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Improved Procedure for Purification of *Clostridium botulinum* Type B Toxin

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Clostridium botulinum Type B 독소의 정제방법에 관한 연구

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ABSTRACT

The neurotoxin of *Clostridium botulinum* type B was purified from a liquid culture. The purification steps consist of ammonium sulfate precipitation of whole culture, treatment of Polymin P (0.15%, v/v), gel filtration on Sephadex G-100 at pH 5.6 and DEAE-Sephadex chromatography at pH 8.0. The procedure recovered 17% of the toxin assayed in the starting culture. The toxin was homogeneous by sodium dodecyl sulfate(SDS)-polyacrylamide gel electrophoresis and had a molecular weight of 163,000. Subunits of 106,000 and 56,000 molecular weight were found when purified toxin was treated with a disulfide-reducing agent and electrophoresed on SDS-polyacrylamide gels.

INTRODUCTION

Clostridium botulinum is a gram positive, spore forming anaerobic bacillus producing a potent neurotoxin which causes the food poisoning of botulism. The species is divided into eight types (A, B, C_α, C_β, D, E, F, G) on the bases of immnological specificity of toxin produced by the culture. Although its incidence is low compared with several other poisoning of bacterial origin, botulism is important because the high toxicity and unique pharmacological action of the toxin cause severe illness.

It is necessary to obtain highly purified toxin for the characterization of the toxin and for the preparation of toxoid and antitoxin of clinical acceptability. Two methods have been reported for purification of *Clostridium botulinum* type B toxin. Both method used at least three successive chromatographic steps, and so was very time consuming procedure. In the first method, dialysis sac culture was precipitated with ammonium sulfate and was subjected to a series of chromatographic steps: DEAE-cellulose and Sephadex G-100 at pH 5.6, Sephadex G-200 at pH 7.3, and finally DEAE-Sephadex at pH 7.3(DasGupta *et al.*, 1968). In the second method, ammonium sulfate precipitated toxin was successively chromatographed on DEAE-cellulose at 5.6, DEAE-cellulose at pH 8.0, and Sephadex G-200 at pH 5.7 (Beers and Reich, 1969). The reason for using many chromatographic steps was to remove

nucleic acid and some acidic proteins from toxin molecule. Polymine P(a polyethylenimine) has been used in purification of DNA-dependent RNA polymerase(Burgess and Jendrisak, 1975; Jendrisak and Burgess, 1975). This very basic agent is known to precipitate acidic molecules including nucleic acds, nucleoproteins, and acidic proteiins. The present study is designed to simplify the purification steps of botulinal toxin using Polymin P.

MATERIALS AND METHODS

Toxin production

Clostridium botulinum type B strain Lamanna was used. Stocks were cultures grown in cooked meat medium(Difco) for 4 days and then stored at 4°C. All incubation were carried out at 37°C. Toxin production medium was 1% trypticase(BBL), 2% proteus peptone(Difco), 1% yeast extract (Difco), and 0.05% sodium thioglycolate. The pH was adjusted to 7.3 with 1.0 N NaOH. After autoclaving(121°C, 20min), 50% glucose solution, sterilized sepeartely, was added to the final concentration of 1%. The inoculum of actively growing culture was prepared by serially subculturing the stock culture into 9 volumes of fresh media at 14~16 hr invervals. An inoculum of 100 *ml* was added for 1700 *ml* of media to obtain a total culture volume of 1.8 liters. Incubation for toxin production was 3 days.

Toxin assay

Toxin was assayed in mice of 20~25g body weight by intravenous (iv) injection method (Boroff and Fleck, 1966). Two to four mice were injected in the tail vein with 0.1 *ml* of sample and the time (minutes) between challenge and death was recorded. The LD_{50} was read from a standard curve that had been obtained by determining death times of mice challenged with known LD_{50} doses.

Concentration of toxin

Solid ammonium sulfate was added to the whole culture of 1.8 liters to the final concentration of 60% saturation(390 *g/l*). After holding 1 day at 4°C, the precipitate was collected by centrifuging at 5,000×g for 20min at 4°C. The pellet was was exacted with about 100 *ml* of 0.067 M citrate-phosphate buffer, pH 5.6 and the turbid fluid was clarified by centrifuging at 7,000×g for 15 min at 4°C. This toxin solution will be called "conc crude toxin."

Polymin P treatment

Polymin P was obtained from Elli Lilly Co. A 10%(w/v) stock solution titrated with concentrated HCl to pH 5.6 was prepared and clarified by centrifuging at 7,000×g for 20 min. This stock solution was added slowly to the "conc crude toxin" with stirring to a predetermined final concentration. After continuing to stir for 5 minutes, the mixture was centrifuged for 15 min at 7,000×g and the supernatant was collected. This toxin fluid was 60% saturated with ammonium sulfate by slowly adding saturated ammonium sulfate solution. After holding 24hr at 4°C, toxin precipitate was collected by centrifugation(7,000×g, 20 min, 4°C) and dissolved in 10 *ml* of 0.067 M citrate phosphate buffer, pH 5.6. This toxin solution will be called "conc toxin."

Gel filtration on Sephadex G-100 column

Sephadex G-100 (Sigma) was allowed to swell at room temperature for 4 days in 0.067 M citrate phosphate buffer, pH 5.6. A 2.0× 100 *cm* column was packed to a bed height of 65 *cm*. A wad of glass wool was placed on top of the packed resin and the column was equilibrated with the above buffer for 24 hr. After application of 5 *ml* of "conc toxin," the column was eluted with the same buffer at a flow rate of 10 *ml*/hr. Fractions of 2.0 *ml* were collected and A_{278} of the fraction was measured using UV spectrophotometer. Toxicity of

appropirate fractions was determinied by iv assay. Pool of toxin fractions from G-100 column was dialyzed against 20 volumes of 0.15 M Tris-HCl buffer, pH 8.0, for 6hr with four changes of the dialyzing buffer at 4°C. This toxin preparation will be called "G-100 toxin."

DEAE-Sephadex chromatography

DEAE-Sephadex A-50 (Sigma), exchange capacity 3.5 meq/*g*, was soaked in 0.15 M Tris-HCl buffer, pH 8.0, for 2 days at room temperature. A 1.0×10 *cm* bed was prepared. Twenty-five *ml* of "G-100 toxin" was applied, and the column was washed with the same buffer. When A_{278} of the fractions dropped to near zero, toxin was eluted by a linear NaCl gradient in the buffer. The NaCl gradient was generated with a gradient mixer in one chamber charged with 100 *ml* of the pH 8.0 buffer and the other chamber with 100 *ml* of the same buffer containing 0.5 M NaCl. Flow rate was 20 *ml*/hr and ffraction volume was 2.5 *ml*. Toxin fractions was pooled and tested for homogeneity and other properties.

SDS-polyacrylamide gel electrophoresis

The purified type B toxin was tested for homogeneity by SDS-polyacrylamide gel electrophoresis. Preparation of gels, electrophoresis, staining, and destaining were according to Weber and Osborn (1969). Samples for electrophoresis were solutions made with 0.2 *ml* of toxin solution, 0.02 *ml* of 10% SDS, and 50 *mg* of urea. For reduction of disulfides in samples, 5 μl of β-mercaptoethanol was added to the toxin-SDS solution; the resulting solution was held in boiling water bath for 5 min, cooled and added urea. Gels (6×100 *mm*) were electrophoresed without sample for 2 hr at 8 *m*A/gel. Samples of 0.05 *ml* were then carefully layered at the buffer-gel interface with a 0.05 *ml* syringe. After electrophoresis for 6 hr at 8 *m*A/gel, gels were extruded and stained with Coomassie brilliant blue.

Molecular weight determination

The method of Weber and Osborn (1969), described above, was used. Molecular weights of proteins in the sample was established by comparing their electrophoretic mobility with those of marker proteins. As marker proteins, Pharmacia High Molecular Weight Electrophoresis Calibration Kit was used. It was dissolved in 0.1m*l* of 0.1M phosphate buffer containing 1% SDS and 1% β-mercaptoethanol and heated at 60°C for 15 min.

RESULTS AND DISCUSSION

Table 1 shows effect of Polymin P concentration on A_{280}/A_{260} ratio and toxicity yield in supernatant solution. Up to final concentration of 0.15%, A_{280}/A_{260} ratio was increased, indicating that nucleic acids were precipitated out from the solution. At this condition, 80% of original toxicity was remained in the supernatant. From this results, final concentration of 0.15% Polymin P was used as optinum concentration in the purification of toxin.

Fig. 1 shows the results of gel filtration of "conc toxin" through Sephadex G-100. Three well seperated protein peaks were obtained. Most of the toxic activity applied to the column was found in the first peak. Fractions across this peak with A_{278} of 0.6 and above were pooled and dialyzed against 0.15 M Tris-HCl buffer, pH 8.0.

Fig. 2 shows the typical elution profile of DEAE-Sephadex chromatography at pH 8.0. A nontoxin protein peak emerged on washing the column with 0.15 M Tris-HCl buffer, pH 8.0. During elution with a NaCl gradient, toxin protein came off first; this was followed by two nontoxin protein peaks. The specific toxicity of the eluate in the toxin peak was a mean value of 1.4×10^7 LD_{50}/A_{278}. Fractions

Table 1. Effect of Polymin P concentration on A_{280}/A_{260} ratio and toxicity yield.

Polymin P concentration(%)	0	0.05	0.1	0.15	0.2	0.25	0.3	0.35	0.4	0.8	0.16
A_{280}/A_{260}	0.54	0.73	0.99	1.03	1.03	1.00	1.01	1.02	1.03	1.03	1.01
Toxicity yield(%)	100	83	80	80	—	—	73	—	—	71	56

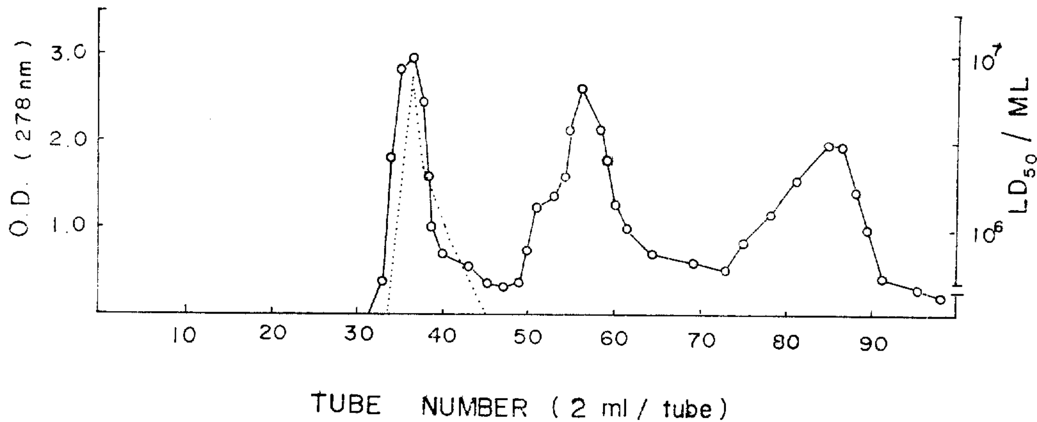

Fig. 1. Gel filtration of "conc, toxin" through the 2.0×100 *cm* Sephadex G-100 column. Sample volumes was 5 *ml*; buffer was 0.067 M citrate phosphate buffer, pH 5.6. —0— Absorbance, ... Toxicity

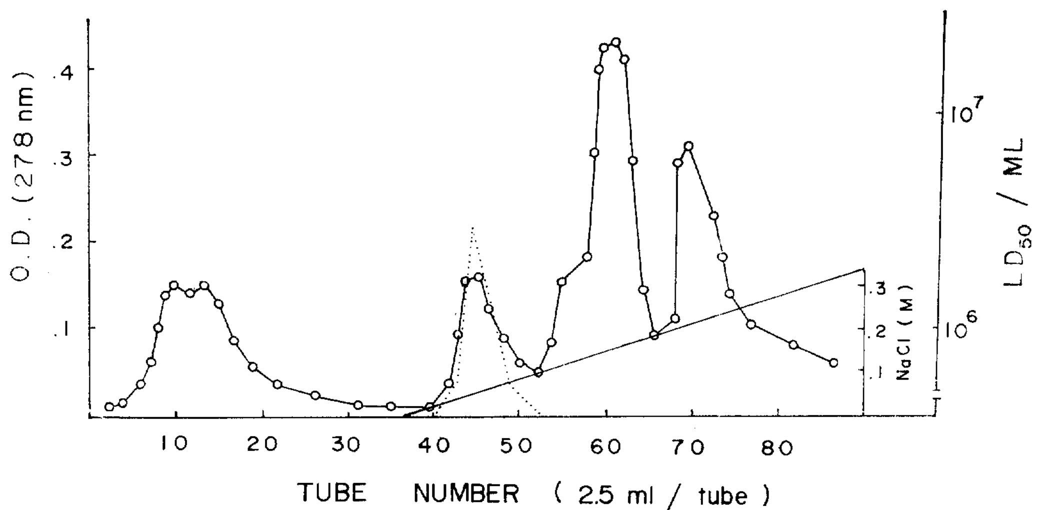

Fig. 2. Elution profile when "G-100 toxin" chromatographed on DEAE-Sephadex. Equilibrating buffer was 0.15 M Tris-HCl, pH 8.0. —0— Absorbance, ... Toxicity, ——Molarity NaCl

across the toxin peak with A_{278} of 0.07 and above were pooled and tested for homogeneity and other properites.

Table 2 shows the recovery and purification attained at each of the purification steps described. They were the average figures obtained during processing of two seperate 1.8 liters cultures. The purified toxin had a specific toxicity about 3,600 fold greater than the starting culture fluid and contained 17% of the original toxicity.

Fig. 3 shows the typical result when purified

Table 2. Summary of purification of *Clostridium botulinum* type B toxin.

Purification step	Volume (*ml*)	Toxicity (LD_{50}/ml)	Specific toxicity (LD_{50}/A_{278})	% Recovery	
				From preceding step	From culture fluid
Culture fluid	1800	1.1×10^5	3.8×10^3	(100)	(100)
$(NH_4)_2SO_4$	100	1.3×10^6	7.2×10^4	67	67
Polymin P	10	9.6×10^6	3.8×10^5	72	48
Sephadex G-100	25	3.0×10^6	2.0×10^6	78	37
DEAE-Sephadex	25	1.4×10^6	1.4×10^7	45	17

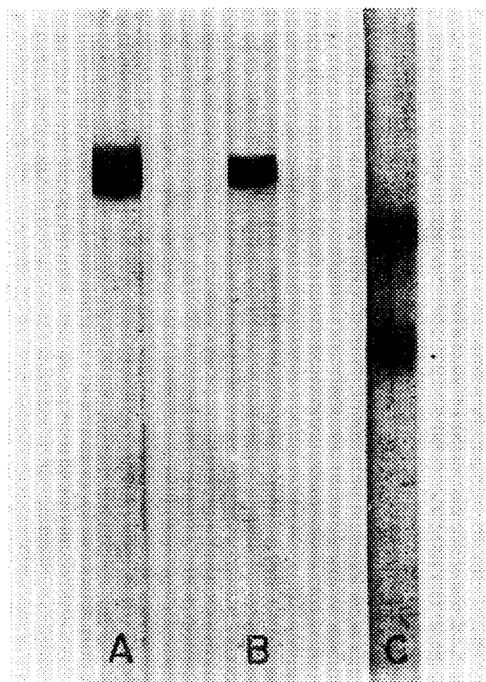

Fig. 3. SDS-polyacrylamide gel electrophoresis of purified toxin. Band migrated from top to bottom (anode). Electrophoresis was done for 6hrs at 8*m*A/gel.
A: 100 *μg* of sample, not reduced
B: 50 *μg* of sample, not reduced
C: 100 *μg* of sample, reduced with β-mercaptoethanol.

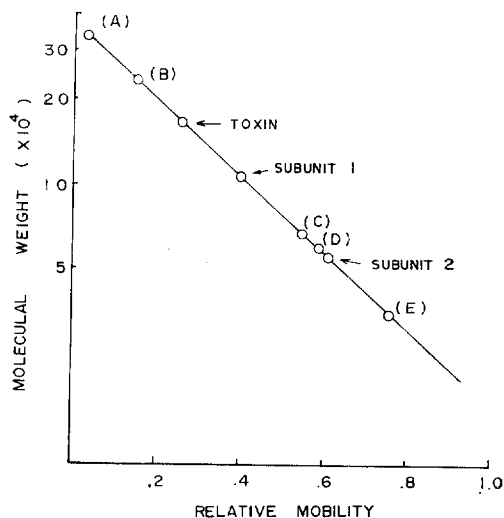

Fig. 4. Plot of molecular weight of marker proteins versus their electrophoretic mobility on gels.
(A) Tyroglobulin, M.W. 330,000
(B) Ferritin, M.W. 220,000
(C) Albumin, M.W. 67,000
(D) Catalase, M.W. 60,000
(E) Lactate dehydrogenase, M.W. 36,000
Mobility of toxin and subunits(arrows).

toxin was electrophoresed in SDS-polyacrylamide gels. Homogeniety of the purified toxin is shown by the single band in gels(Fig.3 A and B). When the toxin was treated with β-mercaptoethanol to reduce disulfides, the band obtained with the unreduced toxin disappeared; in its place were two faster moving bands (Fig. 3 C). This results are in agree with previous reports that botulinum toxins are composed of two polypeptide subunits which are linked by disulfide bond(s) (DasGupta and Sugiyama, 1972; Sugiyama, 1980).

Fig. 4 shows the plot of molecular weight of marker proteins versus their electrophoretic mobility on gels. Molecular weight of toxin was 163,000. This value is in accord with

previous reports of 165,000 (DasGupta *et al.*, 1968) and 167,000 (Beer and Reich, 1969). Toxin reduced with β-mercaptoethanol showed subunits of mol wt 106,000 and 56,000.

The overall significance of current study is that it provides a simplified procedure for purification of type B toxin. The existing methods used three to four chromatographic steps which are inconvinient and time consuming. Meanwhile the procedure presented in this paper reduced the chromatographic steps to two. Efforts to simplify the purification procedure further have been failed so far; elimination of one step or change the order of steps always ended up two or more bands on SDS-polyacrylamide gel electrophoresis. The results of Polymin P treatment suggests Polymin P effectively precipipate out nucleic acids from the toxin solution with minimum loss of toxicity. Whether Polymin P treatment process could applicable to purify other types of botulinal toxin remains to be solved.

摘 要

Clostridium botulinum type B가 생성하는 독소를 정제할 수 있는 방법을 연구하였다. 정제과정은 독소를 ammonium sulfate로 배양액에서 침전시켜 추출한후 Polymin P를 처리하여 핵산 및 기타 단백질을 최대한 제거한후 Sephadex G-100에서 gel filtration을 시키고 DEAE-Sephadex로 이온교환 크로마토그래피를 시켰다. 이러한 과정으로 정제된 독소의 회수율은 17%였으며 SDS-polyacrylamide gel electrophoresis 결과 하나의 선을 나타내 동질성을 증명하였다. 정제된 독소의 분자량은 163,000이었으며 β-mercaptoethanol을 사용하여 환원시킨 결과 분자량 106,000과 56,000의 하위 단위체로 분리되었다.

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