD mL$^{-1}$ of the culture.

The toxin yield of the filtration and chromatographic purification steps are summarized in Table 1. The data presented are the mean values of three consecutive production and purification runs.

#### Table 1

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (mL)</th>
<th>Protein concentration (µg mL$^{-1}$)</th>
<th>Toxin titer (MLD mL$^{-1}$)</th>
<th>Toxin amount (MLD)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fermentor culture</td>
<td>~10,000</td>
<td>6400</td>
<td>$8 \times 10^4$</td>
<td>$8.0 \times 10^8$</td>
<td>100.0</td>
</tr>
<tr>
<td>Microfiltration permeate</td>
<td>~12,000</td>
<td>4400</td>
<td>$4 \times 10^4$</td>
<td>$4.8 \times 10^8$</td>
<td>60.0</td>
</tr>
<tr>
<td>Ultrafiltration retentate</td>
<td>1050</td>
<td>5600</td>
<td>$4 \times 10^4$</td>
<td>$4.2 \times 10^8$</td>
<td>52.5</td>
</tr>
<tr>
<td>Hydrophobic interaction chromatography</td>
<td>640</td>
<td>95</td>
<td>$4 \times 10^3$</td>
<td>$2.6 \times 10^8$</td>
<td>32.5</td>
</tr>
<tr>
<td>Anion exchange chromatography</td>
<td>45</td>
<td>169</td>
<td>$4 \times 10^3$</td>
<td>$1.8 \times 10^8$</td>
<td>22.5</td>
</tr>
<tr>
<td>Cation exchange chromatography</td>
<td>14</td>
<td>320</td>
<td>$8 \times 10^6$</td>
<td>$1.1 \times 10^8$</td>
<td>13.8</td>
</tr>
</tbody>
</table>

Mean values of three consecutive production and purification runs are given.
Fig. 3. Chromatograms of the three purification steps of BoNT/E. The vertical lines limit either the peaks, which were toxin-positive, collected and subject to further purification or the final product (last step). Run details are given in the corresponding text boxes.
the micro- and ultrafiltration steps the total volume of the 10L cultures was reduced to approximately 1L, which was subject to chromatography. The corresponding chromatograms are given in Fig. 3. In the initial hydrophobic interactions chromatography, the toxin eluted at an ammonium sulfate concentration between 550 and 490 mM. Note that most of the DNA and RNA was eluted in the flow through fractions and the first major peak (absorption at 260 nm higher than at 280 nm). From the anion exchange column the fractions of two toxin peaks were collected and applied to the cation exchange column. The fractions of one distinct peak proved to contain BoNT/E (Fig. 4).

A total amount of 4.3 mg of BoNT/E (mean value) could be purified out of a 10L culture within 1 day. This corresponds to a recovery of 13.8% based on the toxicity of the fermentor culture. The purified toxin banded at approx. 150 kD in SDS-PAGE with a purity of > 98.8%. Two distinct bands, corresponding to the heavy and light chain of BoNT/E were found, when reducing conditions were applied after tryptic digestion, only. As expected with BoNT/E, DTT treatment alone did not cleave the molecule in heavy and light chain (s. Fig. 5). The purified toxin had a biological activity of $2.6 \times 10^7$ MLD mg$^{-1}$, again after treatment with trypsin.

4. Discussion

After the initial publication by Kitamura et al. (1968) on the purification of BoNT/E various improvements and novel procedures have been reported (DasGupta and Rasmussen, 1983; Schmidt and Siegel, 1986; Giménez and Sugiyama, 1987; Prabaykaran et al., 2001). These protocols have in common, that the cultures are centrifuged to discard and/or extract the toxins from the bacteria. The supernatants obtained in these steps and/or the bacterial extract(s) are often precipitated by ammonium sulfate treatment (DasGupta and Rasmussen, 1983; Giménez and Sugiyama, 1987; Prabaykaran et al., 2001). This requires a second centrifugation step to collect and wash the precipitate. Since botulinum cultures are highly toxic, transfer of the cultures in an open system has to be done with regard to the strict precautions necessary. These measures
are comparatively easy to follow, if the toxin is purified on the laboratory scale. However, if larger culture volumes need to be purified, these precautions require major technical input. In the purification protocol developed here all precipitation and centrifugation steps were avoided.

4.1. Filtration

The initial purification consisted of a two-step procedure with microfiltration (hollow fibre) and ultrafiltration (spiral cartridge). In the microfiltration step only 60% of the toxicity were recovered. An explanation might be the nominal pore size of the membrane of 0.1 μm. These pores are difficult to pass for molecules with the size and molecular mass of complexed BoNT. A future approach could be to favour the dissociation of the toxin in alkaline conditions prior to the filtration and/or to increase the nominal pore size of the filtration cartridge (0.2–0.45 μm). Since toxin is found intracellularly and bound to the surface of the bacterial cells, a toxin extraction from the cells might increase the total recovery as well (Giménez and Sugiyama, 1987).

The recovery of the ultrafiltration step alone (87.5%) was similar to the one achieved by De Jongh et al. (1989). De Jongh developed a rapid one-step ultrafiltration for the purification of BoNT/D using the supernatant of a centrifuged culture (98% recovery).

The micro-ultra-filtration provided a concentrated, pre-purified culture supernatant within 2 h and already in the buffer needed either for storage or for chromatography. Centrifugation and precipitation of the culture usually needs 1–2 days.

4.2. Hydrophobic interaction chromatography

In the culture supernatant and therefore in the retentate of the ultrafiltration step, the 150 kD BoNT is found as a 125 complex of 300 kD. In this complex BoNT/E is comparatively stable and less susceptible to enzymatic cleavage. Kitamura et al. (1968) and Prabaykaran et al. (2001) purified the toxin complex first. Then the complex was dissociated and the 150 kD BoNT/E was purified in the second stage. Schmidt and Siegel (1986) dissociated the complex already in the second step (anion exchange at pH 7.6) of a three step protocol. In the recently published protocol for the purification of BoNT/C and D (Gessler and Böhnel, 1999) the first step was a hydrophobic interaction chromatography at pH 8.0. At this pH the complex dissociates. For these toxins the complex could already partly be separated within this step. However, BoNT/E bound insufficiently to the resin with these conditions (data not shown). Therefore, the hydrophobic interaction chromatography (HIC) was run at pH 6.0.

Either ribonuclease digestion (Kitamura et al., 1968) or protamine sulfate precipitation (Schmidt and Siegel, 1986) is needed to remove RNA. This enables the toxin to bind to negatively charged resins. Giménez and Sugiyama (1987) succeeded to purify BoNT/E without RNA removal with an initial citrate buffer-DEAE-Sephadex chromatography.

In the method described herein the toxic fractions were sufficiently separated from the ribonucleic acids in the hydrophobic interaction chromatography speeding up the purification procedure.

HIC was also used by Weatherly et al. (2002) for the purification of recombinant heavy chains (Hc) of...
BoNT/A and BoNT/B. The proteins were not stable under these conditions. We could not find any drop in biological activity using this separation technique. However, the complete native toxin was applied.

4.3. Anion and cation exchange chromatography

The second step, anion exchange chromatography was, as well as the third, a modification of the protocol published by Schmidt and Siegel (1986). The separation was run at alkaline conditions to dissociate the complex and to obtain the 150 kD toxin. However, the toxin positive fractions of this run still contained further impurities and were subject to cation exchange chromatography at pH 6.0, the third step.

With this final step BoNT/E with single-band purity (>98.8% as determined in SDS-PAGE) was obtained.

5. Conclusion

To summarize, in three consecutive batches BoNT/E was reproducibly purified. The purification procedure used herein included filtration and chromatography techniques only. The filtration system was self-contained and minimizes the risk of laboratory contamination and/or aerosol formation. Besides that, it speeds up the purification. Purified neurotoxin can be obtained within 1 day depending on the equipment. The purification protocol can easily be scaled up to industrial needs.

Acknowledgements

This study was supported by the German Federal Ministry of Education, Science and Technology (BMBF), grant number 0311128. I am especially grateful to Prof. Korkeala and his group at the University of Helsinki, who provided us with the C. botulinum strain we used in this work. I wish to thank K. Hampe and M. Schmidt for their excellent laboratory work.

References


