Four molecules of the 33 kDa haemagglutinin component of the *Clostridium botulinum* serotype C and D toxin complexes are required to aggregate erythrocytes

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Normally, large-sized botulinum toxin complexes (L-TC) of serotype C and D are composed of a single neurotoxin, a single non-toxic non-haemagglutinin, two HA-70 molecules, four HA-33 molecules and four HA-17 molecules that assemble to form a 650 kDa L-TC. The 540 and 610 kDa TC species (designated here as L-TC₂ and L-TC₃, respectively) were purified in addition to the 650 kDa L-TC from the culture supernatants of serotype D strains (D-4947 and D-CB16) and serotype C strains (C-6814 and C-Yoichi). The 650 kDa L-TC from D-4947, D-CB16 and C-6814 showed haemagglutination and erythrocyte-binding activity, but their L-TC₂ and L-TC₃ species had only binding activity. In contrast, every TC species from C-Yoichi having the C-terminally truncated variant of HA-33 exhibited neither haemagglutination activity nor erythrocyte-binding activity. Four strain-specific HA-33/HA-17 complexes were isolated from the 650 kDa L-TC of each strain. The 650 kDa HA-hybrid L-TCs were reconstituted by various combinations of isolated HA-33/HA-17 complexes and haemagglutination-negative L-TC₂ or L-TC₃ from each strain. HA-hybrid 650 kDa L-TC, including at least one HA-33/HA-17 complex derived from C-Yoichi, lost haemagglutination activity, leading to the conclusion that the binding of four HA-33 molecules is required for haemagglutination activity of botulinum L-TC. The results of the modelling approach indicated that the structure of a variant C-Yoichi HA-33 molecule reveals clear deformation of the β -trefoil domain responsible for the carbohydrate recognition site.

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INTRODUCTION

Clostridium botulinum strains produce seven distinct serotypes (A–G) of neurotoxin (NT; 150 kDa). After ingestion of NT-contaminated food, the NT is absorbed by intestinal epithelial cells, enters the bloodstream and consequently reaches neuromuscular junctions. NT enters nerve cells via receptor-mediated endocytosis and cleaves specific sites on fusion proteins of synaptic vesicles involved in the exocytosis of acetylcholine through its Zn²⁺-dependent endopeptidase activity, leading to the inhibition of

neurotransmitter release (Montecucco & Schiavo, 1993; Li & Singh, 1999). This process causes muscular paralysis in humans 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) through association with non-toxic non-haemagglutinin (NTNHA; 130 kDa) and haemagglutinin (HA) components. HA is composed of three subcomponents: HA-70, -33 and -17 (70, 33 and 17 kDa, respectively). The *C. botulinum* serotypes A–D and G produce different forms of TC, the 280 kDa M-TC (complex of NT and NTNHA), the 650 kDa L-TC (complex of NT, NTNHA and HAs) and the 900 kDa LL-TC (probable

Abbreviations: CBB, Coomassie brilliant blue R-250; CD, circular dichroism; HA, haemagglutinin; NT, neurotoxin; NTNHA, non-toxic non-haemagglutinin; TC, toxin complex.

dimer of L-TC) (Sakaguchi, 1982). Thus, both L-TC (serotypes A–D and G) and LL-TC (serotype A) are haemagglutination-positive, while M-TC (serotypes A–F) is haemagglutination-negative. The NT, NTNHA and HA-70 subunits of the TC produced by serotypes A, C and D strains have been found to be nicked at specific sites due to bacterial proteases, yielding more protein bands on SDS-PAGE than the number of predicted gene products (Oguma *et al.*, 1999; Sagane *et al.*, 1999, 2002; Watanabe *et al.*, 1999). Some strains of serotypes C and D produce haemagglutination-negative L-TC due to the deletion of 31 aa residues from the C-terminal region of HA-33 (Sagane *et al.*, 2001).

We have found that the botulinum toxin of the serotype D unique strain 4947 (D-4947) can be purified from culture supernatant as an L-TC and M-TC, which consists of only unnicked components. Using the isolated components of the D-4947 TC, we have successfully reconstituted *in vitro* the 650 kDa L-TC with properties that are indistinguishable from those of the parent L-TC (Kouguchi *et al.*, 2002). Additionally, we found haemagglutination-negative TC species (M/HA-70, 540 and 610 kDa L-TC), which have a smaller number of HA-33/HA-17 molecules than those of 650 kDa L-TC and are found in the D-4947 culture supernatant. These TC species are considered to be intermediates in the 650 kDa L-TC assembly pathway. We therefore deduced that the complete subunit composition of D-4947 L-TC is a dodecamer assembled by a single NT, a single NTNHA, two HA-70 molecules, four HA-33 molecules and four HA-17 molecules (Mutoh *et al.*, 2003). Additionally, we have proposed a model for the arrangement of the individual components in the botulinum L-TC through tryptic susceptibility of the isolated components and complex forms (Suzuki *et al.*, 2005).

In the gastrointestinal tract of animals, the free NT molecule dissociated from the TC is susceptible to proteolytic and acidic conditions in the digestive environment and is easily degraded into short peptides or amino acids, similar to other proteins. On the other hand, the NTNHA protein and a part of the HA proteins are resistant to proteolytic degradation (Sakaguchi *et al.*, 1984; Fu *et al.*, 1998). Thus, it is postulated that the non-toxic proteins protect the NT from the host digestive system. Additionally, the HA components have been proposed to recognize sugar chains on intestinal microvilli, thereby possibly providing efficient absorption to the intestinal wall for the botulinum TC (Fujinaga *et al.*, 1997, 2000, 2004). In this study, we examined the role of individual HA components in L-TC for recognition of erythrocytes and haemagglutination activity through the *in vitro* reconstitution of the HA-hybrid TC species composed of different HA-33/HA-17 isolated from serotype C and D TC strains. Based on these results, we characterized the HA-33 component responsible for haemagglutination caused by botulinum TC, which may lead to clarification of the configuration of the HA-33 molecules in the subunit structure of the botulinum TC.

METHODS

Production and purification of TC species. *C. botulinum* strains D-4947, D-CB16, C-6814 and C-Yoichi were cultured using the dialysis method as described previously (Ohyama *et al.*, 1995). The culture supernatants were concentrated by ammonium sulfate precipitation and dialysed against 50 mM acetate buffer (pH 4.0) containing 0.2 M NaCl. The sample was then applied to an SP-Toyopearl 650S (Tosoh) cation-exchange column equilibrated with 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 in 50 mM phosphate buffer (pH 6.0) containing 0.15 M NaCl and then further purified by application to a HiLoad 16/60 Superdex 200 pg (Amersham Biosciences) gel-filtration column equilibrated with the same buffer. Eluted fractions were diluted fourfold with 50 mM acetate buffer (pH 5.0). Subsequent to purification of the TC species, the diluents were applied to a Mono S HR 5/5 (Amersham Biosciences) cation-exchange column equilibrated with the dilution buffer and eluted with a linear gradient of NaCl ranging from 0 to 0.5 M.

Isolation of the HA-33/HA-17 complex. Isolation of the HA-33/HA-17 subcomponent complexes from D-4947, D-CB16, C-6814 and C-Yoichi 650 kDa L-TCs were performed according to the method described previously (Kouguchi *et al.*, 2002). A 250 mg pellet of the ammonium sulfate precipitate from the L-TC preparation was dissolved in 0.7 ml 20 mM Tris/HCl (pH 7.8) containing 4 M guanidine hydrochloride (guanidine buffer) and incubated at 21 °C for 4 h. The treated sample was applied to a HiLoad 16/60 Superdex 200 pg gel-filtration column equilibrated with guanidine buffer. The fraction containing HA-33/HA-17 was pooled and diluted to an A_{280} of 0.1 with guanidine buffer. After dialysis against 20 mM Tris/HCl (pH 7.8) at 4 °C, the fraction containing HA-33/HA-17 was concentrated using Vivapure 10 (Sartorius).

Reconstitution of HA-hybrid L-TC with isolated HA-33/HA-17 from each strain. For hybridization of L-TCs containing different types of HA-33/HA-17, purified 610 and 540 kDa L-TC from four strains (D-4947, D-CB16, C-6814 and C-Yoichi) and isolated HA-33/HA-17 were mixed at a 1:5 molar ratio in phosphate buffer (pH 6.0) containing 0.15 M NaCl. After incubation at room temperature for 10 min, the mixture was applied to a Superdex 200 HR 10/30 gel-filtration column (Amersham Biosciences) equilibrated with the same buffer to remove unbound HA-33/HA-17. The resultant samples were examined by native PAGE.

Haemagglutination assay. Haemagglutination assays were performed using U-bottom microtitre plates. Samples (35 µl) of each preparation (0.25 µM) were diluted in serial twofold steps with 0.15 M phosphate buffer (pH 7.0) and mixed with an equal volume of 1% (v/v) equine erythrocytes. After incubation at room temperature for 2 h, the reciprocal haemagglutination titre was determined as 2ⁿ.

Western blot erythrocyte binding assay. The binding of TC species, HA-hybrid TC and isolated HA subcomponents to equine erythrocytes was carried out according to the method described by Inoue *et al.* (1999) with some modifications. A 60 µl sample of the TC species (0.25 µM) was incubated with 20 µl of a 1% suspension of equine erythrocytes at room temperature for 30 min, followed by collection of the erythrocytes by centrifugation. The pellets were subjected to SDS-PAGE. The separated bands bound to erythrocytes were blotted to a polyvinylidene difluoride membrane and were probed with rabbit anti-HA of type C toxin complex IgG. The bound antibodies were detected with anti-rabbit IgG-HRP conjugate using a POD immunostaining set (Wako).

PAGE and densitometric analyses. PAGE under non-denaturing conditions (native PAGE) was carried out at pH 8.8 using a

5–12.5% polyacrylamide linear gradient gel, according to the method of Davis (1964). SDS-PAGE was performed as described by Laemmli (1970) using a 13.6 or 15% polyacrylamide gel in the presence of 1% 2-mercaptoethanol. The molecular size markers were phosphorylase *b* (97 kDa), BSA (66 kDa), egg ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α -lactalbumin (14.4 kDa). The separated protein bands were detected with Coomassie brilliant blue R-250 (CBB). The CBB-staining intensities of the components were analysed with NIH image software, version 1.62 (<http://rsb.info.nih.gov/ni-image/>). The intensity of the protein band was determined by measuring the area of the peak. The protein concentration was determined by using a BCA protein assay kit (Pierce) with BSA as the standard.

Analytical gel filtration. A Superdex 200 HR 10/30 gel-filtration column was equilibrated with 50 mM phosphate buffer (pH 6.0) containing 0.15 M NaCl. The molecular masses of the complexes were estimated from calculations with standard proteins aldolase (158 kDa), BSA (67 kDa), egg ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and RNase A (13.8 kDa). The molecular masses were estimated from the mean of the three experiments.

Isoelectric focusing. Isoelectric focusing under native conditions was carried out using Immobiline DryStrip (pH 3.0–10.0, 7 cm; Amersham Bioscience). The sample (30 μ g) in 50 mM Tris/HCl buffer (pH 7.4) was applied to a strip holder, then run on an Ettan IPGphor II (Amersham Bioscience) electrophoresis apparatus. The separated protein bands were stained with CBB. The isoelectric point (pI) value was estimated by calculations with the Broad pI kit (Amersham Bioscience).

Far-UV circular dichroism (CD) spectroscopy. The secondary structure of the HA-33/HA-17 complex was analysed using a far-UV CD spectropolarimeter (model J-720WI, JASCO). CD spectra were recorded using a 10 mm path length cuvette at room temperature. The sample (450 μ g ml⁻¹) was dialysed against 50 mM Tris/HCl buffer (pH 7.4). The secondary structure content was estimated by the methods of Yang *et al.* (1986) and Manavalan & Johnson (1987) using software provided with the instrument.

Modelling of the C-Yoichi and D-4947 HA-33 structure. Structure models of C-Yoichi and D-4947 HA-33 were constructed with the SWISS-MODEL Protein Homology modelling Server (<http://swissmodel.expasy.org/SWISS-MODEL.html>) (Guex & Peitsch, 1997) using the coordinates of serotype C HA-33 (Protein Data Bank code 1QXM). The images were generated using the Molsoft ICM Browser (available at www.molsoft.com) for displaying stereo ribbon diagrams of the crystal structure of C-Yoichi and D-4947 HA-33 molecules. The models exhibit good geometry according to Ramachandran plots generated using Swiss-PdbViewer (available at <http://ca.expasy.org/spdbv/>) with 90.2, 88.0 and 87.0% of the residues in the most favoured regions for HA-33 molecules of D-4947, C-Yoichi and truncated C-Yoichi, respectively.

RESULTS AND DISCUSSION

Purification of botulinum TC species

Previously, we reported that *C. botulinum* strain D-4947 produces three species of L-TC with a smaller molecular ratio of HA subcomponents (410, 540 and 610 kDa L-TC species) than the 650 kDa L-TC in the culture supernatant (Mutoh *et al.*, 2003). In this study, we also found incomplete TC species (540 and 610 kDa TCs, but not the 410 kDa TC) in the culture supernatants of D-CB16, C-6814 and C-Yoichi, similar to those of D-4947, based on SDS-PAGE

and native PAGE data from chromatographic fractionations. As shown in Fig. 1, the crude toxic fractions of four strains were separated by an SP-Toyopearl 650S column under acidic conditions (pH 4.0), showing different elution profiles depending on the strains. The SP-Toyopearl chromatogram showed four distinct peaks for D-4947 and D-CB16, three peaks for C-6814 and one peak with a shoulder for C-Yoichi. The SDS-PAGE and native PAGE analysis of each toxic fraction from the four strains indicated the existence of TC species similar to the 540, 610 and 650 kDa L-TCs compared to those of D-4947, in which they show different mobilities on native PAGE, but a similar banding pattern on SDS-PAGE.

Accordingly, the purification of the TC species of each strain was undertaken by combinations of various chromatographic methods. First, ammonium sulfate concentrates of corresponding peak fractions were applied to a HiLoad 16/60 Superdex 200 pg gel-filtration column, where M-TC (NT/NTNHA complex) and other L-TC species were separated. The fractions containing TC species were then applied to a MonoS column equilibrated with 50 mM acetate buffer (pH 5.0). The absorbed protein was eluted by a linear gradient of NaCl concentrations ranging from 0 to 0.5 M. The distinct peak fractions were collected separately and their homogeneities were confirmed by native PAGE, as shown in Fig. 2(a). The molecular masses of the three purified L-TC species were determined to be 650, 610 and 540 kDa by analytical gel filtration from all strains tested (data not shown). Under native PAGE conditions at pH 8.8, non-toxic complex (NTNHA/HAs) and NT dissociate from the purified TC species in all four strains. Fig. 2(b) shows the SDS-PAGE banding pattern of each L-TC species from the four strains, with different staining intensities of the HA-33 and HA-17 bands.

Molecular composition of L-TC species

C. botulinum serotype C and D TCs 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*, that produce various TC species by association of each gene product (Hauser *et al.*, 1994; Fujinaga *et al.*, 1994; Nakajima *et al.*, 1998). Normally, the components of the TC species produced by serotype C and D strains are nicked at specific sites in NT and HA-70 (Oguma *et al.*, 1999), but only D-4947 produces TC species, which are composed of unnicked components (Kouguchi *et al.*, 2001). Recently, we have proposed that the complete subunit structure of the D-4947 650 kDa L-TC is deduced to be a dodecamer assembled by a single NT, a single NTNHA, two HA-70 molecules, four HA-33 molecules and four HA-17 molecules. Furthermore, on the basis of titration analysis with the HA-33/HA-17 complex to the 610 and 540 kDa TC species, we also have demonstrated that the stoichiometry of the HA-33/HA-17 molecules in the 650 kDa L-TC, 610 and 540 kDa TC species was 4, 3 and 2, respectively (Mutoh *et al.*, 2003). Considering observed common features among serotype C and D TCs, the stoichiometry

Fig. 1. Separation of *C. botulinum* D-4947, D-CB16, C-6814 and C-Yoichi TC species by SP-Toyopearl 650S column chromatography, and SDS-PAGE and native PAGE banding patterns of the peak fractions. Peak fraction numbers indicated on each chromatogram correspond to the lane numbers from SDS-PAGE and native PAGE.

of the TC components produced by C and D strains appears to be similar to that of D-4947. In this study, we additionally found that NTNHA/HAs complexes generated from the 540, 610 and 650 kDa L-TC of the three strains, D-CB16,

C-6814 and C-Yoichi, on native PAGE under alkaline conditions showed similar electrophoretic mobility to those of D-4947, as shown in Fig. 2(a). However, the mobility of each NT was different because the amino acid sequences of

Fig. 2. Native and SDS-PAGE analysis of purified L-TC species from D-4947, D-CB16, C-6814, and C-Yoichi. (a) The native PAGE at pH 8.8 showed dissociated NT and NTNHA/HAS from each L-TC species. The bands corresponding to the dissociated NT and NTNHA/HAS complex of TC species from each strain are indicated by the appropriately labelled arrow. (b) SDS-PAGE of L-TC species from each strain in the presence of 1% 2-mercaptoethanol using a 13.6% polyacrylamide gel. Except for the D-4947 L-TC species, the 100 kDa Hc and 50 kDa Lc derived from cleavage of NTs, and the 55 kDa and 22–23 kDa HA fragments (HA-55 and HA-22–23, respectively) derived from HA-70 were observed in the banding patterns of the L-TC species from the other three strains. All bands indicated on the gels were identified by N-terminal amino acid sequence analysis (data not shown). Lane M, molecular size markers.

the NTs produced by C and D strains are 51–77 % identical, although those of three HA subcomponents are almost identical (Oguma *et al.*, 1999).

To characterize the molecular composition of HA-33/HA-17 in each L-TC species, the ratios of the band-staining intensities of HA-33/NT and HA-33/NTNHA on SDS-PAGE in each L-TC species were examined. As summarized in Table 1, ratios of HA-33/NT in D-4947 and HA-33/NTNHA in D-CB16, C-6814 and C-Yoichi were roughly

Table 1. Ratios of staining intensity of HA-33/NT and HA-33/NTNHA

Data are ratios of CBB staining intensity after SDS-PAGE of HA-33 to NT or NTNHA as indicated in Fig. 2(b).

TC	HA-33/NT	HA-33/NTNHA			
	D-4947*	D-4947	D-CB16	C-6814	C-Yoichi
L-TC ₂	0.49	0.47	0.38	0.42	0.42
L-TC ₃	0.68	0.69	0.75	0.76	0.71
L-TC	1.36	0.95	0.99	1.15	0.98

*The ratio of HA-33/NT is only shown for D-4947 because the NTs from the other strains always split into 100 kDa Hc and 50 kDa Lc on SDS-PAGE by nicking.

estimated to be 2:3:4, stepwise, with the 540 kDa L-TC, the 610 kDa L-TC and the 650 kDa L-TC, respectively, as observed in D-4947 L-TCs. Accordingly, we tentatively designated the 540 kDa L-TC as L-TC₂ (NT/NTNHA/2. HA-70/2.HA-33/2.HA-17), the 610 kDa L-TC as L-TC₃ (NT/NTNHA/2.HA-70/3.HA-33/3.HA-17) and 650 kDa L-TC as just L-TC (complete form containing four HA-33/HA-17 molecules), in the respective strains used in this study. There was no L-TC₁ (probably around 500 kDa) corresponding to the L-TC species composed of only one HA-33/HA-17 molecule in the culture supernatants of every strain. However, since a small amount of 410 kDa L-TC₀ corresponding to the NT/NTNHA/2.HA-70 complex was found (data not shown), L-TC₁ is assumed to immediately convert to L-TC₂ or L-TC₀ as stable forms in culture supernatants.

SDS-PAGE banding patterns of D-4947 L-TC species revealed that they consist of five intact components, NT, NTNHA, HA-70, HA-33 and HA-17, with different staining intensities, which is dependent on the L-TC species, as illustrated in Fig. 2(b). Unlike D-4947, however, banding patterns of C-6814, C-Yoichi and D-CB16 L-TC species showed seven bands on SDS-PAGE because their NTs separate into 100 kDa heavy chains (Hc) and 50 kDa light chains (Lc), and their HA-70s separate into 55 kDa (HA-55) and 22–23 kDa (HA-22–23) fragments due to nicking by an endogenous bacterial protease (Fig. 2b). Additionally, the band of C-Yoichi HA-33 migrated faster than those of

the other strains and the apparent molecular mass was approximately 30 kDa, due to a deletion of 31 aa residues from its C terminus (Sagane *et al.*, 2001).

Molecular properties of isolated HA-33/HA-17

Gel filtration elution patterns of the HA-33/HA-17 complexes purified from the L-TC of four strains showed a single peak, as illustrated in Fig. 3(a). The molecular sizes of the isolated HA-33/HA-17 of each strain were estimated to be approximately 40 kDa (but 36 kDa for C-Yoichi) by their elution volumes from gel filtration with 50 mM phosphate buffer (pH 6.0) and 0.15 M NaCl. This molecular size was lower than the calculated mass of 50 471 Da. An apparently lower molecular mass might result from differences in hydrodynamic properties, such as shape and/or hydrophobic interaction between the HA-33/HA-17 complex and the Superdex 200 gel matrix. Thus, every HA-33/HA-17 complex might be present as a monomer complex with a 1:1 molecular ratio (Suzuki *et al.*, 2005). Thus, we obtained pure HA-33/HA-17 complexes from each L-TC, although HA-33 and HA-17 could not be separated from the complex as viable forms due to irreversible precipitation during dialysis. Previously, we demonstrated that the HA-33/HA-17 complex is able to bind to the M-TC/HA-70 complex, but free HA-33 is not. Thus, this observation suggested indirectly that the role of the HA-17 molecule is

to adhere to HA-70 and form a linkage between HA-33 and HA-70 (Mutoh *et al.*, 2003).

Isolated HA-33/HA-17 complexes from four strains were separated by SDS-PAGE as shown in Fig. 3(b). The complexes from D-4947, D-CB16 and C-6814 had two bands, 33 and 17 kDa, while the HA-33 from C-Yoichi showed a band corresponding to about 30 kDa, due to a deletion of 31 aa from the C terminus at a specific site, as observed in all C-Yoichi L-TC species. Additionally, the ratio of 1:1 was supported by the stained band intensities of HA-33 and HA-17 from the four strains on SDS-PAGE based on their molecular masses.

As shown in Fig. 3(c), the isolated HA-33/HA-17s from four strains were purified to homogeneity by native PAGE. However, their gel mobilities were different from each other. To characterize the ionization state of HA-33/HA-17, the pI values were examined by the native isoelectric focusing method. As shown in Table 2, the pI values of the HA-33/HA-17 complexes from D-4947 and C-6814 were estimated to be neutral, at 6.8 and 7.3, respectively. On the other hand, those of D-CB16 and C-Yoichi were estimated to be alkaline, at 8.0 and 8.4, respectively. Except for the unique results with C-Yoichi HA-33/HA-17, these data seem to reflect the different banding patterns on native PAGE, as shown in Fig. 3(c). Although the pI values of the complex form cannot be predicted by protein analysis software, the pI

Fig. 3. Molecular properties of HA-33/HA-17 isolated from D-4947, D-CB16, C-6814 and C-Yoichi TC species. (a) Superdex 200 HR 10/30 gel filtration profiles of the isolated HA-33/HA-17s from each strain. The molecular masses of the standard proteins estimated from the elution volume (indicated at the bottom) are indicated along the top of the figure. Arrows on the chromatograms indicate molecular masses of each HA-33/HA-17 complex estimated from their elution volumes. (b) SDS-PAGE banding patterns of the isolated HA-33/HA-17s from each strain in the presence of 1% 2-mercaptoethanol using 15% polyacrylamide gel. (c) Native PAGE banding patterns of the isolated HA-33/HA-17s from each strain.

Table 2. pI values of HA-33/HA-17

Strain	Measured isolated HA-33/HA-17	Predicted*	
		HA-33	HA-17
D-4947	6.8	7.11	5.04
D-CB16	8.0	8.51	5.04
C-6814	7.3	8.07	6.49
C-Yoichi	8.4	9.07†	6.49

*Predicted pI was calculated from amino acid sequences using protein analysis software.

†Calculated based on amino acid sequences of HA-33 with a C terminus lacking 31 aa residues.

values of each complex seemed intermediary between the predicted alkaline pI values (7.11–9.07) of the HA-33 molecule rather than the acidic pI values (5.04–6.49) of the HA-17 molecule. However, only HA-33 among the five components of the botulinum TC was predicted to be a basic protein, while the remaining components were predicted to be acidic, suggesting that the overall TC complex forms will be acidic (Hasegawa *et al.*, 2004).

To compare the secondary structure of the HA-33/HA-17 complexes from four strains, far-UV CD spectra of the isolated HA-33/HA-17 were measured. As shown in Fig. 4, the far-UV CD analysis showed slightly different spectral curves in respective HA-33/HA-17 complexes. Every HA-33/HA-17 complex commonly showed a predominant β -sheet-rich structure (74.7–77.2 %), a random coil (22.8–25.0 %) and a small amount of α -helix (Table 3), values which are considerably higher compared to the β -sheet content in

Fig. 4. Far-UV CD spectra of the isolated HA-33/HA-17 complexes from D-4947 (◆), D-CB16 (■), C-6814 (▲) and C-Yoichi (●) dissolved in 50 mM Tris/HCl buffer (pH 7.4). The spectral recording was performed at 200 nm min⁻¹ with a 0.5 s response time.

Table 3. Secondary structure measurements of the HA-33/HA-17 complex

Secondary structure content was measured using a far-UV CD spectropolarimeter. Each structure is presented as a percentage.

Strain	α -Helix	β -Sheet	Turns	Random coil
D-4947	1.1	74.7	0	24.2
D-CB16	0	77.2	0	22.8
C-6814	0	75.0	0	25.0
C-Yoichi	0.9	75.9	0	23.2

individual components (51.4–52.4 % for HA-33 and 49.3–52.1 % for HA-17) predicted by a protein analysis server (http://fasta.bioch.virginia.edu/fasta_www/garnier.htm). However, the β -sheet-rich structure is probably a common feature among HA subcomponents of the botulinum TC, as measured by far-UV CD [79.3 % for D-4947 HA-70 (Suzuki *et al.*, 2005), 71.1 % for HA-33 of C-6814 (Kouguchi *et al.*, 2001) and 74–77 % for serotype A Hn-33 (corresponding to our HA-33) (Sharma *et al.*, 1999)].

Haemagglutination and binding activities to erythrocytes of reconstituted HA-hybrid L-TC with HA-33/HA-17 from different strains

The L-TCs containing a full number of HA-33/HA-17 complexes from D-4947, D-CB16 and C-6814 are haemagglutination-positive with titre values of 2⁵–2⁶ (Table 4), but L-TC₂ and L-TC₃ from these three strains are haemagglutination-negative due to a smaller number of HA-33/HA-17 complexes. On the other hand, any L-TC species from C-Yoichi containing a truncated variant HA-33 molecule in the HA-33/HA-17 complex showed no haemagglutination activity. The HA-33/HA-17 complexes from the

Table 4. Haemagglutination titre of each L-TC species

–, Negative at 0.25 μ M (in 35 μ l). All data for C-Yoichi L-TCs were negative.

Strain	TC	No addition	Addition of HA-33/HA-17*			
			D-4947	D-CB16	C-6814	C-Yoichi
D-4947	L-TC	2 ⁵	2 ⁵	2 ⁵	2 ⁵	2 ⁵
	L-TC ₃	–	2 ⁵	2 ⁴	2 ⁴	–
	L-TC ₂	–	2 ⁵	2 ⁴	2 ⁴	–
D-CB16	L-TC	2 ⁴	2 ⁴	2 ⁴	2 ⁴	2 ⁴
	L-TC ₃	–	2 ⁴	2 ⁴	2 ⁴	–
	L-TC ₂	–	2 ⁴	2 ⁴	2 ⁴	–
C-6814	L-TC	2 ⁴	2 ⁴	2 ⁴	2 ⁴	2 ⁴
	L-TC ₃	–	2 ⁴	2 ⁴	2 ⁴	–
	L-TC ₂	–	2 ⁴	2 ⁴	2 ⁴	–

*Each HA-33/HA-17 complex showed negative haemagglutination activity.

four strains exhibited very weak or negative haemagglutination activity with titre values below 2^1 at $0.25 \mu\text{M}$ each, as previously demonstrated when both isolated HA-33 and HA-33/17 complex from serotype C and D strains exhibited very weak haemagglutination activity (titre 2^2) even at high concentrations (Kouguchi *et al.*, 2001, 2002). In this study, we attempted to reconstitute an HA-hybrid L-TC with L-TC₂ and L-TC₃ from the four strains assembled with different combinations of the isolated HA-33/HA-17 complex from each of the four strains. After mixing L-TC₂ and L-TC₃ at the appropriate ratios for the HA-33/HA-17 complex under phosphate buffer (pH 6.0) containing 0.15 M NaCl, the mixtures were subjected to a gel filtration column equilibrated with the same buffer. As shown in Fig. 5, when the newly generated peaks were applied to native PAGE, the NTNHA/HA bands of every L-TC₂ and L-TC₃ at the original position disappeared and migrated completely to a single band at the position of the NTNHA/HAs corresponding to the respective 650 kDa L-TC. Additionally, gel filtration elution patterns of

HA-hybrid TCs under acidic conditions (50 mM acetate buffer, pH 2.5) gave a single peak at 650 kDa (data not shown), suggesting that no components dissociated from the respective HA-hybrid L-TC. These results imply that all L-TC₂ and L-TC₃ lacking two and one molecules, respectively, of the HA-33/HA-17 complex convert to the stable L-TC with four HA-33/HA-17 complexes by the addition of HA-33/HA-17 complexes from different strains.

The haemagglutination activities of the HA-hybrid L-TC reconstituted with HA-33/HA-17 complexes from different strains were examined using equine erythrocytes. As shown in Table 4, the haemagglutination titres of HA-hybrid D-4947, D-CB16 and C-6814 L-TCs with HA-33/HA-17 from each strain were determined to be 2^4 – 2^5 , which are similar to those of the respective parent L-TCs. In contrast, neither of the HA-hybrid L-TCs of C-Yoichi with the HA-33/HA-17 complexes from the four strains showed haemagglutination activity at $0.25 \mu\text{M}$. Likewise, every HA-hybrid L-TC reconstituted with C-Yoichi HA-33/HA-17 showed negative

(a) D-4947 TC species

(b) D-CB16 TC species

(c) C-6814 TC species

(d) C-Yoichi TC species

Fig. 5. Native PAGE analysis of various HA-hybrid L-TCs constructed by the isolated HA-33/HA-17 complexes from each strain and each L-TC₂ and L-TC₃. HA-hybrid L-TCs were prepared by mixing L-TC₂ and L-TC₃ with the HA-33/HA-17 complex. After gel filtration of the mixtures, samples were subjected to native PAGE. (a) D-4947 HA-hybrid L-TCs; (b) D-CB16 HA-hybrid L-TCs; (c) C-6814 HA-hybrid L-TCs; (d) C-Yoichi HA-hybrid L-TCs. The HA-33/HA-17s added to construct the HA-hybrid L-TC are indicated by the strain names above the L-TC lane indicators. L-TCs of each strain at the left of each panel show the complete 650 kDa complex.

haemagglutination activity. However, when the C-Yoichi HA-33/HA-17 was added to the L-TCs of the other three strains exhibiting full haemagglutination activity, no decrease in activity was detectable. The difference observed in haemagglutination activity among the HA-hybrid L-TCs is likely to result from the HA-hybrid L-TCs containing at least the C-Yoichi variant HA-33/HA-17 lacking 31 aa residues in the HA-33 C terminus. In other words, a combination of four unmodified HA-33 molecules is required for full haemagglutination activity of the botulinum TC.

Binding activities of the L-TC species to equine erythrocytes were also analysed by immunoblotting bands, separated by SDS-PAGE, from washed erythrocytes after incubation. As summarized in Table 5, both haemagglutination-negative L-TC₂ and L-TC₃ species from D-4947, D-CB16 and C-6814 could bind to erythrocytes. Accordingly, the HA-hybrid L-TCs of these strains reconstituted with HA-33/HA-17 molecules from four strains containing C-Yoichi bound to erythrocytes. On the other hand, only when C-Yoichi L-TC₂ and L-TC₃ were repaired with active HA-33/HA-17 complexes from the other three strains did binding activity appear. However, C-Yoichi L-TC occupied with four truncated HA-33 molecules no longer exhibited binding activity, even if an active HA-33/HA-17 complex was added. Contrary to haemagglutination, it is likely that botulinum TC species containing at least one complete HA-33 molecule can bind to erythrocytes. These results strongly suggest that the lack of the C-terminal region of the HA-33 molecule plays a definite role in the botulinum TC functions of haemagglutination and erythrocyte binding. Kouguchi *et al.* (2001) demonstrated that the isolated serotype C HA subcomponents, HA-70, HA-33 and the HA-33/HA-17 complex, bound to erythrocytes, but that each single component showed no aggregation of erythrocytes. They also concluded that a combination of at least HA-33, HA-17 and HA-70 was required for full haemagglutination activity of the botulinum L-TC.

Currently, recognition and binding by plural HA components to specific carbohydrates on the surface of

erythrocytes are thought to be essential for the complicated cross-linkage between each erythrocyte, leading to the appearance of haemagglutination. In this regard, it has been suggested that the binding of haemagglutination-positive L-TC from serotype C and D strains to erythrocytes would be significantly inhibited by the presence of 100 mM of *N*-acetylneuraminic acid (Sagane *et al.*, 2001; Inoue *et al.*, 1999). Previous studies suggest that the concanavalin A dimer and tetramer species composed of identical subunits, possess different binding affinities to target cells (McKenzie & Sawyer, 1973). Soybean agglutinin, belonging to a plant lectin family, is also a tetramer composed of approximately 30 kDa identical subunits (Lotan *et al.*, 1974), HA of an influenza virus is a trimer of identical subunits (Wilson *et al.*, 1981) and amaranth grain agglutinin is a tightly associated homodimer of 66 kDa (Transue *et al.*, 1997), which is essential for cell recognition. Recently, Mancheno *et al.* (2005) have demonstrated that the haemolytic lectin from the parasitic mushroom *Laetiporus sulphureus* is a hexamer composed of 35 kDa subunits containing two distinct modules, an N-terminal lectin module and a pore-forming module, yielding strong haemagglutination activity and haemolytic activity. Accordingly, an oligomeric structure that provides multiple binding sites for aggregation by cross-linkage of erythrocytes has been observed across species. Although four prominent HA-33s on the botulinum L-TC implies four binding sites against the erythrocytes via carbohydrate chains, the spatial distance, position and the configuration among four HA-33s required for cross-linkage of erythrocytes are still unknown. X-ray crystallographic and electron microscopic analysis, which will be performed in the near future, may provide the mechanism for the haemagglutination caused by the four HA-33 molecules in the subunit structure of the botulinum TC.

Molecular modelling of the C-terminal truncated HA-33 of C-Yoichi

Since the X-ray crystallographic analyses of serotypes C and A have been elucidated, the complete HA-33 structure is available (Inoue *et al.*, 2003; Arndt *et al.*, 2005), thus enabling our efforts in molecular modelling of the C-terminal truncated HA-33 of C-Yoichi and D-4947 using the serotype C HA-33 structure as the modelling template. Both serotype C and A HA-33 models have a dumbbell-shaped structure ($70 \times 40 \times 37$ Å) with two β -trefoil domains connected by a short α -helix. The HA-33s of serotypes A to D are well known to be members of a carbohydrate recognition protein family, as shown by search results from a characteristic β -trefoil fold previously described in many lectins (Hazes, 1996; Rutenber *et al.*, 1987; Transue *et al.*, 1997). Indeed, the HA-33s of serotype C and D strains employed here were also shown to have two β -trefoil domains, suggesting HA activity and binding activity to the erythrocytes through recognition of *N*-acetylneuraminic acid or sialylglycolipid (Inoue *et al.*, 1996). According to the profile created using the hidden Markov model method with Pfam software (Bateman *et al.*, 2000), one of the putative

Table 5. Binding of each L-TC species to equine erythrocytes

Binding of TC species to equine erythrocytes was determined by Western blotting. Any TC species band that was identified from Western blotting was determined as binding positively at 0.25 μ M (in 60 μ l). All data were positive for all L-TCs of strains D-4947, D-CB16 and C-6814.

Strain	TC species	No addition	Addition of HA-33/HA-17			
			D-4947	D-CB16	C-6814	C-Yoichi
C-Yoichi	L-TC	—	—	—	—	—
	L-TC ₃	—	+	+	+	—
	L-TC ₂	—	+	+	+	—

(a) D-4947 HA-33 intact form

(b) C-Yoichi HA-33 intact form

(c) C-Yoichi HA-33 truncated form

Fig. 6. Computer modelled structure of C-Yoichi and D-4947 HA-33. The structures were generated with the Molsoft ICM Browser. In (a) and (b), the model shows the full-length D-4947 and C-Yoichi HA-33, respectively, showing the two domains connected by a short α -helix. The β -structure and α -helix are labelled. In (c), the model shows the C-terminal truncated HA-33. The missing 31 aa residues are indicated in the full-length model (b) by a red colour. The N and C terminals are labelled as N and C, respectively. The putative carbohydrate recognition site is indicated with an arrow (Arndt *et al.*, 2005).

carbohydrate-binding sites is located in the C-terminal domain. HA-33 molecules commonly consist of a single polypeptide chain of approximately 290 residues across the serotype. However, the amino acid sequence of C-Yoichi HA-33 deduced from the nucleotide sequence was identical to that of the consensus from the N terminus up to residue 145, but showed 46% aa identity with 76 residue substitutions in the remaining C-terminal region (residues 146–285). Despite low sequence identity in this region of the C terminus, Fig. 6(b) demonstrates a normal structure consisting of two β -trefoil domains that are highly similar to each other.

The processing site of C-Yoichi HA-33 was predicted to be between L254 and D255 in the C-terminal region, probably cut by an endogenous protease (Sagane *et al.*, 2001). According to the serotype A HA-33 model (Arndt *et al.*, 2005), the cleavage site could be positioned between β 21 and the counterpart of the anti-parallel β 22 sheets by a β -turn

structure. As presented in Fig. 6(c), the ribbon diagram for truncated C-Yoichi HA-33 displays the structure of two unsymmetrical domains caused by the destruction of the C-terminal β -trefoil structure due to the absence of 31 aa from the C terminus. According to the crystal structure model of serotype A HA-33, putative carbohydrate-binding sites are located at Q263, Y265, Q268, Q276, F278 and Q286 (Arndt *et al.*, 2005), which are contained in the sequence deleted in C-Yoichi HA-33. Thus, these data can explain the behaviour unique to the C-Yoichi L-TC species in which C-Yoichi L-TC can neither bind to erythrocytes nor aggregate with the erythrocytes.

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