Procedures for large-scale production and purification of Clostridium botulinum C1 toxin for preparation of toxoid

(Purification of C1 toxin; fortified cooked meat medium; acid precipitation)

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1. SUMMARY

Fortified cooked meat medium containing calcium carbonate (CaCO₃-FCM) supported toxin production of a strain of Clostridium botulinum type C to a level of 2 × 10⁶ mouse i.p. LD₅₀/ml. C1 toxin was purified by sequential steps of acid precipitation from 5-fold diluted culture supernatant in the presence of RNA, 2nd acid precipitation by dialysis, removal of RNA by protamine treatment, removal of excess protamine and bufferisation by ultrafiltration through Amicon PM-30 membrane, sulphopropyl-Sephadex chromatography, and Sephadex G-200 gel filtration. By these procedures, 25 mg or more of highly purified C1 toxin was constantly obtained from a lot of 600-ml culture.

2. INTRODUCTION

Varieties of birds, particularly wild ducks and other water birds, broiler chickens, turkeys and pheasants are affected by type C botulism [2]. In Japan, outbreaks of type C botulism have been reported among wild ducks [3], broiler chickens [4] and pheasants [5]. On a game pheasant farm in Hiroshima Prefecture, 50 to 100% of approximately 8000 pheasants annually died of type C botulism for three consecutive years from 1978. Vaccination of the pheasants with partially purified C1 toxoid conducted since 1981 effectively protected them from death [5]. It appeared that the purer the toxoid, the higher the immunogenicity and a simple method for larger scale production and purification of C1 toxin was urgently needed for production of the toxoid.

Sterne and Wentzel [6] and Cardella et al. [7] reported procedures for fairly large-scale production and purification of type C toxin. Our procedures, in contrast with theirs, are characteristic of the use of cooked meat medium for toxin production without such a laborious method as the sac culture and of rather simple steps of purification procedures.

3. MATERIALS AND METHODS

3.1. Strain

C. botulinum type C strain 003-9, isolated from the caecal contents of a broiler in 1980 and capable of producing a large quantity of C1 toxin, was used [5]. Two-day cultures of this strain in CaCO₃-fortified cooked meat medium (CaCO₃-FCM, see below) were kept frozen for seeding.
3.2. Culture for toxin production

For production of C1 toxin, CaCO₃-FCM was used. This medium consisted of 12.5% cooked meat medium (Difco Laboratories), 0.5% CaCO₃, 1.0% yeast extract (Oriental Yeast Kogyo), 1.0% ammonium sulphate, 0.8% glucose, 0.5% soluble starch and 0.1% L-cysteine hydrochloride. The pH was adjusted to 7.6. The frozen seed culture was defrosted and heat-treated for 15 min at 80°C. A 10-ml portion of the heat-treated culture was inoculated into 600 ml of CaCO₃-FCM, which was incubated for 2 days at 37°C in an anaerobic jar.

3.3. Chemicals and reagents

Sulphopropyl-Sephadex C-50 (medium) and Sephadex G-200 (particle size 40 to 120 μm) were purchased from Pharmacia Fine Chemicals; RNA (from yeast) was from P-L Biochemicals; protamine sulphate (from salmon sperm) were from Wako Pure Chemical Industries; Coomassie brilliant blue was from Nakarai Chemical Industries. Type C goat antitoxin of > 320 IU/ml (containing both anti-M and anti-L toxins of Clostridium botulinum type C strain 573) was a gift from Chiba Serum Institute. Rabbit antitoxin against C1-M toxin of the strain 003-9 was prepared in this laboratory.

3.4. Assays

The toxin was titrated by the time-to-death method in mice, and mouse i.p. LD₅₀/ml was calculated with a calibration curve made with purified C1 toxin [8]. Protein contents were determined by the method of Lowry et al. [9] with bovine serum albumin as the standard.

3.5. Polyacrylamide gel electrophoresis

Polyacrylamide gel of 4.5% and the tray buffer were prepared according to Reisfeld et al. [10]. Electrophoresis was carried out by applying a current of 2 mA per column at room temperature. After electrophoresis, the gel columns were stained with 0.25% Coomassie brilliant blue.

3.6. Agar gel double diffusion

About 4 ml of 1.0% agar (Agarose-1; Wako Pure Chemical Industries) gel in 0.05 M acetate buffer, pH 6.0, was spread over a glass plate (5 by 5 cm) with 7 stainless steel cylinders (2 by 10 mm) placed at the center and each angle of a hexagon of 7 mm in one side. The cylinders were removed after the gel solidified. After each well was filled, the agar plates were incubated in a moist chamber for 2 days at 20°C.

3.7. Purification of C1 toxin

The starting material was the culture supernatant obtained by centrifugation of the gauze filtrate of the whole culture at 3800 × g for 15 min. The flow sheet for purification of C1 toxin is given in Fig. 1.

For step 1 (acid precipitation), the culture supernatant was 5-fold diluted with distilled water, added with RNA (0.4 mg/ml) [11], adjusted to pH 3.9 with 3N H₂SO₄, and kept standing overnight under refrigeration.

For step 2 (extraction), the precipitate obtained by centrifugation after siphoning off the supernatant was extracted twice with 0.1 M Tris–HCl buffer, pH 7.5, containing 0.5 M NaCl, by centrifugation for 15 min at 10000 × g.

For step 3 (2nd acid precipitation and extraction), the extract was dialysed against 0.05 M...
acetate buffer, pH 4.0, for 2 days at 4°C. The precipitate formed during dialysis was extracted twice with 0.05 M acetate buffer, pH 4.0, containing 1 M NaCl, by centrifugation for 15 min at 10000 × g.

For step 4 (protamine treatment and ultrafiltration) were added to the extract: an equal volume of 0.05 M citrate buffer, pH 4.0, containing 1 M NaCl and a 2% protamine solution to an amount enough to reduce the $A_{260}/A_{280}$ to 1.0 or smaller [12]. The supernatant obtained by centrifugation for 20 min at 10000 × g was concentrated through an Amicon PM-30 membrane to one-eighth volume. The concentrate was diluted to the original volume with 0.05 M acetate buffer, pH 4.0, containing 0.2 M NaCl. Ultrafiltration and dilution were repeated twice more to bring the sodium chloride concentration down to 0.2 M and to remove the excess protamine. The bufferised material was clarified by centrifugation for 20 min at 10000 × g.

For step 5 (SP-Sephadex chromatography), the supernatant material was applied to an SP-Sephadex C-50 column (2 by 10 cm) equilibrated with the same buffer containing 0.2 M NaCl. The toxin, which was adsorbed onto the column, was eluted with linear gradient of NaCl concentration from 0.2 to 1 M in 1000 ml of the same buffer (Fig. 2).

For step 6 (gel filtration on Sephadex G-200), the toxic fractions eluted from SP-Sephadex were pooled and concentrated by filtration through Amicon PM-30 membrane. The concentrate was applied to a Sephadex G-200 column (2.5 × 95 cm) and eluted with 0.05 M acetate buffer, pH 4.0, containing 0.2 M NaCl.

RESULTS

4.1. Purification of CI toxin

The purity and recovery at each step of purification are given in Table 1. The culture supernatant gave a toxin titer of 2.20 × 10⁶/ml. Only upon dilution of the culture supernatant, acid precipitation was possible in the presence of RNA with a high recovery of CI toxin. The excess RNA was removed by the subsequent protamine treatment. The excess protamine was removed by the subsequent ultrafiltration through Amicon PM-30 membrane. In Sephadex G-200 gel filtration, two toxin peaks were eluted (Fig. 3); the former representing large-sized (L) and the latter medium-sized (M) toxins [1].

![Fig. 2. Chromatography of protamine-treated toxin from an SP-Sephadex column (2 by 10 cm) equilibrated with 0.05 M acetate buffer, pH 4.0, containing 0.2 M NaCl. Elution was made with a linear gradient of sodium chloride in the buffer. The sample contained 65.5 mg protein and a toxicity of 5.3 × 10⁸ mouse i.p. LD₅₀. Symbols: (●) mg protein/ml; (○) mouse i.p. LD₅₀/ml; (----) NaCl concentration.](https://academic.oup.com/femsle/article-abstract/30/1-2/47/539210)

![Fig. 3. Gel filtration of SP-Sephadex eluate on a Sephadex G-200 column (2.5 × 95 cm) with 0.05 M acetate buffer, pH 4.0, containing 0.2 M NaCl as an eluant. The sample contained 36.3 mg protein and a toxicity of 5.3 × 10⁸ mouse i.p. LD₅₀. Symbols: (●) mg protein/ml; (○) mouse i.p. LD₅₀/ml.](https://academic.oup.com/femsle/article-abstract/30/1-2/47/539210)
Table 1

Purification of *C. botulinum* C1 toxin from 600 ml culture supernatant

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>LD$_{50}$ ($\times 10^8$)</th>
<th>LD$_{50}$/mgN ($\times 10^7$)</th>
<th>Recovery of LD$_{50}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture supernatant</td>
<td>445</td>
<td>13</td>
<td>1.8</td>
<td>100</td>
</tr>
<tr>
<td>Extract of 1st acid precipitate</td>
<td>411</td>
<td>12</td>
<td>1.8</td>
<td>92</td>
</tr>
<tr>
<td>Extract of 2nd acid precipitate</td>
<td>166</td>
<td>8</td>
<td>3.0</td>
<td>62</td>
</tr>
<tr>
<td>Protamine-treated</td>
<td>65.5</td>
<td>5.3</td>
<td>5.1</td>
<td>41</td>
</tr>
<tr>
<td>SP-eluate</td>
<td>36.3</td>
<td>5.3</td>
<td>9.1</td>
<td>41</td>
</tr>
<tr>
<td>Sephadex G-200 effluent</td>
<td>27.0</td>
<td>4.3</td>
<td>10</td>
<td>33</td>
</tr>
<tr>
<td>1st toxin</td>
<td>(19.4)</td>
<td>(2.5)</td>
<td>(15.1)</td>
<td>(19.2)</td>
</tr>
<tr>
<td>2nd toxin</td>
<td>(7.6)</td>
<td>(1.8)</td>
<td>(13.8)</td>
<td></td>
</tr>
</tbody>
</table>

The overall recovery in the toxicity was 33%, which corresponded to 27 mg of pure toxin, from 600 ml of culture.

4.2. Polyacrylamide gel electrophoresis of purified C1 toxin at pH 4.0

Purified L toxin formed a major band and some minor bands; purified M toxin migrated in virtually a single band (Fig. 4).

4.3. Agar gel double diffusion tests with purified C1-L and M toxins at pH 6.0

Both toxins each formed one or two precipitin lines against rabbit antitoxin type C and the lines fused each other. Against goat antitoxin type C, both toxins formed a precipitin line (or two) and the lines fused each other, in addition, L toxin formed another distinct precipitin line.

5. DISCUSSION

Type C botulism has been reported in domestic animals and fowls. Vaccination of the animals and fowls in danger of botulism with type C toxoid, mostly crude preparations, has effectively protected them [6,13].

Type C organisms are known to produce three toxic factors, C1, C2 and D. Miyazaki and Sakaguchi [14] suggested that C2 toxin might play an important role in chicken death. Later on, both pheasants and chickens proved to be highly susceptible to C1 toxin but not to C2 nor D toxin by oral or intraduodenal administration. Vaccination of the pheasants on the farm where type C botulism had been prevalent in preceding years with partially purified C1 toxoid effectively protected them [5]. These results indicate that C1 toxin is the primary cause of death in avian type C botulism.

C1 toxin has been purified by several investigators in this and other laboratories [6,7,11,15]. Their methods, however, are too laborious for large-scale
production and purification of C1 toxin. The procedures reported herein are simple to perform and efficient for purifying C1 toxin. We adopted C. botulinum type C strain 003-9, which produces C1 toxin to a level of $2 \times 10^6$ mouse ip LD$_{50}$/ml of culture. The level is equivalent to those attained by good toxin producers among type A and B strains. In addition, we used CaCO$_3$-fortified cooked meat medium instead of peptone-yeast extract-glucose medium or other commonly used clear media for production of C1 toxin. The disadvantage of this medium is the large amount of proteinous substances present in the initial culture supernatant, but the present procedures for purification of C1 toxin overcame this disadvantage. The procedures are characterized by the following three points: (1) Dilution of the culture supernatant with distilled water allowed acid precipitation of C1 toxin in the presence of RNA. This step, simple and safe to perform, allowed a high recovery and purification. (2) The acid precipitate was extracted with a buffer of pH 7.5 instead of that of pH 6.0 or below. Only at this pH, extraction was satisfactory. Molecular dissociation of C1 toxin at pH 7.5 was anticipated but all the subsequent steps were performed at pH 4.5 or below, which allowed reassociation of the dissociated components. (3) The toxic material after protamine treatment was concentrated by ultrafiltration through Amicon PM-30 membrane and diluted with the buffer for chromatography. This process allowed both removal of the excess protamine, which can not be removed by dialysis, and bufferisation of the toxic material.

By the present methods, at least 25 mg of C1 toxin was constantly obtained from 600-ml culture. The C1 toxin thus purified appeared to be highly pure on the basis of the specific toxicity and the patterns of agar gel double diffusion and polyacrylamide gel electrophoresis. The two precipitin lines, if there were common to L and M toxins, might represent those of the toxic and the nontoxic components as observed with progenitor toxins of other types. If the dose of 21 μg of toxoid is enough for a pheasant [5], a lot of 5-l culture would be sufficient for the production of 10000 pheasant doses of C1 toxoid.

REFERENCES