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Isolation of the components of progenitor toxin produced by *Clostridium botulinum* type C strain Stockholm

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SUMMARY

Clostridium botulinum type C strain Stockholm (C-St) produced two types of progenitor toxins (M and L). The purified L toxin consisted of neurotoxin (NT), nontoxic-nonhemagglutinin (NTNHA) and hemagglutinin (HA) components which could be separated into four subcomponents, HA-33, HA-17, HA-22-23 and HA-55. On the other hand, purified M toxin lacked HA components. We isolated all components from the progenitor L toxin by following chromatographic methods. The L toxin was separated into an NT and NTNHA/HAs complex via Mono Q anion exchange column chromatography. Using gel filtration with a TSK-gel HW-55 S column in the presence of 6 M guanidine hydrochloride, the NTNHA/HAs complex derived from the L toxin could be separated into three fractions, NTNHA single component, HA-55/33 mixture and HA-22-23/17 mixture. Then, the HA-55/33 mixture was separated into each component using Mono S cation exchange column chromatography in the presence of 8 M urea. The HA-22-23/17 mixture was also separated from each other with Superose 12 gel filtration column chromatography in the presence of 8 M urea. The separated NT, NTNHA and HA subcomponents were homogenous on SDS-PAGE, and were identified to be C-St progenitor toxin gene products based on their N-terminal amino acid sequence analysis. This study presents for the first time an attempt to isolate components from the progenitor toxin. Although reassociation of the isolated components was also attempted via mixing of NTNHA, HA-55, HA-33, HA-22-23 and HA-17, HA activity of the mixture was lower than that of parent NTNHA/HAs complex.

Key words: botulinum toxin, type C-St, progenitor toxin, hemagglutinin, isolation.

INTRODUCTION

Botulinum toxin, which is one of the most toxic materials found in nature, acts primarily on cholinergic nerve terminals where it inhibits the release of acetylcholine at the neuro-muscular junctions and synapses. *Clostridium botulinum* has been classified into seven types (A to G) based on its antigenecities against the neurotoxin (NT) produced. Most NTs are produced as a single chain except type E and some strains of types A and D, and are nicked in the molecule by endogenous protease(s) produced by the bacteria.¹⁾ The nicked NT

can be separated into two fragments, designated as light chain (Lc; 50-kDa) and heavy chain (Hc; 100-kDa) by a reduction in the disulfide bond. This proteolytic NT modification is much more potent than the single chain structure.²⁾

In culture supernatants, the NT associates with the nontoxic nonhemagglutinin (NTNHA) components and/or hemagglutinin (HA) components, then exists as stable and large complexes. These complexes, designated progenitor toxin, are found in three forms with molecular weights of 900-kDa (LL toxin for type A), 500-kDa (L toxin for types A, B, C, D and G) and

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Abbreviations: NT, neurotoxin; HA, hemagglutinin; NTNHA, nontoxic-nonhemagglutinin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Lc, light chain; Hc, heavy chain; PVDF, polyvinylidene difluoride; kDa, kilo dalton.

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300-kDa (M toxin for types A, B, C, D, E and F).^{3,4)} The LL and L toxins show hemagglutinin activity, but the M toxin does not.

Of the seven toxin types, types C and D have had their toxigenicity determined by specific bacteriophages^{5,6)} and the presence of common antigenic sites in both types has been determined previously.⁷⁾ Recently, progenitor toxins from some strains of the type C strains Stockholm (C-St) and 6813 (C-6813), and type D strains CB-16 (D-CB 16) and 1873 (D-1873) were purified and their molecular composition were determined based on nucleotide sequences and protein chemical analysis.⁸⁻¹¹⁾ The homology of the amino acid sequences of the type C and D NTs was 52.2%, but that of NTNHA and HAS was almost identical. Furthermore, the purified M toxins from types C and D commonly consisted of the NT (molecular weight of 150-kDa) and nicked NTNHA on the N-terminal region of which the banding profile gave two bands, 115- and 15-kDa, on SDS-PAGE. On the other hand, the purified L toxins from both types were also formed by association with NT, NTNHA and three HA subcomponent (HA-17, -33 and -70) gene products. The NT was split into a Lc and Hc, and the HA-70 gene product was split into HA-55 (55-kDa) and HA-22-23 (22- and 23-kDa) by processing after translation.

The nontoxic components of the progenitor toxin are considered to play an important role in the onset of botulism caused by oral ingestion. NTNHA is considered to protect the NT from acidity and proteases in the stomach,¹²⁾ whereas HAS plays an essential role in the effective absorption of the progenitor toxins in the small intestine.¹³⁾ Of the components, the HA-33 subcomponent except NT and NTNHA have been isolated from the progenitor toxin and characterized.^{14,15)} However, complete purifying and separation of all the components of the progenitor toxin have not yet achieved. In this paper, we first demonstrate that all components could be separated from the C-St progenitor toxin using chromatographic methods in the presence of denaturant reagents and reassociation of the HA subcomponents showed partially restored HA activity.

MATERIALS AND METHODS

Materials

The following materials were obtained from the sources indicated. Polypeptone (Nihon Pharmaceutical Co., Ltd., Osaka, Japan); peptone (Kyokuto, Tokyo, Japan); yeast extract (Difco Lab., Detroit, MI, USA); lactalbumin hydrolysate (Wako Ltd., Osaka, Japan); TSK-gel HW-55 S, SP-Toyopearl 650 S (Tosoh, Tokyo, Japan); molecular weight size marker for SDS-PAGE, Mono S HR 5/5, Mono Q HR 5/5, Superose 12 and HiTrap desalting column (Pharmacia, Uppsala, Sweden); Vivapore 10 (VivaScience, Ltd., Lincoln, UK). All other reagents used were of commercially

available analytical grade.

Production of the progenitor toxin

Production of the progenitor toxin from *Clostridium botulinum* type C strain Stockholm (C-St) was performed by essentially the method as described by Ohya et al.¹⁰⁾ Toxins were produced using the dialyzing cultivation method reported previously¹⁶⁾ with some modifications. The outer medium (1,000 ml) of the dialysis tube consisted of 2% polypeptone, 2% lactalbumin, 1% peptone and 1% yeast extract. The inner medium (50 ml) consisted of 20% glucose, 4% NaCl and 1% L-cysteine hydrochloride. The cells were precultured at 37°C overnight in 100 ml of TYG medium with 3% trypticase pepton, 2% yeast extract, 0.5% glucose and 0.1% L-cysteine hydrochloride (pH 7.2). 10 ml of the culture from the TYG medium was inoculated into the inner medium of the dialysis tube, and the bottle was incubated at 37°C for 5 days. For one batch of toxin preparation, 8 bottles were used. The inner medium was centrifuged at 8,000 *g* for 20 min and the resulting supernatants were used as crude preparations for the purification of the progenitor toxin components.

SDS-PAGE

SDS-PAGE was performed as described by Laemmli¹⁷⁾ using a 12.5% acrylamide gel. Protein samples containing 10 mM Tris-HCl (pH 6.8), 1% SDS, 1% 2-mercaptoethanol and 20% glycerol were treated in a boiling bath for 5 min. Electrophoresis was performed with RAPIDAS mini slab gel electrophoresis apparatus (ATTO, Tokyo, Japan). After electrophoresis, the gels were stained with 0.25% Coomassie Brilliant Blue (CBB) R-250 and then destained. The molecular weight standards were phosphorylase b (94-kDa), bovine serum albumin (67-kDa), egg ovalbumin (43-kDa), carbonic anhydrase (30-kDa), trypsin inhibitor (20.1-kDa) and α -lactalbumin (14.4-kDa).

N-terminal amino acid sequence analysis

All progenitor toxin components separated by SDS-PAGE were electroblotted onto a polyvinylidene difluoride (PVDF) membrane using semi-dry blotting apparatus (Nippon Eido, Tokyo, Japan) according to the method of Hirano and Watanabe.¹⁸⁾ Protein transfer was completed within 60 min at a constant current of 100 mA. The membrane was briefly stained with 0.1% CBB R-250 to visualize protein bands and stained bands corresponding to each component were cut out. The N-terminal amino acid sequences of the components were determined using a pulsed-liquid phase sequencer (Model 477 A, Applied Biosystems, Foster City, CA, USA) equipped with a on-line high-performance liquid chromatograph (Model 120 A, Applied Biosystems).

Hemagglutination assay

A hemagglutination assay was performed with U-bottom microtiter plates. 25 μ l of each preparation (250 μ g/ml) were diluted in serial two fold steps with 0.15 M

of a sodium phosphate buffer (pH 7.0) and mixed with an equal volume of a 2% suspension of washed horse erythrocytes. After incubation at room temperature for 2 hrs, the reciprocal hemagglutination titer was denoted. Protein concentration was calculated using the absorbance at 280 nm (absorbance 0.7 corresponds to 1 mg of protein/ml).

RESULTS

Purification of the progenitor toxin

The progenitor toxin was purified from the culture supernatant of *Clostridium botulinum* C-St according to the flow sheet as illustrated in Fig. 1. The culture supernatant from the cells was precipitated with ammonium sulfate at 60% saturation. The precipitate was dissolved and dialyzed against a 50 mM acetate buffer (pH 4.0) containing 0.2 M NaCl. The crude toxin preparation was applied to an SP-Toyopearl 650 S cation exchange column (1.6×40 cm) equilibrated with the same buffer. The absorbed toxin was eluted with a gradient of NaCl (0.2 to 0.8 M) in the same buffer. The two different sized molecular toxins, M and L, were demonstrated by cation exchange column chromatography under acidic conditions (pH 4.0) as shown in Fig. 2. The fractions containing the L and M toxins were collected separately, and then concentrated with ammonium sulfate at 80% saturation to a minimum volume.

Fig. 1. Flow sheet of isolation and purification procedure for the components from the *Clostridium botulinum* type C strain Stockholm progenitor toxin.

Each chromatographic step, (a) to (e), described in materials and method was corresponding to those in Fig. 3.

The concentrated L toxins were dissolved and dialyzed against a 20 mM Tris-HCl buffer (pH 7.8) to change pH condition for the next Mono Q column chromatography. **SDS-PAGE and N-terminal amino acid sequence analysis of the progenitor toxins**

The L progenitor toxin preparation showed seven major bands with molecular weights of 130-, 100-, 55-, 50-, 33-, 22-23- and 17-kDa on SDS-PAGE in the presence of a reducing reagent (Fig. 2, right profile). Based on a previous report,⁸⁾ it could be concluded that the 130-kDa bands was the NTNHA, and those of 100- and 50-kDa were the Hc and Lc of the NT, respectively. The remaining proteins of 55-, 33-, 22-23- and 17-kDa were components of HA. The N-terminal amino acid sequences of the protein bands constituting the progenitor toxin were determined using the direct protein microsequencing procedure (Table 1). The sequences exactly corresponded to those of the deduced amino acid sequence from the nucleotide sequence of the C-St progenitor toxin gene⁸⁾ reported previously. The HA-55 and HA-22-23 components were derived from the splitting of the HA-70 gene product due to endogenous protease after translation.

The M progenitor toxin preparation demonstrated three major bands with molecular weights of 115-, 100-, 50- and 15-kDa on SDS-PAGE in the presence of a reducing reagent (Fig. 2, left). The bands of 100- and 50-kDa were the Hc and Lc of the NT as shown by their respective N-terminal amino acid sequences (Table 1), as well as those of the L toxin preparation. The bands of 115-kDa and 15-kDa were fragments derived from the NTNHA protein by cleavage between Thr 140 and Ser 141 based on N-terminal amino acid sequence analysis. Recently, the NTNHAs of type A^{19,20)} and D¹⁰⁾ M toxins have also been shown to have cleavage on their N-terminal regions and therefore they are separated into two parts on SDS-PAGE with or without a reducing reagent.

Thus, all protein components of the progenitor toxin preparation were characterized by their N-terminal amino acid sequences, and each component in the subsequent dissociation procedure was ascertained by the molecular size of its protein bands developed on SDS-PAGE.

Dissociation and purification of the components from the progenitor L toxin

The L toxin prepared by dialyzing against 20 mM Tris-HCl buffer (pH 7.8) was applied to an FPLC system (Pharmacia, Uppsala, Sweden) fitted with the Mono Q HR 5/5 anion exchange column equilibrated with the dialysis buffer. The absorbed proteins were eluted with a step gradient of NaCl (0 to 0.3 M) in the same buffer. Two peaks appeared as shown in Fig. 3a; the first peak corresponded to NT and the second to the NTNHA/HAS complex, judged by SDS-PAGE analysis

Fig. 2. Purification of M and L toxins produced by *C. botulinum* type C strain Stockholm on SP-Toyopearl 650S column chromatography (a) and the SDS-PAGE banding profile of the purified toxin (b).

(a) The solid line indicates absorbance at 280 nm and the dotted line indicates NaCl concentration. (b) Electrophoresis of each toxin was performed on 12.5% acrylamide gels in the presence of 2-mercaptoethanol. Each component of the progenitor toxin is indicated by arrows.

Table 1. N-terminal amino acid sequences of the separated components from the M and L toxin preparations produced by *C. botulinum* type C strain Stockholm on SDS-PAGE as illustrated in Fig. 2 and corresponding sequences deduced from the nucleotide sequences of *C. botulinum* type C strain Stockholm genes.⁸⁾

Protein band	N-terminal sequence determined	Amino acid sequence deduced from the nucleotide sequence	Gene product
M Toxin			
NTNHA (115kDa)	1 5 10 15 20 S T I P F P E G G Y R E T N Y I E S Q N	141 145 150 155 160 V T S T I P F P E G G Y R E T N Y I E S Q N N	NTNHA
Hc (100kDa)	1 5 10 15 20 S L Y N K T L D X R E L L V K N T D L P	432 435 440 445 450 D G R S L Y N K T L D C R E L L V K N T D L P	NT (Hc)
Lc (50kDa)	1 5 10 15 20 P I T I N N F Y S D P V D N K N I L Y L	1 5 10 15 20 M P I T I N N F Y S D P V D N K N I L Y L D T	NT (Lc)
NTNHA (15kDa)	1 5 10 15 20 M D I N D D L N I N S P V D N K N V V I	1 5 10 15 20 M D I N D D L N I N S P V D N K N V V I V R A	NTNHA
L Toxin			
NTNHA (130kDa)	1 5 10 15 20 M D I N D D L N I N S P V D N K N V V I	1 5 10 15 20 M D I N D D L N I N S P V D N K N V V I V R A	NTNHA
Hc (100kDa)	1 5 10 15 20 S L Y N K T L D X R E L L V K N T D L P	432 435 440 445 450 D G R S L Y N K T L D C R E L L V K N T D L P	NT (Hc)
HA-55 (55kDa)	1 5 10 15 20 N S N N N I N F N Y S S Q T I L P Y P N	191 195 200 205 210 S K N S N N N I N F N Y S S Q T I L P Y P N G	HA-70
Lc (50kDa)	1 5 10 15 20 P I T I N N F Y S D P V D N K N I L Y L	1 5 10 15 20 M P I T I N N F Y S D P V D N K N I L Y L D T	NT (Lc)
HA-33 (33kDa)	1 5 10 15 20 S Q T N A N D L R N N E V F F I S P S N	1 5 10 15 20 M S Q T N A N D L R N N E V F F I S P S N N T	HA-33
HA-22-23 (23kDa)	1 5 10 15 20 E L Y Y T K D K S I N N V N L A D	1 5 10 15 20 M S L S I K E L Y Y T K D K S I N N V N L A D	HA-70
HA-22-23 (22kDa)	1 5 10 15 20 S I N N V N L A D G N Y V V N R G D G W	15 20 25 30 D K S I N N V N L A D G N Y V V N R G D G W I	HA-70
HA-17 (17kDa)	1 5 10 15 20 S S E R T F L P N G N Y K I K S L F S D	1 5 10 15 20 M S S E R T F L P N G N Y K I K S L F S D S L	HA-17

Fig. 3. Isolation and purification of each component of the progenitor toxin on successive chromatograms corresponding to the procedures in Fig. 1 and their SDS-PAGE banding analyses.

(a) Mono Q cation exchange column chromatography. (b) HW-55 gel filtration column chromatography in the presence of 6 M guanidine hydrochloride. (c) Mono S cation exchange column chromatograms in the presence of 8 M urea. (d) Superose 12 gel filtration column chromatography in the presence of 8 M urea. (e) Mono S cation exchange column chromatography in the presence of 8 M urea. To compare the electrophoretic mobility of the components, the SDS-PAGE pattern of the NTNHA/HAs complex preparation was aligned in the left lane. Each component is indicated an arrow.

in the presence of 2-mercaptoethanol. The separated fractions of NT and the NTNHA/HAs complex were collected respectively, and then the NTNHA/HAs fraction was concentrated with ammonium sulfate at 80% saturation for further separation.

The concentrated NTNHA/HAs preparation was treated in 3 ml of 20 mM Tris-HCl buffer (pH 7.8) containing 6 M guanidine hydrochloride (guanidine buffer), and then incubated for 2.5 hrs at 21°C. The sample was applied to a gel filtration column (2.6×90 cm) with TSK-gel HW-55S equilibrated with a guanidine buffer, and eluted with the same buffer at a flow rate of 7.5 ml/hr. The elution profile demonstrated 4 major peaks (peaks 1 to 4) as shown in Fig. 3b. Each peak fraction was collected separately. Based on SDS-PAGE analysis as illustrated in Fig. 3b, the peak 1 fraction contained a single component NTNHA. In this

step, the NTNHA was isolated as a single component. On the other hand, SDS-PAGE analysis showed that fractions of both peaks 2 and 3 contained an HA-55/33 mixture, and the peak 4 fraction contained an HA-22-23/17 mixture. These fractions were further purified in the next step.

Fractions of peaks 2 and 3 were combined and concentrated to a minimum volume using an Amicon YM 10 membrane filter. The solvent of the concentrate was displaced by a 20 mM Tris-HCl buffer (pH 7.8) containing 8 M urea (urea buffer) via passage through a HiTrap desalting column equilibrated with the urea buffer. The sample was applied to an FPLC system fitted with a Mono S HR 5/5 cation exchange column equilibrated with urea buffer. The absorbed protein was eluted with a linear gradient of NaCl (0 to 0.3 M) in the same buffer, as indicated in Fig. 3c. The HA-55 and HA-

Fig. 3. Continued.

33 were separated and each component was confirmed by SDS-PAGE. Fractions containing each component were collected separately.

Fractions of peak 4 containing an HA-22-23/17 mixture in urea buffer were concentrated to about 1 ml using an Amicon YM 10 membrane. The sample solution (0.2 ml) was applied to a Superose 12 gel filtration column equilibrated with an urea buffer. The sample was eluted with the same buffer at a flow rate of 0.3 ml/min. The elution profile gave two peaks as indicated in Fig. 3d. SDS-PAGE analysis showed that the first peak contained a single HA-22-23 component.

However, the second peak showed a mixture of HA-17 and other impurities (possibly HA-33 and others). The second peak fraction was further purified via Mono S HR 5/5 column chromatography in the presence of urea buffer. The HA-17 component was almost pure as judged by SDS-PAGE, with slight impurity protein in the bands (Fig. 3e). Attempt to reassociate of each HA subcomponent was made by reducing the denaturant concentration of the mixture to a minimum which was consisted of isolated NTNHA, HA-55, HA-33, HA-22-23

and HA-17, with molecular ratios of 1: 2.4: 5.7: 2.0: 2.0, respectively, based on densitometric analysis of SDS-PAGE pattern of the parent NTNHA/HAs complex. The mixture (11.2 ml) was dialyzed against a 20 mM Tris-HCl buffer (pH 7.8) containing 10 mM 2-mercaptoethanol and 0.1 mM EDTA overnight at 4°C. The dialyzed mixture was centrifuged at 15,000 *g* for 15 min to remove any inert protein, and then concentrated to 2 ml with Vivapure 10. The hemagglutinin activity of the reassociated NTNHA/HAs preparation was 2⁴, whereas that of the parent NTNHA/HAs complex was 2⁹.

DISCUSSION

We intended to isolate and purify all components from the progenitor toxin of *C. botulinum* C-St, whose individual components had not been obtained in a pure form. To dissociate the molecular composition, we tried several chromatographic techniques in the presence of denaturants, such as guanidine hydrochloride and urea. As the first purification step, cation exchange column chromatography at pH 4.0 seemed to be efficient for

separation of M and L toxins in a pure form from a batch of culture supernatant. Inoue et al.¹⁵⁾ reported that two progenitor toxins of type D were easily separated by dialyzing against a phosphate buffer (pH 6.0), resulting in good recovery of the purified toxins. In the preliminary experiment, however, this procedure could not be applied to our type C progenitor toxin separation, because type C-St L toxin did not precipitate at pH 6.0, as suggested by Inoue et al.¹⁵⁾

The banding profile of the L toxin on SDS-PAGE was somewhat different from that of C-St reported by Fujinaga et al.⁹⁾ with respect to the splitting of the HA-70 gene product. They demonstrated that HA-70 gene product was split into at least five subcomponents designated as HA-53, HA-23 a, HA-23 b, HA-22 a and HA-22 b. However, our results indicated three components designated HA-55 and HA-22-23 (22- and 23-kDa). In both protein preparations, the cleavage sites were at the carboxyl sides of Lys or Tyr, indicating that proteolysis may be catalyzed by trypsin or chymotrypsin-like proteases and that different sized fragments depended upon the preparation employed. Watanabe et al.⁹⁾ also reported that multi cleavage sites occurred at not only HA-20-23, but also near the N-terminal region of the HA-70 protein of the progenitor toxin produced with the type C strain 6813. Consequently, long cultivation may give multiple fragments of the HA-70 gene product. In our L toxin preparation, we could not separate the HA-22 and HA-23 mixture with chromatographic method employed because of a slight difference in molecular weight. The concentration of HA-22 was much more dominant than that of HA-23, as judged by SDS-PAGE band analysis.

The botulinum NT has been purified for all toxin types. DasGupta et al.²¹⁾ first isolated the type A NT chromatographically from the crystalline progenitor toxin. NTNHA was also isolated in a purified form on SDS-PAGE from the M progenitor toxin produced by the type D strain CB 16.²²⁾ On the other hand, the first attempt by Tsuzuki et al.²³⁾ to purify the subcomponents of HA from the type C progenitor toxin failed, but a partially purified HA-55/33 complex was obtained. Recently, HA-33 protein existing in a free state in *C. botulinum* type A^{14,24)} and type D¹⁵⁾ culture supernatants was purified using an ion exchange and gel filtration chromatographic method in the absence of detergent. However, no attempt has been made to isolate all the components from the progenitor toxin. In this study, we succeeded in isolating all components from the type C L toxin and partial reassociation of the NTNHA/HAS complex by mixing the separated components. However, the HA activity of reassociation was lower than that of the parent complex. This suggests that refolding of isolated components offer removal of denaturant appeared to be incomplete, and further examinations

such as molecular chaperone are necessary for the complete reconstitution of botulinum progenitor toxin.

The HA components have been postulated to be important proteins in the botulinum progenitor toxin, protecting the NT from adverse environmental conditions¹²⁾ or specific interaction to translocation of the whole toxin across the small intestine mucosal layer of the digestive tract.⁸⁾ Recently, preliminary study on crystallographic analysis of the type A HA-33 protein²⁵⁾ has been undertaken. Further molecular characterization of the isolated HA components is therefore needed to understand the relationship between the structure and function of the botulinum progenitor toxin.

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