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Purification and Characterization of Neurotoxin Produced by *Clostridium botulinum* Type C 6813

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The toxin produced by *Clostridium botulinum* type C 6813 (C-6813) was purified 1,009-fold from the culture supernatant in an overall yield of 30%. The specific toxicity was 1.1×10^7 mouse minimum lethal doses per mg of protein. The toxin had a molecular weight of 144,000, composed of the light and heavy chains with molecular weights of 52,000 and 92,000, respectively, linked by one or two disulfide bond(s). The purified C-6813 toxin heavy and light chains reacted strongly with anti-type D heavy chain immunoglobulin G and anti-type C_1 light chain immunoglobulin G, respectively. The amino acid compositions of C-6813 toxin heavy and light chains were more similar to those of type D heavy chain and type C_1 light chain than to those of type C_1 heavy chain and type D light chain, respectively. These results suggest that in the toxin produced by the type C strain at least two subtypes exist.

Clostridium botulinum produces immunologically distinct toxins, A, B, C_1, C_2, D, E, F, and G (23). Each of type C and D strains produced different amounts of toxins C_1, C_2, and D in the proper medium (3, 10); however, C_2 toxin differed from the others in its structure and biological activity (9, 11, 20, 21). The presence of common antigenic sites in both types C_1 and D toxin has been determined previously (14, 16–19). Furthermore, the toxin produced by type C strain 6813 was found to be a hybrid-like molecule composed of C_1-type light chain and D-type heavy chain from the results of cross-reaction between the partially purified C-6813 toxin and antibodies against C_1-type light chain and D-type heavy chain (15). To further clarify these antigenic and molecular properties of C-6813 toxin, we described the purification of C-6813 toxin and its heavy and light chains. The comparison between C-6813 and C_1 and D toxins was discussed with respect to their molecular structure and their cross-reaction with antibodies specific for these three toxins and their subunits.

MATERIALS AND METHODS

Organism and toxin production. Three strains of *C. botulinum*, 6813 (C-6813) and Stockholm (C-ST) as type C, and 1873 (D-1873) as type D, were used throughout this study. Toxins were produced by the dialyzing cultivation method reported previously (24).

Purification of toxins and their subunits. Toxins of C-6813, C-ST, and D-1873 were purified by gel filtration with Sephadex G-75, ion-exchange chromatography with DEAE-cellulose and quarternary aminoethyl-Sephadex A-50, and a final gel filtration with Sephadex G-200 super fine at pH 8.0 and 10 to 15°C (14, 25). The heavy and light chains from toxins C-ST and D-1873 were purified by the method reported previously (14, 25). For the purification of C-6813 toxin subunits, the methods used for those of subunits from toxins C-ST and D-1873 were slightly modified. C-6813 toxin was absorbed on a column of quarternary aminoethyl-Sephadex A-50 and aged overnight with 10% 2-mercaptoethanol in 27.5 mM Borax–45 mM sodium dihydrogen phosphate buffer (buffer A) at pH 8.0 and 0°C. After the light chain was eluted with the same solution, the undissociated toxin and the heavy chain were eluted stepwise with 0.12 NaCl–10% 2-mercaptoethanol in buffer A and 0.2 M NaCl–10% 2-mercaptoethanol in buffer A, respectively.

Preparation of anti-toxin IgG and anti-subunit IgG. Anti-

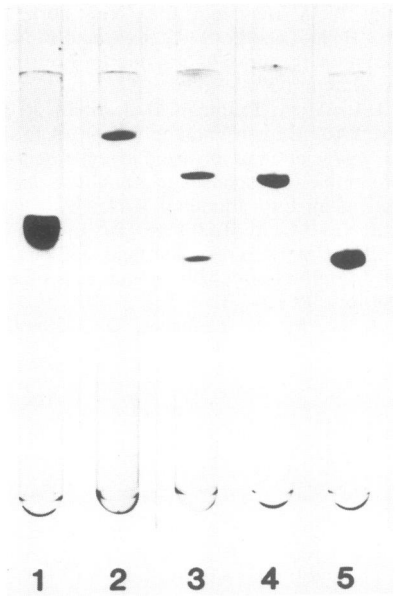

FIG. 1. Disc and SDS-polyacrylamide gel electrophoresis of toxin, heavy, and light chains. A 20-μg sample was applied per gel. Disc electrophoresis of toxin was carried out by the method of Davis (4) with 6.0% polyacrylamide gel at pH 8.9 (lane 1). SDS-polyacrylamide gel electrophoresis of toxin was carried out by the method of Weber et al. (26) with 7.0% polyacrylamide gel in the absence (lane 2) or presence (lane 3) of 2-mercaptoethanol. SDS-polyacrylamide gel electrophoresis of heavy chain (lane 4) and light chain (lane 5) were carried out under the same conditions as described for lane 3.

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TABLE 1. Purification of C-6813 toxin

Fraction	Vol (ml)	Protein (mg/ml)	Total toxicity (MLD × 10^7)	Yield (%)	Specific toxicity (MLD × 10^7/mg)	Purification (fold)
I Culture supernatant	1,225	83.2[a]	108	100	0.0011	1
II. $(NH_4)_2SO_4$ precipitate	48	21.6[a]	91.2	84	0.088	80
III. Three-column chromatography	82	0.902[b]	74.6	69	1.01	918
IV. Sephadex G-200	45	0.661[b]	32.9	30	1.11	1,009

[a] Protein concentrations were estimated by the method of Lowry et al. (13).
[b] Protein concentrations were estimated by using a value of $A^{1\%}_{278nm} = 10.14$.

sera against the purified toxins of C-6813, C-ST, and D-1873 were prepared by the method reported previously (18). The antiserum obtained was applied to the column of toxin-coupled Sepharose 4B. After the column was washed with 10 column volumes of buffer A to remove the unbound serum proteins, anti-toxin immunoglobulin G (IgG) was eluted with 3 M KSCN and then dialyzed against buffer A. Anti-heavy chain IgG (or anti-light chain IgG) was similarly prepared by affinity chromatography with the heavy chain (or light chain) -coupled Sepharose 4B. The purified immunoglobulins contained little IgM.

Protein determination. Protein was routinely estimated by the method of Lowry et al. (13). with bovine serum albumin as the standard. The concentration of heavy and light chains in the chromatographic fractions was densitometrically estimated by the method of Kahn and Rubin (12) after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The concentrations of purified C-6813 toxin and the heavy and light chains at neutral pH were estimated by using values of $A^{1\%}_{1\,cm,\ 278\ nm} = 10.14$, 16.80, and 9.01, respectively, which were calculated from the amino acid compositions. The concentrations of the other purified toxins and their subunits were estimated by using values of $A^{1\%}_{1\,cm,\ 278\ nm} = 14.18$, 12.02, and 9.03 for C-ST toxins and its heavy and light chains (25), respectively, and 11.40, 16.10, and 9.59 for D-1873 toxin and its heavy and light chains (14), respectively.

Amino acid analyses. Amino acid analyses of C-6813 toxin and the heavy and light chains were carried out by using a Nihon Denshi JIC-6AH amino acid analyzer. Hydrolyses were performed in glass-distilled HCl at 110°C for 24 h on the heavy and light chains and for 24, 48, and 72 h on the toxin. Sulfhydryl group of toxin was determined with 5,5′-dithiobis(2-nitrobenzoic acid) in the presence of 0.2% SDS (1).

Enzyme-linked immunosorbent assay. Enzyme-linked immunosorbent assay was performed in microtiter plates (Nunc model 239454). A 30-μl sample of toxins or subunits (1 to 500 ng) in buffer A and 60 μl of 0.01 M carbonate-bicarbonate buffer at pH 9.6 were successively added in each well, and the plate was kept in a refrigerator at 5°C overnight. After washing the wells four times with 0.01 M phosphate buffer containing 0.5% bovine serum albumin, 0.05% Tween 20, and 0.02% NaN_3 at pH 7.2 (buffer B), a 100-μl sample of antibody (300 ng) in buffer A was added to each well; the plate was then incubated at 37°C for 2 h. The wells were washed four times with buffer B, and then a 200 μl sample of alkaline phosphatase-goat anti-rabbit IgG conjugate was added to each well. The plate was incubated again at 37°C for 2 h and washed four times with buffer B. The enzymatic reaction was started by adding 200 μl of 2.5 mM *p*-nitro-phenyl phosphate dissolved in 50 mM carbonate-bicarbonate buffer containing 1 mM $MgCl_2$ at pH 9.8. After 1 h of incubation at 37°C, the reaction was terminated by the addition of 25 μl of 3 M NaOH. The absorbance of the reaction mixture was determined at 405 nm with a Titertek Multiskan (Flow Laboratories, Inc.).

Toxicity assay. White mice (dd line) weighing about 20 g

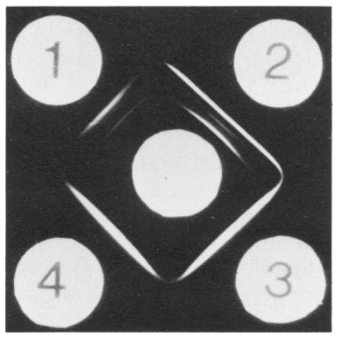


FIG. 2. Immunodiffusion of toxic fractions from purification steps in Table 1. Center well, rabbit anti-C-6813 toxin serum. Peripheral wells: 1, fraction I; 2, fraction II; 3, fraction III; 4, fraction IV. Gel contained 0.8% agarose in Veronal buffer at pH 8.6, and a 50-μl amount of each fraction was applied to the wells.

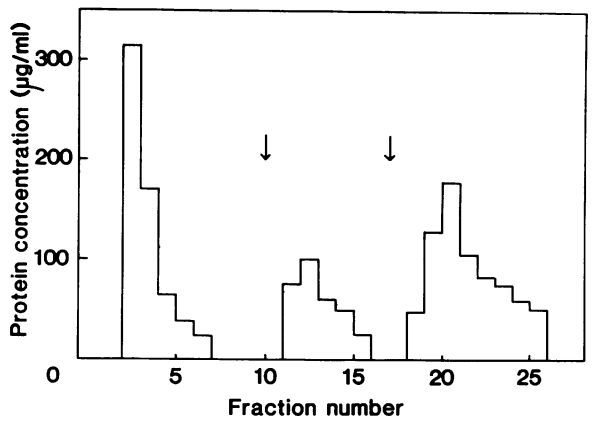
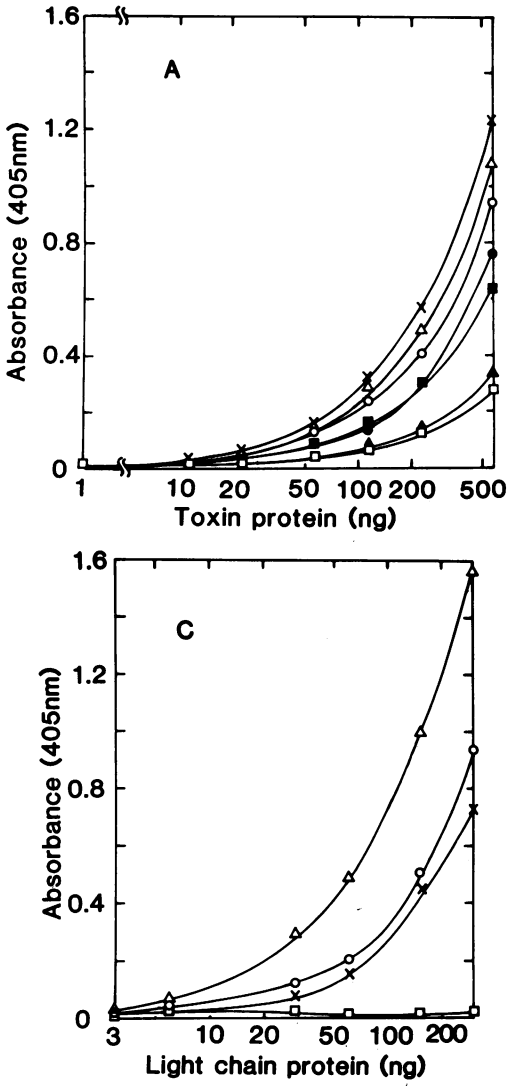


FIG. 3. Chromatographic separation of heavy and light chains from toxin. The toxin (5.6 mg) was applied to a column (1.6 by 5 cm) of quarternary aminoethyl-Sephadex A-50 equilibrated previously with buffer A at 0°C. The column was aged with 6 ml of buffer A containing 10% 2-mercaptoethanol overnight at 0°C. The light chain was then eluted with 40 ml of the same buffer. Undissociated toxin was eluted with 40 ml of 0.12 M NaCl–10% 2-mercaptoethanol in buffer A. The buffer was changed at the first arrow position. Heavy chain was eluted with 40 ml of 0.2 M NaCl–10% 2-mercaptoethanol in buffer A. The buffer was changed again at the second arrow position. The flow rate was 10 ml/h. Each fraction was 5 ml.



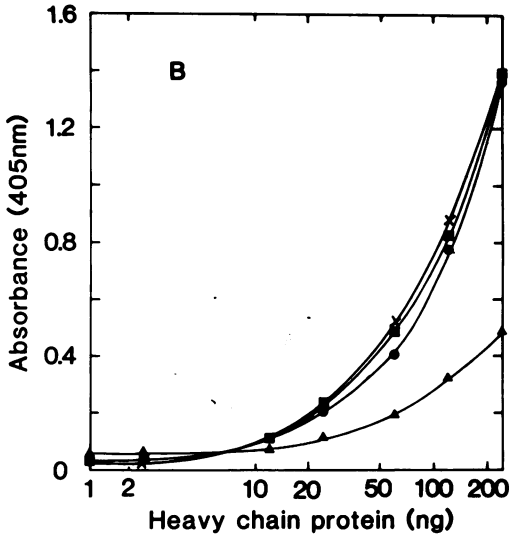


FIG. 4. Titration curves of antibodies to C-6813 toxin and its subunits. Various concentration of C-6813 toxin (A), C-6813 toxin heavy chain (B), and C-6813 toxin light chain (C) were coated to each well of the microtiter plate. Each antibody (300 ng) was reacted with toxin and its subunit was prebound onto the plate. After the enzyme conjugate and the substrate were allowed to react, the yellow color of *p*-nitrophenol released was measured. Symbols: ×, anti-C-6813 toxin IgG; ○, anti-C-6813 toxin light chain IgG; △, anti-C-ST toxin light chain IgG; □, anti-D-1873 toxin light chain IgG; ●, anti-C-6813 toxin heavy chain IgG; ▲, anti-C-ST toxin heavy chain IgG; ■, anti-D-1873 toxin heavy chain IgG.

were injected intravenously with a 0.1-ml portion of the toxin solution in 0.1 M sodium phosphate buffer at pH 6.9. The time-to-death method (2) was applied. Toxicity was measured as the minimum lethal dose (MLD).

Neutralization test. Neutralizing activities of polyclonal antibodies were examined by intravenously injecting the mixture of toxin and antibody into three white mice. A 1-ml sample of mixture was made up of 0.5 ml of toxin (1 μg) in buffer A at pH 8.0 and 0.5 ml of anti-toxin IgG (10 μg) in the same buffer. After 30 min of incubation at room temperature, a 0.1-ml portion of the mixture was injected into a mouse, and the average survival time was calculated.

RESULTS

Purification of C-6813 toxin. The specific toxicity and yield of C-6813 toxin in each step of the purification procedure are summarized in Table 1. The toxin was purified 1,009-fold from the culture supernatant with an overall yield of 30%. The purified toxin was homogeneous by SDS-polyacrylamide gel electrophoresis (Fig. 1) and the double diffusion test (Fig. 2) and had a specific toxicity of 1.1×10^7 MLD per mg of protein, which was lower than that of C-ST toxin (4.4×10^7 MLD per mg of protein) and D-1873 toxin (5.8×10^7 MLD per mg of protein).

Separation of heavy and light chains from C-6813 toxin. The purified toxin had a molecular weight of 144,000, consisting of heavy and light chains with molecular weights of 92,000 and 52,000, respectively. Figure 3 shows the separation profile of heavy and light chains from C-6813 toxin by quarternary aminoethyl-Sephadex A-50 column chromatography. The first and last peaks contained only the light- and heavy-chain fractions, respectively, after determining these fractions by SDS-polyacrylamide gel electrophoresis. The heavy and light chains were homogeneous by SDS-polyacrylamide gel electrophoresis.

Reaction of antibodies with C-6813 toxin and its subunits. Figure 4 shows the reactivities of C-6813 toxin and its subunits with anti-C-6813 toxin IgG, anti-C-ST toxin IgG, anti-D-1873 toxin IgG, anti-C-6813 toxin subunit IgGs, and anti-C-ST toxin subunit IgGs, and anti-D-1873 toxin subunit IgGs in enzyme-linked immunosorbent assay. C-6813 toxin showed the different reactivities with these antibodies; the order of strength of reactivity was anti-C-6813 toxin IgG, and anti-C-ST light chain IgG, anti-C-6813 light chain IgG, anti-C-6813 heavy chain IgG, anti-D-1873 heavy chain IgG, anti-C-ST heavy chain IgG, and anti-D-1873 light chain IgG (Fig. 4A). To make clear reactivity of C-6813 toxin with antibodies against C-ST toxin subunits and D-1873 toxin

TABLE 2. Amino acid composition of C-6813 toxin, heavy chain, and light chain

Amino acid	Toxin (M_r, 144,000)			Heavy chain (M_r, 92,000)			Light chain (M_r, 52,000)		
	Residues per mol		% to total residues	Residues per mol		% to total residues	Residues per mol		% to total residues
	Measured	Integer		Measured	Integer		Measured	Integer	
Aspartic acid	210.3	210	16.8	134.5	135	16.9	71.0	71	15.5
Threonine	67.4[a]	67	5.3	42.8	43	5.4	26.2	26	5.7
Serine	78.3[a]	78	6.2	54.8	55	6.9	28.9	29	6.3
Glutamic acid	112.1	112	8.9	76.7	77	9.6	34.7	35	7.7
Proline	49.6	50	4.0	21.9	22	2.7	27.0	27	5.9
Glycine	53.5	54	4.3	35.8	36	4.5	21.0	21	4.6
Alanine	50.6[b]	51	4.1	26.9	27	3.4	24.8	25	5.5
Valine	69.4[c]	69	5.5	46.8	47	5.9	24.4	24	5.3
Methionine	18.8	19	1.5	12.9	13	1.6	6.8	7	1.5
Isoleucine	137.9[c]	138	11.0	90.6	91	11.4	46.1	46	10.1
Leucine	103.1	103	8.2	66.7	67	8.4	36.1	36	7.9
Tyrosine	53.5	54	4.3	38.8	39	4.9	15.6	16	3.5
Phenylalanine	64.5	65	5.2	36.8	37	4.6	24.9	25	5.5
Histidine	10.9	11	0.9	4.9	5	0.6	4.4	4	0.9
Lysine	105.1	105	8.4	65.7	66	8.2	38.1	38	8.3
Arginine	46.6	47	3.8	24.9	25	3.1	23.4	23	5.0
Tryptophan	11.9[d]	12	1.0	9.9[d]	10	1.2	1.9[d]	2	0.4
Half-cystine	7.9[e]	8	0.6	6.0[e]	6	0.7	2.0[e]	2	0.4
Total residues		1,253			801			457	
Polar amino acid									
Acidic and neutral			46.4			48.9			43.7
Basic			13.1			11.9			14.2
Nonpolar amino acid			40.5			39.2			42.1

[a] Values extrapolated to zero time.
[b] Values for 48-h hydrolysis.
[c] Values for 72-h hydrolysis.
[d] Values determined by the method of Goodwin and Morton (5).
[e] Values determined as cysteic acid (7).

subunits, each of the C-6813 toxin subunits was used to determine cross-reactivity instead of the parent toxin. C-6813 toxin heavy chain cross-reacted with anti-D-1873 heavy chain IgG to the same degree as that of anti-C-6813 toxin IgG and anti-C-6813 toxin heavy chain IgG, but not C-ST heavy chain IgG (Fig. 4B). On the other hand, C-6813 toxin light chain was more reactive to anti-C-ST toxin light chain IgG than were anti-C-6813 toxin light chain IgG and anti-C-6813 toxin IgG, and it did not react to anti-D-1873 toxin light chain IgG (Fig. 4C).

Amino acid compositions of C-6813 toxin and its subunits. Table 2 shows the amino acid compositions of the heavy chain, the light chain, and the parent toxin. The heavy chain contained predominantly acidic and neutral amino acids, whereas the light chain contained more of the basic amino acids. There were 6 mol and 2 mol of half-cysteine residues in the heavy and light chains, respectively. On titration with 5,5′-dithiobis(2-nitrobenzoic acid) in the presence of SDS, the toxin had 4 mol of sulfhydryl group in the molecule. Therefore, the parent toxin had a di-chain structure consisting of heavy and light chains linked by one or two disulfide bond(s). Table 3 shows the comparison of amino acid composition between C-6813 toxin subunits and subunits of C-ST toxin or D-1873 toxin. Differences were estimated by the sum of the square of the difference in each amino acid content between C-6813 toxin subunits and subunits of C-ST toxin or D-1873 toxin. From the results of the above estimation, it was found that the differences in amino acid composition between C-6813 toxin heavy chain and D-1873 toxin heavy chain and between C-6813 toxin light chain and C-ST toxin light chain were smaller than those between C-6813 toxin heavy chain and C-ST toxin heavy chain and between C-6813 toxin light chain and D-1873 toxin light chain.

Toxin neutralization by antibodies. Table 4 shows neutralization titer of two kinds of anti-toxin IgGs. Anti-C-6813 toxin IgG had high neutralizing activity against both C-6813 toxin and C-ST toxin, whereas anti-C-ST toxin IgG had very low neutralizing activity against C-6813 toxin.

DISCUSSION

The toxin of *C. botulinum* type C 6813 was purified 1,009-fold from the culture supernatant in an overall yield of 30% by the same purification procedure used for other type C and D toxins (14, 24). The specific activity was 1.1×10^7 MLD per mg of protein, which was lower than that of C-ST and D-1873 toxins (4.4×10^7 MLD per mg of protein and 5.8×10^7 MLD per mg of protein, respectively). This suggests that the active structure of these three toxins may not be the same and each toxin may have its own active structure. However, C-6813 toxin had a dichain molecular structure composed of a light chain (M_r, 52,000) and a heavy chain (M_r, 92,000) like other botulinum toxins (23), and these subunits were purified essentially by the same separation procedure used for the preparation of C-ST and D-1873 toxin subunits (14, 24). Thus, the physicochemical properties and

the fundamental structure may be similar in nearly all types of toxins.

Botulinum neurotoxins have been classified by their distinct antigenicities. However, Oguma et al. (16–19) determined the existence of common antigenic sites between C-ST and D toxins, and Ochanda et al. (15) proposed a hybrid molecular structure in C-6813 toxin, which was composed of C-ST-type light chain and D-type heavy chain, on the basis of the cross-reaction between the partially purified C-6813 toxin and antibodies against subunits of both C-ST and D toxins. In this study, the nature of the hybrid-like molecule was shown by the cross-reaction, in which the purified C-6813 toxin heavy and light chains reacted with anti-type D heavy chain IgG and anti-C-ST light chain IgG, respectively. The antigenic characteristics of C-6813 toxin was consistent with the amino acid compositions of the subunits, and by comparing the sum of the square of the difference in each amino acid content, it might be suggested that the amino acid compositions of C-6813 toxin heavy chain was similar to that of type D toxin heavy chain rather than to that of C-ST toxin heavy chain. In contrast, the amino acid composition of C-6813 toxin light chain was more similar to that of C-ST toxin light chain than to that of type D toxin light chain. Consequently, the present data on amino acid composition partially confirm that C-6813 toxin is a hybrid-like molecule between type D toxin heavy chain and C-ST toxin light chain. However, to confirm the complete identity of this hybrid-like molecule, further sequence analysis of amino acid to detect homology and heterogeneity within the toxins is necessary.

Pfenninger (22) and Gunnison and Meyer (6) described anti-C_α toxin neutralization of both the C_α and C_β toxins, whereas anti-C_β toxin neutralized only C_β toxin. In our neutralization test, anti-C-6813 toxin IgG neutralized both the C-ST and C-6813 toxins, and anti-C-ST toxin IgG neutralized C-ST toxin completely (99.7%) (Table 4) but C-6813 toxin only partially (20.0%) (Table 4). Therefore, C-6813 toxin and C-ST toxin may correspond to C_α toxin and C_β toxin, respectively.

These results lead us to conclude that type C_1 neurotoxin contains at least two subtypes which are different in biological activity, amino acid composition, and antigenic characteristics and that the existence of the hybrid-like molecule and toxin subtype may be explained if the determination of toxigenicity in type C and D toxins is controlled by the phage genetic code (8).

TABLE 3. Comparison of amino acid composition of C-6813 toxin subunits with C-ST and D-1873 toxin subunits

Amino acid	Heavy chain (% to total residues)			Light chain (% to total residues)		
	C-ST[a]	C-6813	D-1873[b]	C-ST[a]	C-6813	D-1873[b]
Aspartic acid	18.8	16.9	16.9	17.4	15.5	14.8
Threonine	5.5	5.4	5.1	6.4	5.7	6.5
Serine	7.6	6.9	7.8	6.8	6.3	9.1
Glutamic acid	10.2	9.6	10.3	8.9	7.7	9.1
Proline	2.4	2.7	3.5	5.7	5.9	6.1
Glycine	5.1	4.5	4.3	4.6	4.6	5.5
Alanine	3.2	3.4	3.5	4.1	5.5	3.0
Valine	5.3	5.9	5.9	4.8	5.3	6.1
Methionine	1.9	1.6	1.6	1.1	1.5	1.1
Isoleucine	9.3	11.4	9.9	8.7	10.1	8.0
Leucine	7.3	8.4	8.5	7.3	7.9	8.4
Tyrosine	5.8	4.9	5.4	5.0	3.5	3.8
Phenylalanine	5.3	4.6	4.3	6.0	5.5	6.1
Histidine	0.6	0.6	0.7	1.1	0.9	1.5
Lysine	6.7	8.2	7.3	6.4	8.3	6.3
Arginine	3.6	3.1	2.4	4.7	5.0	3.4
Tryptophan	0.9	1.2	1.6	0.5	0.4	0.8
Half-cystine	0.5	0.7	0.8	0.5	0.4	0.4
Square of difference[c]	14.96	6.16		16.78	31.02	

[a] Values published in reference 25.
[b] Values published in reference 14.
[c] Values calculated by using the equation $\Sigma(N_i - N_{i6813})^2$; where N_i is the percentage of the residues of a particular amino acid i in C-ST or D-1873 toxin subunit and N_{i6813} is the percentage of the residues of a particular amino acid i in C-6813 toxin subunit.

TABLE 4. Neutralization test with antibodies[a]

Antibody	Toxin	
	C-ST (%)	C-6813 (%)
Anti-C-ST toxin IgG	99.7	20.0
Anti-C-6813 toxin IgG	99.7	99.9

[a] The neutralization test was performed by mixing anti-toxin IgG and toxin with a weight ratio of 10 to 1. Values were obtained by the following equation (1 − remaining toxicity of mixture [MLD per ml]/toxicity of sample [MLD per ml]) × 100.

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