Complete Subunit Structure of the *Clostridium botulinum* Type D Toxin Complex via Intermediate Assembly with Nontoxic Components‡

Shingo Mutoh,§ Hirokazu Kouguchi,|| Yoshimasa Sagane,§ Tomonori Suzuki,§ Kimiko Hasegawa,§ Toshihiro Watanabe,§ and Tohru Ohyama*,§

Department of Food Science and Technology, Faculty of Bioindustry, Tokyo University of Agriculture, 196 Yasaka, Abashiri 099-2493, Japan, and Hokkaido Institute of Public Health, N19, W12, Kita-Ku, Sapporo 060-0819, Japan

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ABSTRACT: *Clostridium botulinum* serotype D strains usually produce two types of stable toxin complex (TC), namely, the 300 kDa M (M-TC) and the 660 kDa L (L-TC) toxin complexes. We previously proposed assembly pathways for both TCs [Kouguchi, H., et al. (2002) *J. Biol. Chem. 277,* 2650−2656]: M-TC is composed by association of neurotoxin (NT) and nontoxic nonhemagglutinin (NTNHA); conjugation of M-TC with three auxiliary types of hemagglutinin subcomponents (HA-33, HA-17, and HA-70) leads to the formation of L-TC. In this study, we found three TC species, 410, 540, and 610 kDa TC species, in the culture supernatant of type D strain 4947. The 540 and 610 kDa TC species displayed banding patterns on SDS−PAGE similar to that of L-TC but with less staining intensity of the HA-33 and HA-17 bands than those of L-TC, indicating that these are intermediate species in the pathway to L-TC assembly. In contrast, the 410 kDa TC species consisted of M-TC and two molecules of HA-70. All of the TC species, except L-TC, demonstrated no hemagglutination activity. When the intermediate TC species were mixed with an isolated HA-33/17 complex, every TC species converted to 650 kDa L-TC with full hemagglutination activity and had the same molecular composition of L-TC. On the basis of titration analysis with the HA-33/17 complex, the stoichiometry of the HA-33/17 complex molecules in the L-TC, 610 kDa, and 540 kDa TC species was estimated as 4, 3, and 2, respectively. In conclusion, the complete subunit composition of mature L-TC is deduced to be a dodecamer assembled by a single NT, a single NTNHA, two HA-70, four HA-33, and four HA-17 molecules.

Clostridium botulinum strains produce seven distinct serotypes (A−G) of neurotoxin (NT).[1] After ingestion of NT-contaminated food, the NT is absorbed from intestinal epithelial cells into the bloodstream and, consequently, reaches the neuromuscular junctions. NT enters into nerve cells via receptor-mediated endocytosis and cleaves specific sites on its target proteins through Zn^{2+}-dependent endopeptidase activity and then blocks the docking and fusion of synaptic vesicles, leading to the inhibition of neurotransmitter release (*1*, *2*). This process causes muscular paralysis in human and animals, leading to the botulism disease state.

In culture supernatants and naturally contaminated foods, the NT molecules exist as part of a large complex [toxin complex (TC)], which is often referred to as progenitor toxin. This TC consists of NT and auxiliary nontoxic proteins, designated as nontoxic nonhemagglutinin (NTNHA) and hemagglutinin (HA). The HA is composed of three subcomponents with molecular masses of 70, 33, and 17 kDa (HA-70, HA-33, and HA-17, respectively). In the gastrointestinal tract of animals, the free NT molecule, dissociated from the TC, is susceptible to the proteolytic and acidic conditions of digestive juices and easily degrades into short peptides or amino acid in the same manner as other general proteins. On the other hand, NTNHA proteins and some HA proteins are resistant to proteolytic degradation (*3*, *4*). Thus, it is postulated that the nontoxic proteins protect the NT from the digestive system of animals. In addition, it has been proposed that the HA component recognizes sugar chains on intestinal microvilli, whereby it might facilitate efficient absorption of botulinum TC via the intestinal wall (*5*, *6*).

C. botulinum serotype A, B, C, and D toxins are encoded by two gene clusters in close proximity to each other; cluster 1 contains the *nt* and *ntnha* genes, and cluster 2 contains three genes, *ha-70*, *ha-33*, and *ha-17* (*7*). According to the gene organization, it was expected that botulinum TC would consist of five components. Nevertheless *C. botulinum* strains having two cluster genes produce both hemagglutination-positive and -negative TCs with different molecular masses. The hemagglutination-positive TC is the L-TC (serotypes A−D and G), or a mixture of L- and LL-TC (serotype A), with the hemagglutination-negative M-TC (serotypes A−F) (*8*−*12*). Serotypes E (*11*) and F (*12*) are encoded by just two genes, *nt* and *ntnha*, and therefore produce hemagglu-

‡ The nucleotide sequence reported in this paper has been submitted to the GenBank/EBI Data Bank with accession number AB037920.

* To whom correspondence should be addressed. Phone: +81 (152) 48-3838. Fax: +81 (152) 48-2940. E-mail: t-oyama@bioindustry.nodai.ac.jp.

§ Tokyo University of Agriculture.

|| Hokkaido Institute of Public Health.

[1] Abbreviations: NT, neurotoxin; NTNHA, nontoxic nonhemagglutinin; HA, hemagglutinin; TC, toxin complex; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride.

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tination-negative M-TC. The M-TC consists of NT and NTNHA, the L-TC is formed by association of M-TC with three HA subcomponents, and the LL-TC is formed as a dimer of L-TC linked by HA-33 (*13*). Accordingly, the L- and LL-TC exhibit hemagglutination activity, but the M-TC does not. However, the number of each HA subcomponent responsible for hemagglutination activity remains undetermined for the subunit structure of botulinum TC.

The NT, NTNHA, and HA-70 subunits of the TCs produced by the A, C, and D strains have always been found to be nicked at unique sites due to bacterial protease(s), leading to more bands on SDS−PAGE than the number of their predicted gene products (*14*−*16*). Recently, we reported a unique TC, which consisted only of unnicked components (intact L- and intact M-TC), from a culture of the type D strain 4947 (D-4947) (*17*) whose proteolytic activity is lacking or very weak. Using the isolated components of the D-4947 TC, we have demonstrated, for the first time, in vitro reconstitution of L-TC molecules whose properties are indistinguishable with respect to those of the parent L-TC. On the basis of the results of reconstitution by various combinations of components, sequence-specific binding of the components for construction of the TC was proposed as follows: mixing of M-TC and isolated HA-70 components yields the M/HA-70 complex, and the reconstituted L-TC is then formed by addition of the HA-33/17 complex to M/HA-70. Due to the inability to isolate HA-33 and HA-17 from their complex, the number of HA subcomponents on the subunit structure of TC and the specific subcomponent that binds directly to M/HA-70, HA-33, or HA-17 on completion of assembly is yet to be elucidated. Further, there has been no attempt to clarify the subunit composition of the botulinum TC.

During the purification procedure of D-4947 TC, we found that a partially purified L-TC preparation obtained by cation-exchange chromatography displayed five bands corresponding to whole components of the L-TC on SDS−PAGE but with different mobility on native PAGE. This finding strongly suggests that the TC species likely exists as an intermediate leading to complete L-TC in culture supernatant. In this study, we separated the botulinum TC species having different molecular masses and no hemagglutination activity in the culture of D-4947. We clarified here the complete subunit composition of the mature botulinum L-TC and the L-TC assembly pathway with binding order of the HA subcomponents by titration-based reconstitution experiments of the intermediate TC species.

EXPERIMENTAL PROCEDURES

Production and Purification of TC Species. *C. botulinum* D-4947 was cultured using the dialysis method as previously described (*17*). The culture supernatant was concentrated by ammonium sulfate precipitation and dialyzed against 50 mM acetate buffer (pH 4.0) containing 0.2 M NaCl. The sample was then applied to an SP-Toyopearl 650S (Tosoh, Tokyo, Japan) cation-exchange column equilibrated with the dialysis buffer. The absorbed materials were eluted with a linear gradient of NaCl ranging from 0.2 to 0.8 M. Each peak fraction was pooled separately and concentrated by ammonium sulfate precipitation. The resultant precipitates were dissolved with 50 mM phosphate buffer (pH 6.0) containing 0.15 M NaCl and then further purified by applying to a HiLoad 16/60 Superdex 200 pg (Pharmacia Biotech, Uppsala, Sweden) gel filtration column equilibrated with the same buffer. The fraction containing free HA-33 was concentrated to 5 mL using a YM10 membrane filter (Amicon, Beverly, MA) and was applied to a HiLoad 26/60 Superdex 200 pg column equilibrated with the 50 mM phosphate buffer (pH 6.0) containing 0.15 M NaCl. Two peaks were eluted; the first peak corresponding to the TC species and the second peak to the HA-33 single component. Both peak fractions were concentrated using VIVAPORE 10 (Sartotrius, Goettingen, Germany).

Isolation of HA Subcomponents. Isolation of the HA subcomponents from D-4947 TC was performed with the methods described previously (*17*). A 250 mg pellet of ammonium sulfate precipitation of the 650 kDa L-TC preparation was dissolved in 0.7 mL of 20 mM Tris-HCl (pH 7.8) containing 4 M guanidine hydrochloride (Gdn buffer) and incubated at 21 °C for 4 h. The treated sample was applied to a HiLoad 16/60 Superdex 200 pg column equilibrated with the Gdn buffer. After elution, two peak fractions containing HA-70 and HA-33/17 were pooled separately. The fractions were diluted to 0.05 absorbance unit at 280 nm for the HA-70 fraction and 0.1 absorbance unit for the HA-33/17 fraction with Gdn buffer, and both were then dialyzed against 20 mM Tris-HCl (pH 7.8) at 4 °C for 15 h. The fraction containing HA-70 was concentrated to 1.5 mL using VIVAPORE 10.

Reconstitution of M/HA-70 and M/HA-70/HA-33 Complexes. For preparation of the M/HA-70 complex, the M-TC and isolated HA-70 were mixed at a molar ratio of 1:4, and then the reconstitution buffer was added to the mixture to a final concentration of 5 mM sodium phosphate (pH 6.0), 350 mM KCl, 20 mM $MgCl_2$, 6 mM 2-mercaptoethanol (2-ME), and 0.5 mM PMSF. The final protein concentration of the mixture ranged from 0.4 to 0.5 mg/mL. After incubation at 27 °C for 21 h, the reconstituted M/HA-70 complex was separated by gel filtration using a HiLoad 16/60 Superdex 200 pg column. To examine whether the reconstituted M/HA-70 and free HA-33 assemble, they were mixed at a 1:12 molar ratio and incubated at room temperature for 1 h. After incubation, the sample was examined by gel filtration using a Superdex 200 HR 10/30 (Pharmacia Biotech) column and native PAGE.

Hemagglutination Assay. A hemagglutination assay was performed by the microtitration method using a multiwell titer plate. Samples (35 μL) of each preparation (0.25 μM) were diluted in serial 2-fold steps with 0.15 M phosphate buffer (pH 7.0) and mixed with an equal volume of 1% (v/v) horse erythrocytes. After incubation at room temperature for 2 h, the reciprocal hemagglutination titer was determined as 2^n.

Analytical Gel Filtration for Estimation of Molecular Mass. Each sample (20 μg) was applied to a Superdex 200 HR 10/30 column equilibrated with 50 mM phosphate buffer (pH 6.0) containing 0.15 M NaCl. Molecular size markers were thyroglobulin (669 kDa), aldolase (158 kDa), BSA (67 kDa), and ribonuclease A (13.7 kDa). Molecular sizes were estimated from the mean of five experiments from a plot of the log of molecular mass vs elution volume.

PAGE and Densitometric Analyses. PAGE under nondenaturing conditions (native PAGE) was carried out using

the method of Davis (*18*) at pH 8.8 using a 5−12.5% polyacrylamide linear gradient gel. SDS−PAGE was performed as described by Laemmli (*19*) using a 13.6% polyacrylamide gel in the presence of 2-ME. The separated protein bands were detected with Coomassie Brilliant Blue R-250 (CBB). The CBB-staining intensities of the components were analyzed with NIH image software, version 1.62 (available from URL: http://rsb.info.nih.gov/nih-image/). The intensity of the protein band was determined by measuring the area of the peak. The protein concentration was determined by using the BCA protein assay kit (Pierce, Rockford, IL) with BSA as the standard.

N-Terminal Amino Acid Sequence Analysis. The components of TC separated by SDS−PAGE were transferred onto a poly(vinylidene difluoride) membrane using a semidry blotting apparatus (Nippon Eido, Tokyo, Japan) according to the method of Hirano and Watanabe (*20*). The N-terminal amino acid sequences of the components were determined using a protein sequencer (Model 492HT; Applied Biosystems, Foster City, CA).

Titration of TC Species with HA-33/17. To determine the binding capacities of the TC species, with isolated HA-33/17 as a titrant, the titration experiment was performed by addition of HA-33/17 to the purified TC species. The titration mixtures containing 0.25 μM TC species and several concentrations of HA-33/17 were incubated for 1 h at room temperature. After incubation, the mixtures were examined by native PAGE and hemagglutination assay.

RESULTS

Purification of the Botulinum TC Species. The crude toxic fraction of D-4947 in the culture supernatant was applied to an SP-Toyopearl 650S column under acidic conditions (pH 4.0). As shown in Figure 1A, four distinct peaks designated as I−IV were eluted, and each pooled peak fraction was subjected to SDS−PAGE. The peak II fractions showed three major bands corresponding to NT, NTNHA, and the GroES homologue (*21*), based on their N-terminal amino acid sequences, indicating that the TC species in the peak II fraction is M-TC, which consists of NT and NTNHA.

On the other hand, peak I, III, and IV fractions demonstrated banding patterns on SDS−PAGE similar to that of L-TC as shown in our previous data (*17*), corresponding to NT, NTNHA, HA-70, HA-33, and HA-17. However, their native PAGE banding profiles exhibited different behaviors, as shown in Figure 1B. One band having common electrophoretic mobility among all peak fractions corresponded to NT. Since the botulinum NT has been known to separate easily from TC (both M- and L-TC) under alkaline conditions (*3*), the NT obtained from each fraction was likely generated from M-TC or L-TC during electrophoresis under alkaline conditions (pH 8.8).

Peak I, III, and IV fractions gave two bands with common and different electrophoretic mobilities on native PAGE. Upon SDS−PAGE of each band isolated from the gel, one of the bands was determined to be NT while the other dominant band split into four bands corresponding to NTNHA, HA-70, -33, and -17. It is likely that the peak I, III, and IV fractions contain TC species consisting of NT and NTNHA/HA complexes with different molecular ratios. Furthermore, SDS−PAGE of a weak band found in the peak

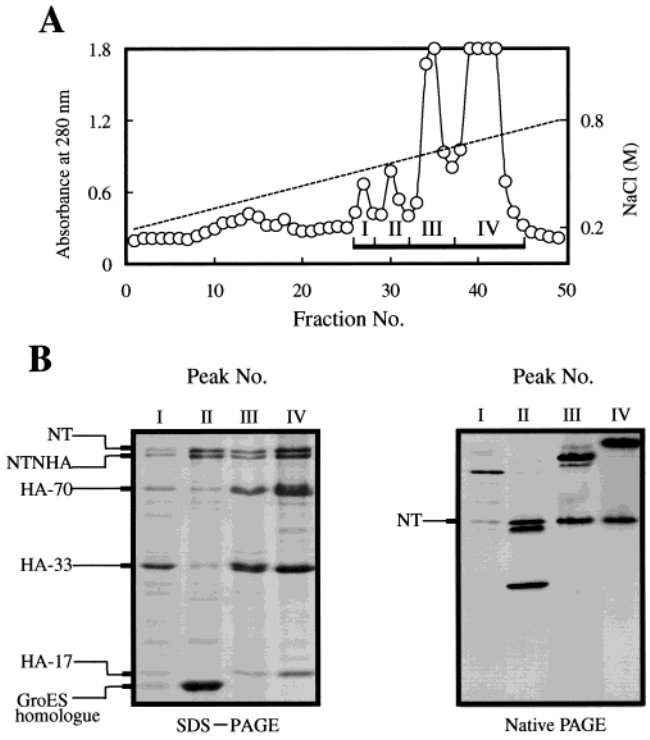


FIGURE 1: Separation of *C. botulinum* D-4947 TC species by SP-Toyopearl 650S column chromatography (A) and SDS−PAGE and native PAGE banding patterns of the peak fractions corresponding to each TC species (B). In panel A, four distinct peaks are designated as I−IV. In panel B, the numbers of the lanes in SDS−PAGE and native PAGE correspond to fraction numbers 27 for I, 30 for II, 36 for III, and 42 for IV on the chromatogram as illustrated in (A). The native PAGE at pH 8.8 gave dissociated NT and NTNHA/HAs complexes from the TC species.

I fraction exhibited two bands corresponding to NTNHA and HA-70. This finding indicates that a very small amount of the complex consisting of NT, NTNHA, and HA-70 (M/HA-70) also exists in the culture fluid.

To further purify, the peak I, II, III, and IV fractions were applied to a HiLoad 16/60 Superdex 200 pg column. From the peak II fraction, M-TC was purified, and the molecular mass was determined to be 280 kDa by gel filtration (Table 1). However, the other peak I, III, and IV fractions still gave several bands on native PAGE after gel filtration, as shown in Figure 2. Each fraction was then applied to a Mono S HR 5/5 (Pharmacia Biotech) column equilibrated with 50 mM acetate buffer (pH 5.0). The absorbed protein was eluted by a linear gradient of NaCl with concentrations ranging from 0 to 0.5 M. The distinct peak fractions were collected separately, and their purities were confirmed with native PAGE, and then the molecular mass was measured by gel filtration. Accordingly, the 540 kDa TC from the peak I fraction, the 610 kDa TC from the peak III fraction, and the 650 kDa TC from the peak IV fraction were purified to homogeneity on native PAGE, as shown in Figure 3. The purification of M/HA-70 by chromatographic procedures failed because of the small amount of complex present. Therefore, the 410 kDa M/HA-70, composed of M-TC and isolated HA-70, was reconstituted in vitro as in our previous report (*17*) and was used for the subsequent experiments.

When the peak I fraction was applied to a gel filtration column, the free HA-33 subcomponent was separated from the TC. The HA-33 subcomponent was nearly homo-

Table 1: Molecular Masses, Hemagglutination Titer of the TC Species, and Ratio of Staining Intensity of the HA Components

	molecular mass		hemagglutination	ratio of staining intensity of individual components[c]			
	estd[a] (kDa)	calcd[b] (Da)	(titer)	NT/HA-70	NTNHA/HA-70	HA-33/HA-70	HA-17/HA-70
M-TC	280	285786	negative[d]				
M/HA-70	410	426399	negative	0.64	0.70		
540 kDa TC	540	527342	negative	0.78	0.77	0.57	0.10
610 kDa TC	610	577813	negative	0.74	0.78	0.82	0.16
650 kDa TC (L-TC)	650	628285	2^6	0.64	0.77	1.12	0.19

[a] Estimated by analytical gel filtration. [b] Sum of the molecular mass of each component calculated from their amino acid sequences. [c] Given by CBB staining intensity of the each component per that of HA-70 on SDS−PAGE indicated in Figure 4. [d] Negative at 0.25 μM.

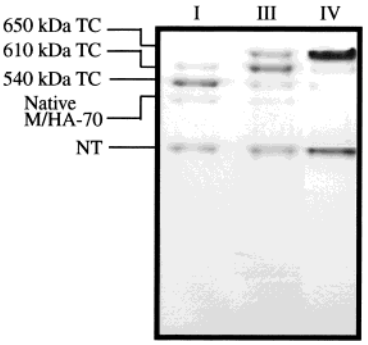


FIGURE 2: Native PAGE banding pattern of partially purified TC species after gel filtration. Peak I, III, and IV fractions correspond to those in Figure 1.

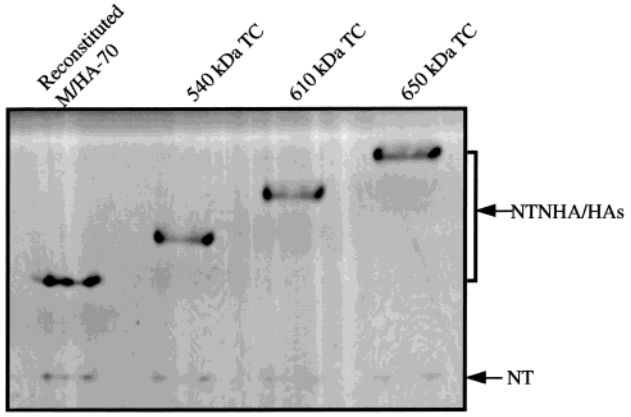
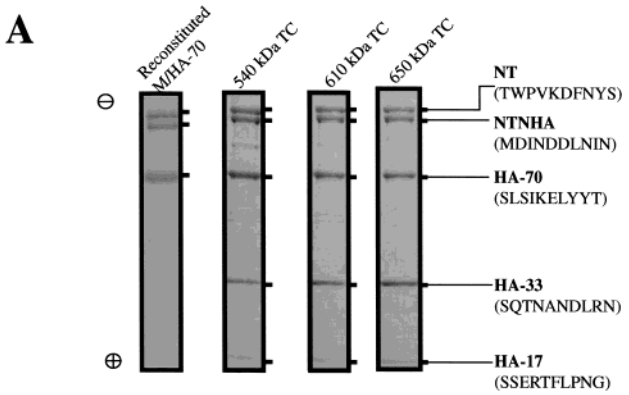


FIGURE 3: Native PAGE banding patterns of the in vitro reconstituted M/HA-70 complex and the purified 540, 610, and 650 kDa TC species preparations (4 μg of protein each) from the peak fractions shown in Figure 1A. Bands corresponding to dissociated NT and the NTNHA/HA complex of the TC species are indicated by arrows.



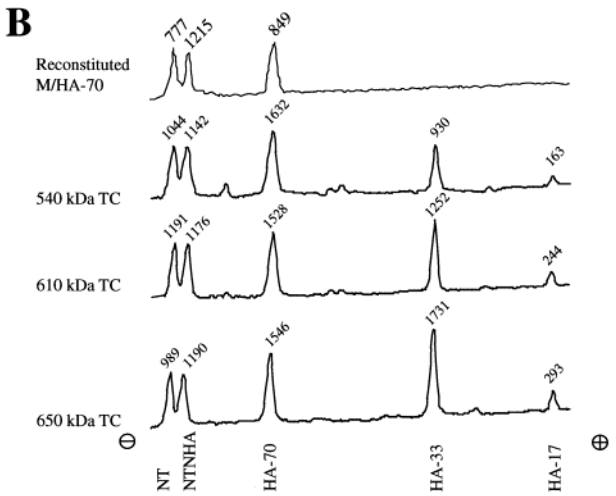


FIGURE 4: SDS−PAGE banding patterns indicating their N-terminal amino acid sequences (A) and their densitometric trace of the CBB-staining bands from the 540, 610, and 650 kDa TC species (B). In panel B, the numerical values indicated on each peak were determined by densitometric tracing of the peak area.

geneous on SDS−PAGE, and its N-terminal sequence (SQTNANDLRN) was identical to that deduced from the nucleotide sequence of the D-4947 *ha-33* gene.

Molecular Composition of TC Species. As indicated in Table 1, the difference of the molecular masses between M-TC and M/HA-70 is 130 kDa, suggesting strongly that M/HA-70 possesses approximately two HA-70 molecules. On the other hand, the 540, 610, and 650 kDa TC species showed the same banding pattern on SDS−PAGE. Comparing the staining intensities of the bands corresponding to NT, NTNHA, HA-70, HA-33, and HA-17 (Figure 4), the ratios of intensities for NT:HA-70 and NTNHA:HA-70 exhibited similar values among the four TC species as shown in Table 1. The ratio of staining intensities for HA-33:HA-70 was, however, given as 0.57, 0.82, and 1.12, and that for HA-17:HA-70 was given as 0.10, 0.16, and 0.19 for the 540, 610, and 650 kDa TC species, respectively. Therefore, the integral proportion for the ratios of staining intensity of HA-33 and HA-17 were represented as 2:3:4 among the TC species. Consequently, the differences observed in the molecular masses of the three forms of TC species were derived from the number of the HA-33 and -17 subcomponents. Furthermore, the 650 kDa TC was determined to be the complete L-TC form among the TC species, because no larger complex was identified from the culture supernatant, and the molecular mass corresponds to the value obtained by summing the molecular masses of the constituent subunits. However, the discrepancy between the estimated molecular masses by gel filtration and the calculated molecular masses of these TC species (around 600 kDa) may probably be due

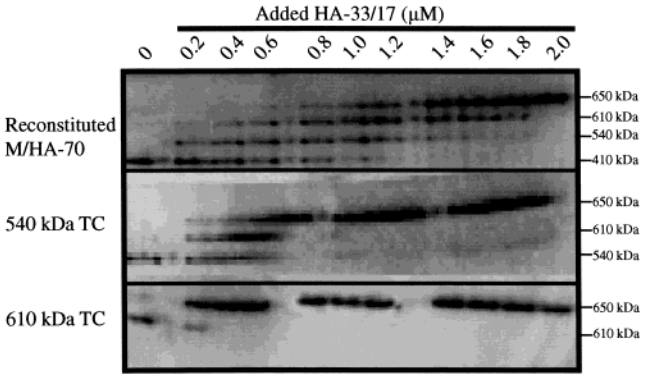


FIGURE 5: Conversion from the TC species to 650 kDa L-TC by the serial addition of the isolated HA-33/17 complex on native PAGE. NTNHA/HAs complexes dissociated from the TC species are presented, but the NTs are omitted. The molar concentration of isolated HA-33/17 was determined on the basis of the calculated molecular mass of the sum of each subcomponent from their amino acid sequences and the determined protein concentration.

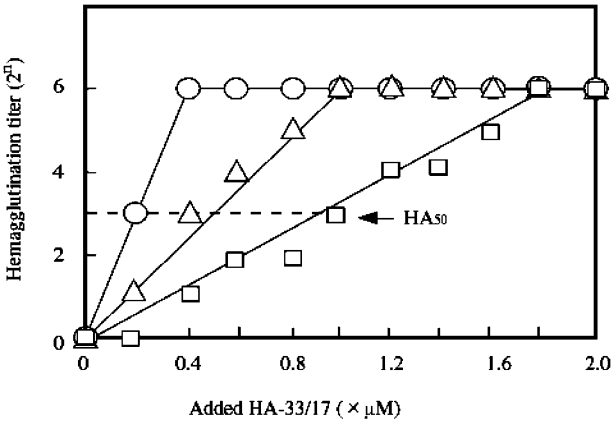


FIGURE 6: Hemagglutination titer based titration with HA-33/17. Each titer was determined after incubation for 1 h of the TC species with various concentrations of HA-33/17. L-TC exhibited the maximum titer of 2^6. Key: (○) 610 kDa TC; (△) 540 kDa TC; (□) M/HA-70 complex. The concentrations of HA-33/17 resulting in the half-maximum hemagglutination titer, designated HA_{50}, are indicated by the arrows.

to either experimental error using a single size marker or the interaction between the TC species and the column resin.

Native PAGE Based Titration of TC Species with HA-33/17. As shown in Figure 3, the M/HA-70, 540 kDa TC, 610 kDa TC, and 650 kDa TC (L-TC) demonstrate two bands corresponding to the NT and NTNHA/HAs complexes on native PAGE. The NTNHA/HAs developed from each TC demonstrated a different electrophoretic mobility depending upon the TC species. When HA-33/17 was gradually added to M/HA-70, the NTNHA/HA band at the original position disappeared and migrated to the position of the NTNHA/HAs of L-TC (650 kDa), passing through those of the 540 and 610 kDa TC species with increasing concentration of HA-33/17 added (Figure 5). Finally, every band disappeared and migrated completely to the single band at the position corresponding to that of the 650 kDa L-TC, with a concentration of 2.0 μM HA-33/17 added.

Similarly, the bands corresponding to the NTNHA/HAs of the 540 and 610 kDa TC also disappeared at concentrations of 0.8−1.2 and 0.4−0.6 μM HA-33/17 added, respectively. Moreover, the molar ratio of HA-33/17 required for completion of the conversion to L-TC was estimated at roughly 1, 2, and 4 for 610 kDa TC, 540 kDa TC, and M/HA-70, respectively.

In the addition of HA-33/17 to M/HA-70 or other TC species, the question of which subcomponent, HA-33 or HA-17, binds directly to the TC species was examined using free HA-33; HA-17 could not be used because its isolation failed. When M/HA-70 and free HA-33 were mixed, no new peaks at larger molecular sizes than that of M/HA-70 appeared on the gel filtration elution profile, indicating that the combination of these components did not assemble into complexes (data not shown), while HA-33/17 binds M/HA-70 and the other TC species. These results indirectly suggest that HA-17 is required for the formation of links between M/HA-70 and HA-33, and it is likely that the HA-33 subcomponent is exposed on the surface of the TC species.

Hemagglutination Activity Based Titration of TC Species with HA-33/17. The hemagglutination activity of the L-TC was determined to be 2^6 (Table 1). In contrast, no hemagglutination titer could be detected in the other TC species. These results imply that the TC must contain the full number of HA-33/17 complexes for hemagglutination activity. Therefore, we also examined whether the hemagglutination activity of the M/HA-70, 540 kDa, and 610 kDa TC species arises by binding of HA-33/17 to unoccupied sites. As shown in Figure 6, the hemagglutination activities of these TC species increased with added HA-33/17. At the end point of titration every TC species exhibited the hemagglutination titer of 2^6, which is similar to that of L-TC. The M/HA-70, 540 kDa, and 610 kDa TC (0.25 μM each) required 1.0, 0.5, and 0.2 μM HA-33/17 complex, respectively, to give half-maximum hemaggutination activity and also 1.8, 1.0, and 0.4 μM HA-33/17 complex, respectively, to exert full hemagglutination activity.

DISCUSSION

Recently, we proposed a sequence-specific assembly pathway of D-4947 TC in which the M-TC forms first by assembly of NT with NTNHA and is subsequently converted to L-TC by assembly with HA-70 and HA-33/17. This pathway was based on reconstitution experiments of TC from various combinations of the isolated components (*17*). Mixing of M-TC and isolated HA-70 components under adequate conditions yielded a novel M/HA-70 complex, and addition of HA-33/17 to the M/HA-70 complex led to the formation of L-TC, which showed a full hemagglutination titer at the same level as the parent L-TC. Furthermore, both native and reconstituted L-TCs are very stable complexes and never dissociate into M-TC or intermediate TC through treatment with proteases and various physical conditions, such as freezing and thawing. Accordingly, it seemed likely to suppose that the L-TC might be constructed from each component via intermediates composed of M/HA-70 and HA-33/17. However, so far just two TC species, M- and L-TC, have been found in the culture supernatants of types C and D *C. botulinum* strains. In this work, we found the L-TC species corresponding to the intermediates from M/HA-70 to L-TC in the minor peak fractions on cation-exchange column chromatography of D-4947 culture supernatant.

On the basis of the native PAGE and hemagglutination activity titration analyses of the intermediate TC species with

the HA-33/17 molecule, we clearly demonstrated that complete hemagglutination-positive L-TC is achieved by the binding of four HA-33/17 molecules to the M/HA-70 complex. Neither HA-33/17 nor isolated HA-33 (*17*, *22*) aggregates horse erythrocytes. As to the hemagglutination activity of HA-33, Fu et al. (*4*) reported that isolated HA-33 from type A *C. botulinum* exists predominantly in a dimeric form in the aqueous solution and has full hemagglutination activity in human blood. On the other hand, Inoue et al. (*23*) reported that purified HA-33 from type C and D strains formed a 200 kDa polymer (corresponding to 6−7mer) and did not agglutinate human erythrocytes. The likely reasons for the discrepancy could be the different *C. botulinum* strains used and erythrocyte source tested. Previously, evidence has been presented that soybean agglutinin of plant lectin is a tetramer composed of identical subunits (*24*). Similarly, hemagglutinin of influenza viruses is a trimer that is essential for cell recognition (*25*), and concanavalin A dimer and tetramer species have significantly different binding affinity to cells (*26*). Thus, the importance of the oligomeric structure for hemagglutination activity has been observed across several species.

From many lines of evidence for hemagglutination activity by the botulinum TC (*5*, *17*, *27*, *28*), it has been postulated that the HA-33 molecule of the HA subcomponents is most likely responsible for aggregation of the erythrocytes. Recently, Sagane et al. (*29*) suggested that the short C-terminal region of the HA-33 plays an essential role in the hemagglutination activity of the type C and D botulinum TC and has a carbohydrate recognition subdomain. We have demonstrated that L-TC is completed by binding of the HA-33/17 complex to the HA-70 subdomain of the M/HA-70 complex at the final stage of the assembly (*17*). However, there has been no examination of which molecule, HA-33 or HA-17, adheres to the HA-70 molecule. In this paper, we show that free HA-33 is not able to bind to M/HA-70, while HA-17 was not examined because of the separation of HA-17 from the complex due to irreversible precipitation during dialysis. This observation suggests indirectly that the HA-17 molecule, not the HA-33 molecule, binds to HA-70 and makes a linkage between HA-33 and the M/HA-70 complex, leading to the configuration in which four HA-33 molecules occupy surface positions in the subunit structure of TC. Such observations are consistent with the hemagglutination activity of the botulinum TC and the possibility that HA-33 plays a role in anchoring the complex at the epithelial cell surface (*5*, *6*).

Most research papers have been published on the pharmacology, cell biology, and application of botulinum TC as a therapeutic agent for the treatment of human disease, while few studies have addressed the exact subunit structure of the botulinum TC. There has been no attempt to clarify subunit composition, especially the number of HA subcomponents, of the botulinum TC. Preliminarily, Oguma et al. (*30*) speculated that the molecular ratio of HA-70 (split into HA-55 and -22-23), HA-33, and HA-17 subcomponents of type C and D 16S TC (corresponding to our L-TC) might be 1:2:1, based on the densitometry analysis of the band on SDS−PAGE. More recently, similar work by Sharma et al. (*31*) demonstrated the relative amounts of the components of type A botulinum TC determined densitometrically by detailed SDS−PAGE analyses and expressed as a percentage of staining intensity, although it has been said that composition analysis based on CBB staining could suffer from inaccurate estimation because of the way dye binds differentially to proteins (*4*).

In this work, the complete subunit structure of botulinum type D 4947 L-TC has been elucidated by the following observations: (1) the difference of the molecular mass between M-TC and M/HA-70 is 130 kDa, which corresponds to exactly two HA-70 molecules; (2) from the relative staining intensities of HA-33/17 molecules of each intermediate TC species on SDS−PAGE, the minimum molar ratios of HA-33/17 in the TC species were determined as 2:3:4 for 540, 610, and 650 kDa TC, respectively; (3) the titration curve with HA-33/17 for each hemagglutination-negative intermediate TC species yielded hemagglutinination-positive L-TC; and (4) conversion to complete L-TC was achieved by addition of four HA-33/17 molecules to one M/HA-70 molecule. Finally, we have determined the complete subunit structure of the botulinum type D-4947 TC, composed of one NT, one NTNHA, two HA-70, four HA-33, and four HA-17 molecules, via intermediates constituted of various HA subcomponents. The identification of the subunit structure of the botulinum TC will help in understanding the molecular structure and the auxiliary proteins and their interactions with NT in the toxico-infection process of the botulism diseased state, as well as the effectiveness of the NT-based therapeutic reagents. Our observation may provide information for X-ray crystallographic analyses of the three-dimensional subunit structure of the botulinum toxin complex, which we will attempt soon.

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REFERENCES

1. Montecucco, C., and Schiavo, G. (1993) *Trends Biochem. Sci. 18*, 324−327.
2. Li, L., and Singh, B. R. (1999) *J. Toxicol., Toxin Rev. 18*, 95−112.
3. Sakaguchi, G., Kozaki, S., and Ohishi, I. (1984) in *Bacterial Protein Toxins* (Alouf, J. E., Fehrenbach, F. J., Freer, J. H., and Jeljaszewicz, J., Eds.) pp 435−443, Academic Press, London.
4. Fu, F. N., Sharma, S. K., and Singh, B. R. (1998) *J. Protein Chem. 17*, 53−60.
5. Fujinaga, Y., Inoue, K., Watanabe, S., Yokota, K., Hirai, Y., Nagamachi, E., and Oguma, K. (1997) *Microbiology 143*, 3841−3847.
6. Fujinaga, Y., Inoue, K., Nomura, T., Sasaki, J., Marvaud, J. C., Popoff, M. R., Kozaki, S., and Oguma, K. (2000) *FEBS Lett. 467*, 179−183.
7. Hauser, D., Eklund, M. W., Boquet, P., and Popoff, M. R. (1994) *Mol. Gen. Genet. 243*, 631−640.
8. East, A. K., Bhandari, M., Stacey, J. M., Campbell, K. D., and Collins, M. D. (1996) *Int. J. Syst. Bacteriol. 46*, 1105−1112.
9. Fujinaga, Y., Inoue, K., Shimazaki, S., Tomochika, K., Tsuzuki, K., Fujii, N., Watanabe, T., Ohyama, T., Takeshi, K., Inoue, K., and Oguma, K. (1994) *Biochem. Biophys. Res. Commun. 205*, 1291−1298.
10. Ohyama, T., Watanabe, T., Fujinaga, Y., Inoue, K., Sunagawa, H., Fujii, N., Inoue, K., and Oguma, K. (1995) *Microbiol. Immunol. 39*, 457−465.
11. Fujii, N., Kimura, K., Yokosawa, N., Yashiki, T., Tsuzuki, K., and Oguma, K. (1993) *J. Gen. Microbiol. 139*, 79−86.

12. East, A. K., and Collins, M. D. (1994) *Curr. Microbiol. 29*, 69−77.
13. Inoue, K., Fujinaga, Y., Watanabe, T., Ohyama, T., Takeshi, K., Moriishi, K., Nakajima, H., Inoue, K., and Oguma, K. (1996) *Infect. Immun. 64*, 1589−1594.
14. Sagane, Y., Watanabe, T., Kouguchi, H., Sunagawa, H., Inoue, K., Fujinaga, Y., Oguma, K., and Ohyama, T. (1999) *J. Protein Chem. 18*, 885−892.
15. Watanabe, T., Sagane, Y., Kouguchi, H., Sunagawa, H., Inoue, K., Fujinaga, Y., Oguma, K., and Ohyama, T. (1999) *J. Protein Chem. 18*, 753−760.
16. Sagane, Y., Watanabe, T., Kouguchi, H., Sunagawa, H., Obata, S., Oguma, K., and Ohyama, T. (2002) *Biochem. Biophys. Res. Commun. 292*, 434−440.
17. Kouguchi, H., Watanabe, T., Sagane, Y., Sunagawa, H., and Ohyama, T. (2002) *J. Biol. Chem. 277*, 2650−2656.
18. Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404−427.
19. Laemmli, U. K. (1970) *Nature 227*, 680−685.
20. Hirano, H., and Watanabe, T. (1990) *Electrophoresis 11*, 573−580.
21. Sagane, Y., Hasegawa, K., Mutoh, S., Kouguchi, H., Suzuki, T., Sunagawa, H., Nakagawa, T., Kamaguchi, A., Okasaki, S., Nakayama, K., Watanabe, T., Oguma, K., and Ohyama, T. (2003) *J. Protein Chem. 22*, 99−108.
22. Kouguchi, H., Watanabe, T., Sagane, Y., and Ohyama, T. (2001) *Eur. J. Biochem. 268*, 4019−4026.
23. Inoue, K., Fujinaga, Y., Honke, K., Yokota, K., Ikeda, T., Ohyama, T., Takeshi, K., Watanabe, T., Inoue, K., and Oguma, K. (1999) *Microbiology 145*, 2533−2542.
24. Lotan, R., Siegelman, H. W., Lis, H., and Sharon, N. (1974) *J. Biol. Chem. 249*, 1219−1224.
25. Wilson, I. A., Skehel, J. J., and Wiley, D. C. (1981) *Nature 289*, 366−373.
26. McKenzie, G. H., and Sawyer, W. H. (1973) *J. Biol. Chem. 248*, 549−556.
27. Tsuzuki, K., Kimura, K., Fujii, N., Yokosawa, N., Indoh, T., Murakami, T., and Oguma, K. (1990) *Infect. Immun. 58*, 3173−3177.
28. Inoue, K., Fujinaga, Y., Honke, K., Arimitsu, H., Mahmut, N., Sakaguchi, Y., Ohyama, T., Watanabe, T., Inoue, K., and Oguma, K. (2001) *Microbiology 147*, 811−819.
29. Sagane, Y., Kouguchi, H., Watanabe, T., Sunagawa, H., Inoue, K., Fujinaga, Y., Oguma, K., and Ohyama, T. (2001) *Biochem. Biophys. Res. Commun. 288*, 650−657.
30. Oguma, K., Inoue, K., Fujinaga, Y., Yokota, K., Watanabe, T., Ohyama, T., Takeshi, K., and Inoue, K. (1999) *J. Toxicol., Toxin Rev. 18*, 17−34.
31. Sharma, S. K., Ramzan, M. A., and Singh, B. R. (2003) *Toxicon 41*, 321−331.

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