Study on three-dimensional structure and sugar recognition characteristics of HA-33 of toxin complex from *Clostridium botulinum* serotype C strain Yoichi

2016

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INTRODUCTION

*Clostridium botulinum* produces a botulinum neurotoxin (BoNT; 150 kDa) that is the causative agent of human and animal botulism. After the absorption from intestine, BoNT enters nerve cells via receptor-mediated endocytosis and cleaves specific sites on target proteins through its zinc protease activity. This results in the inhibition of acetylcholine release from peripheral cholinergic nerves, leading to flaccid muscle paralysis (Montecucco and Schiavo 1993). BoNT is serologically classified into seven serotypes A through G. Serotype A, B, E and F are mainly responsible for human botulism, whereas serotypes C and D causes in animal and avian botulism. Food borne botulism occurs every 2 or 3 years in Japan, although the case of death has never been reported since 1986. On the other hand, sporadic and massive outbreaks of cattle botulism have occurred worldwide in recent years. In Japan, one to five cases of cattle botulism have been reported every year, causing large economic losses in dairy and beef cattle farming (Cobb et al. 2002; Martin 2003; Steinman et al. 2007).

In culture supernatant and naturally contaminated foods, BoNT molecule is associated with auxiliary proteins including nontoxic nonhemagglutinin (NTNHA; 130 kDa) and hemagglutinin (HA) subcomponents having molecular mass of 70, 33 and 17 kDa (HA-70, HA-33 and HA-17), yielding botulinum toxin complex (TC). *C. botulinum* produces different forms of TC depending on serotypes. Serotype A-D strains produce the M-TC (a complex of single BoNT and single NTNHA) and L-TC (a complex of M-TC and some HA components). Serotype A strain also produce LL-TC that is thought to be a dimer of L-TC (Oguma et al. 1999). On the other hand, serotype E and F strains produce only M-TC. Although the three dimensional (3D) structure of L-TC has long been obscure, a recent study by Hasegawa et al. (2007) clarified that L-TC is 14-mer.
protein consists of a single BoNT, a single NTNHA, three HA-70, three HA-17 and six HA-33 molecules, and that L-TC has an ellipsoidal-shaped structure with three extended “arms” of the HA-33/HA-17 (2:1) trimer.

When ingested orally, pure BoNT molecule is easily degraded and loses its toxicity, whereas an association of BoNT with NTNHA molecule amazingly enhances the stability against gastric acid and proteolytic conditions, enabling BoNT molecules to reach the intestine. Thus NTNHA protects BoNT molecule from digestion with harsh conditions of gastrointestinal tract. On the other hand, HA-33/HA-17 trimer is thought to contribute to the effective transport of the toxin across the intestinal epithelia. It has been shown that HA-33 binds to cells such as erythrocyte and intestinal cells via sugar chains on the cell surface. Fujinaga et al. (2000) showed that type A L-TC (L-TC/A) recognizes galactose residues of these cells. It was also reported that HA-33 of L-TC/A binds to erythrocytes via galactose moiety (Inoue et al. 2001). A recent report showed that HA-33 of type B L-TC (HA-33/B) bound to porcine gastric mucin, whose carbohydrate moiety is devoid of sialic acid, but not to bovine submaxillary mucin, which is rich in sialic acid (Sugawara et al. 2014). Thus HA-33/A and B predominantly recognizes galactose. In contrast, it has been shown that HA-33 of serotype C and D L-TC (HA-33/C and D) preferentially binds to sialic acid moiety but not galactose moiety on the cell surface. Niwa et al. (2010) showed that both binding and permeation of HA-33/HA-17 trimer of serotype D were potently inhibited by N-acetyl neuraminic acid in the cell culture medium or by treatment of the cells with neuraminidase. However, neither galactose, lactose nor N-acetyl galactosamine inhibited binding or permeation of the complex. Similar results were observed in HA-33/HA-17 trimer of serotype C (Inui et al. 2010).
It has been shown that L-TC of a few strains of serotype C and D has poor hemagglutination activity despite possessing HA proteins. Sagane et al. (2001) demonstrated that hemagglutination activity of serotype C strain Yoichi (C-Yoichi) was much lower than that of the reference serotype C strains. They analyzed N- and C-terminal sequence of HA-33 and found a deletion of 31 amino acid residues from the C-terminus at a specific site. Furthermore, when compared with a reference serotype C strain, the C-Yoichi HA-33 (HA-33/C-Yoichi) showed 46% amino acid identity with 76 amino acid substitutions in the C-terminal region (residues 146-285). A recent study reported that C-Yoichi L-TC (L-TC/C-Yoichi) showed full HA and binding activity towards neuraminidase treated erythrocytes that were completely inhibited in the presence of galactose or lactose (Matsuo et al. 2011). Furthermore, the HA-33/HA-17 trimer isolated from L-TC/C-Yoichi bound to neuraminidase-treated cells and the binding was inhibited in the presence of galactose or lactose. Thus HA-33/C-Yoichi dominantly recognizes galactose moiety like HA-33/A and B, even though the most serotype C strains preferentially interact with sialic acid moiety.

Although it is likely that the unusual recognition of the galactose moiety of HA-33/C-Yoichi could be due to a variation and/or a truncation in the C-terminal-half of the protein, the mechanism by which this protein recognizes galactose unlike other serotype C strains is still unknown. The understanding of this mechanism may help the elucidation of pathogenesis of food borne botulism in human and animals. In this study, the author focused on the structure of HA-33/C-Yoichi and investigated its unique sugar recognition mechanism. This thesis consists of three chapters.

In the Chapter I, the author investigated the binding of HA-33/C-Yoichi to several types of cells to confirm the HA-33/C-Yoichi interacts with cells by recognizing not
sialic acid but galactose. In the Chapter II the author attempted crystallization of HA-33/C-Yoichi to perform X-ray analysis. In the Chapter III, 3D structures of HA-33/C-Yoichi and reference strain in a crystal and solution were determined to distinguish the characteristic structure of HA-33/C-Yoichi. Finally, the specific site responsible for the carbohydrate recognition of HA-33/C-Yoichi was predicted based on the arrangement of amino acids in 3D structure.
Chapter I

Host-cell and sugar-binding specificity of HA-33 from

Clostridium botulinum serotype C strain Yoichi
1. Introduction

Botulinum neurotoxin (BoNT) causes flaccid muscle paralysis in humans and animals by inhibiting neurotransmitter release at nerve endings. Epidemiological studies have shown that BoNT serotypes A, B, E and F cause human botulism, whereas serotypes C and D appear to be causative toxins for animal and avian botulism (Montecucco and Schiavo 1993). In naturally contaminated foods, BoNT (150 kDa) associates with non-toxic non-hemagglutinin (NTNHA; 130 kDa) and three types of hemagglutinin (HA-70, HA-33 and HA-17; 70, 33 and 17 kDa, respectively), forming a large toxin complex (L-TC; 750 kDa) (Hasegawa et al. 2007; Inoue et al. 1993). The first step in foodborne botulism is the trafficking of L-TC from the intestinal epithelial cells to the bloodstream (Sakaguchi et al. 1983).

A recent study showed that L-TC has a unique ellipsoidal-shaped structure with three extended “arms” of the HA-33/HA-17 (2:1) trimer and that two HA-33 molecules were located at the outermost region of each arm of D-4947 L-TC (Hasegawa et al. 2007). Accordingly, HA-33 is likely to have important roles in the interaction between toxin and the cells. In fact, previous studies have shown that HA-33 contributes to the binding of L-TC to intestinal cells by recognizing carbohydrate residues on the cell surface. On the other hand, types of carbohydrates recognized by HA-33 molecule differ depending on serotypes of toxin. In general, HA-33/A and B preferentially recognizes galactose, whereas type C and D predominantly bind to sialic acid. HA-33/C-Yoichi, however, has been shown to have unique properties as to sugar recognition. Matsuo et al. (2011) demonstrated that C-Yoichi HA-33/HA-17 trimer bound to neuraminidase-treated cells and the binding was inhibited in the presence of galactose using a cultured rat intestinal cell line IEC-6. Although these results suggest that
HA-33/C-Yoichi dominantly recognizes galactose moiety on IEC-6 unlike HA-33 of major strain C, it is unknown whether this is the case in other types of epithelial cells.

Accordingly, in this chapter the author investigated the binding of HA-33/C-Yoichi to several types of epithelial cells to confirm that HA-33/C-Yoichi generally recognizes galactose moiety on the cells.

2. Material and methods

2.1 Production and purification of botulinum L-TC and isolation of HA-33/HA-17 trimer

*C. botulinum* serotype C strains Yoichi (C-Yoichi) and Stockholm (C-St) were cultured using a dialysis method reported previously (Miyata *et al.* 2012). The L-TC in the culture supernatants was precipitate with 60% saturation of ammonium sulfate. The precipitate was dialyzed against 50 mM acetate buffer (pH 4.0) with 0.2 M NaCl and applied to a TOYOPEARL SP-650S (Tosoh, Tokyo, Japan) equilibrated with dialysis buffer. Bound protein was eluted with a liner gradient of NaCl (0.2 - 0.8 M). The elution fraction containing the L-TC, judged by SDS- and Native- PAGE, was collected, concentrated with 80% saturation of ammonium sulfate and further purified with a HiLoad 16/60 Superdex 200 pg (GE Healthcare, Little Chalfont, UL) equilibrated with 50 mM acetate buffer (pH 5.0) with 0.15 M NaCl, The elution fraction containing the L-TC was precipitated with 80% saturation of ammonium sulfate.

Isolation of the HA-33/HA-17 trimer from the L-TC was performed as previously reported (Kouguchi *et al.* 2002). The purified L-TC (250 mg) was incubated in 20 mM Tris-HCl (pH 7.8) containing 4.0 M guanidine hydrochloride (Gdn buffer) at 21°C for 4 h. Treated sample was applied to a HiLoad Superdex 200 pg 16/60 gel-filtration column
equilibrated with the Gdn buffer. The fraction containing HA-33/HA-17 trimer was collected, diluted to 0.05 absorbance at 280 nm with Gdn buffer and then dialyzed against 20 mM Tris–HCl (pH 7.8) at 4°C and then applied to a Mono S HR 5/5 (GE Healthcare) with elution by a linear gradient of NaCl (0 - 0.5 M).

2.2 Cell culture

The rat small intestine epithelial cell line IEC-6 and the human colon carcinoma Caco-2 were obtained from RIKEN BioResouse Center (Tsukuba, Japan). The bovine aortic endothelial cell line (BAEC) was obtained from Cell Applications Inc. (San Diego, CA, USA). IEC-6 cells were grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 IU/ml) and streptomycin (100 μg/ml). When Caco-2 cells were cultivated, the medium was additionally supplemented with MEM non-essential amino acid solution. BAEC were cultured with bovine endothelial cell growth medium obtained from Cell Applications Inc. Cells were maintained in a humidified environment of 5% CO₂ at 37°C. The culture medium was renewed every 2 - 3 days.

2.3 Antibodies

The rabbit polyclonal antibodies recognizing HA-33 was used (Niwa et al. 2010).

2.4 Toxin binding assay

The binding of C-Yoichi and C-St HA-33/HA-17 trimer to IEC-6, Caco-2 and BAEC cells were assayed, as described previously (Niwa et al. 2007) with minor modifications. Cells were prepared in 24 well dishes (Corning, Corning, NY) and grown
to confluence. HA-33/HA-17 trimer was suspended in 300 μl of DMEM at the indicated concentrations and added to culture dishes at 4°C. Cells were then incubated for 30 min at 4°C, rinsed three times with cold PBS and lysed with 150 μl SDS buffer. Proteins bound to cells in 15 μl of the sample were separated on SDS-PAGE and detected by Western blot analysis.

2.5 Western blot

Samples from the binding assay were electrophoresed on 13.6% SDS gels. The separated proteins were blotted onto a nitrocellulose membrane (GE Healthcare) and incubated with antibodies diluted 1 : 1000 in TBST (20 mM Tris-HCl buffer, pH 7.5, 150 mM NaCl, and 0.1% Tween) with 5% skimmed milk at 4°C overnight. After rinsing three times with TBST, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Dallas, TX) diluted 1 : 1000 in TBST containing 5% skimmed milk for 1 h at room temperature. After rinsing, proteins were visualized by chemiluminescence detection. Goat anti-actin antibody and donkey anti-goat IgG antibody conjugated with HRP (Santa Cruz Biotechnology) were used to detect actin. The intensity of detected bands was analyzed with Image J 1.38 v software (http://rsb.info.nih.gov/ij/). The intensity of the band corresponding to HA-33 was divided by that to actin to express the normalized values of HA-33 binding.

3. Results and discussion

To compare the binding properties of HA-33/C-Yoichi and reference strain to cells, HA-33/HA-17 trimer of each strain at the same concentration was incubated with three
kinds of epithelial cells: rat intestinal epithelial cells, human colon carcinoma cells, and bovine aortic endothelial cells. Fig. 1 shows the binding amount of HA-33/HA-17 trimer to each cell cultured in 24 well culture plates. The binding of C-Yoichi HA-33/HA-17 trimer to IEC-6 cells was about 30% of that of C-St. Matsuo et al., (2011) reported a similar observation that binding of L-TC/C-Yoichi was very smaller than that of C-St using horse erythrocytes. By contrast, C-Yoichi HA-33/HA-17 trimer showed a larger binding to Caco-2 cells than C-St. The binding of C-Yoichi HA-33/HA-17 trimer was about 170% of that of C-St. Furthermore, HA-33/HA-17 trimer of both C-Yoichi and C-St bound to BAEC to the same extent. These results indicate that the mechanism for the interaction of HA-33/C-Yoichi with epithelial cells is distinct from that of C-St.

To investigate whether the difference in the binding property of HA-33/HA-17 trimer between C-Yoichi and C-St is due to sugar chains on the cell surface, the binding of the trimer to neuraminidase-treated cells were examined. Fig 2 shows the effects of neuraminidase at several concentrations on the binding of HA-33/HA-17 trimer in IEC-6 cells. The binding of C-St HA-33/HA-17 trimer was abrogated depending on the concentration of neuraminidase, suggesting that the binding was mediated via sialic acid. On the other hand, neuraminidase treatment increased the binding of C-Yoichi HA-33/HA-17 trimer in a concentration-dependent manner. These results suggest that the sugar recognized by HA-33/C-Yoichi dominantly is not sialic acid.

The present results suggested that HA-33/C-Yoichi recognizes not sialic acid but galactose in different types of epithelial cells, i. e. rat intestinal cells and bovine aortic endothelial cells. Additionally, a previous report showed that HA-33/C-Yoichi recognizes galactose on horse erythrocytes (Matsuo et al. 2011). Thus it is strongly
suggested that HA-33/C-Yoichi generally interacts with any types of cells by recognizing galactose. The difference in the binding ratio of C-Yoichi and C-St HA-33/HA-17 trimer among cell types may be due to a variation of carbohydrates expressed on the cells, although the author did not determine the kind of carbohydrate in the present study. It has been reported that sugar chains are differentially expressed in cells and are subject to change during development, differentiation, and oncogenic transformation and that the terminal glycosylation sequences produced by a cell are presumed to reflect the expression of the corresponding glycosyltransferases. (Paulson and Colley 1989). To understand the mechanisms underlying the interaction between HA-33/C-Yoichi and epithelial cells more precisely, analysis of carbohydrate on the surface of cells exploited in this study may be required.
Fig. 1. Binding amount of C-Yoichi and C-Stockholm HA-33/HA-17 trimer on IEC-6, Caco-2 and BAEC cells. Cells were incubated with both C-Yoichi and C-St HA-33/HA-17 trimer for 30 min at 4°C. The cell lysates were applied to SDS-PAGE and bands were detected by a Western blot immunostaining and quantified by densitometric tracing as described in Materials and methods. Experiments were repeated in triplicate, and error bars represent the SE of the mean.
Fig. 2. Binding amount of C-Yoichi and C-St HA-33/HA-17 trimer on
neuraminidase-treated IEC-6 and BAEC cells. IEC-6 and BAEC Cells were
pretreated with the indicated concentrations of neuraminidase for 18 h at 37°C in a CO₂
incubator and then incubated with either C-Yoichi HA-33/HA-17 trimer (A and C) or
C-St HA-33/HA-17 trimer (B and D) for 30 min at 4°C. The cell lysates were applied to
SDS-PAGE and bands were detected by a Western blot immunostaining and quantified
by densitometric tracing as described in Materials and methods. Experiments were
repeated in triplicate, and error bars represent the SE of the mean.
Summary

In this chapter the author investigated the binding of HA-33/C-Yoichi and reference strain C-St to several types of epithelial cells to confirm that HA-33/C-Yoichi generally recognizes galactose moiety on the cells. The binding of HA-33/C-Yoichi and C-St to cultured epithelial cells including a rat intestinal cell line (IEC-6), bovine aortic endothelial cells (BAEC) and human coron carcinoma (Caco-2) was examined. The cells were incubated with HA-33/HA-17 trimer in a CO\textsubscript{2} incubator for 30 min at 4°C, and the toxin bound to the cells were detected by Western blot using an antibody against HA-33. HA-33/HA-17 trimer of both C-Yoichi and C-St bound to IEC-6 cells with C-Yoichi HA-33/HA-17 trimer exhibiting larger binding. When sialic acid residues were removed from the surface of IEC-6 cells by neuraminidase treatment, the binding of C-St HA-33/HA-17 trimer was decreased dependent on neuraminidase concentrations. In contrast, neuraminidase treatment of the cells largely enhanced the binding of C-Yoichi HA-33/HA-17 trimer, suggesting that C-Yoichi HA-33/HA-17 trimer did not recognize sialic acid. The binding amount of C-Yoichi HA-33/HA-17 trimer was larger than that of C-St in Caco-2 cells, whereas HA-33/HA-17 of both C-Yoichi and C-St bound to BAEC to almost the same extent. Thus it is strongly suggested that HA-33/C-Yoichi generally interact with any types of cells by recognizing galactose.
Chapter II.

Crystallization and preliminary X-ray analysis of HA-33 from

*Clostridium botulinum* serotype C strain Yoichi
1. Introduction

The botulinum toxin complex (TC), produced by the anaerobic Gram-positive bacterium *Clostridium botulinum*, causes food borne botulism. The TC is composed of the botulinum neurotoxin (BoNT; 150 kDa) and nontoxic proteins including nontoxic nonhemagglutinin (NTNHA; 130 kDa) and three types of hemagglutinin (HA) (HA-70, HA-33, and HA-17; 70, 33, and 17 kDa, respectively). BoNT is serologically classified into seven serotypes, A–G, and these are also used as descriptors of bacterial strains. Human botulism is caused predominantly by serotypes A, B, E and F and animal and avian botulism by serotypes C and D.

After ingestion of food contaminated with the TC, the complex is exposed to acidic (pH 2) gastric juices, containing pepsin, in the stomach, and to several other proteases in the intestine. Despite such challenges, BoNT and other nontoxic components can nevertheless be detected in blood, wherein BoNT becomes detached from the other peptides and reaches neuromuscular junctions. BoNT is next internalized by nerve endings and cleaves specific sites in target proteins, inhibiting neurotransmitter release (Montecucco and Schiavo 1993). BoNT alone is easily degraded into short peptides when exposed to digestive enzymes of the stomach and intestine. Thus, the NTNHA and HAs attached to BoNT are thought to function as delivery vehicles for BoNT (Miyata et al. 2009).

The first event in food-borne botulism is toxin absorption by the upper small intestine. It has been reported that L-TC binds and passes through intestinal epithelial monolayers much more efficiently than does BoNT, because HA-33 binds to cells with high affinity (Niwa et al. 2007; Ito et al. 2011). Thus, HA-33 is thought to play an important role in binding of the TC to intestinal epithelial cells.
Fujinaga et al. (2000) showed that HA-33/A bound to intestinal microvilli of guinea pig upper small intestinal sections and that binding was inhibited by addition of lactose or galactose, but not by removal of sialic acid moieties by neuraminidase. Kojima et al. (2005) reported similar results. Thus, binding of L-TC/A to the human intestinal cell line Intestine-407 was inhibited by N-acetyllactosamine, lactose, and galactose, but not by N-acetylneuraminic acid. These results suggested that L-TC and HA-33/A preferentially bound to galactose. On the other hand, it has been reported that binding of L-TC and HA-33/D to the mouse intestinal cell line IEC-6 was inhibited by N-acetylneuraminic acid but not by either galactose or lactose (Niwa et al. 2010). Inhibition of L-TC and HA-33/C binding by N-acetylneuraminic acid has also been reported. Thus, it appears that HA-33/C and D bind preferentially to sialic acid (Inui et al. 2010). The specificities of binding of HA-33 to sugars thus differ, depending on the toxin serotype. HA-33/A predominantly recognizes galactose whereas HA-33/C and D predominantly recognize sialic acid.

Recently, Matsuo et al. (2011) showed that HA-33/C-Yoichi exhibits a unique property in terms of sugar recognition. Binding of C-Yoichi L-TC (L-TC/C-Yoichi) to erythrocytes and IEC-6 cells was only about 10% that of the L-TC of the Stockholm serotype C strain (L-TC/C-St). However, when the cells were treated with neuraminidase, binding of L-TC/C-St fell whereas that of L-TC/C-Yoichi increased. These changes were sensitive to the neuraminidase concentration. Further, binding of the HA-33/Ha-17 trimer to neuraminidase-treated IEC-6 cells fell upon addition of galactose and lactose, suggesting that HA-33/C-Yoichi preferentially recognizes galactose, in contrast to the HA-33 of C-St or D-4947.

To date, the crystal structures of HA-33/C and the serotype D HA-33/Ha-17 trimer
have been determined (Inoue et al. 2003; Hasegawa et al. 2007); both recognize sialic acid preferentially. Additionally, the crystal structure of HA-33/A (preferentially recognizing galactose) is available (Arndt et al. 2005). However, the structure of HA-33/C-Yoichi, which (uniquely) preferentially recognizes galactose, although belonging to serotype C, has never been determined. Herein, the author describes the crystallization of, and give preliminary X-ray diffraction data on, HA-33/C-Yoichi.

2. Materials and methods

2.1 Production and purification of HA-33/C-Yoichi

The C-Yoichi ha-33 gene (AB061780) sequence (857 bp) was amplified by PCR using genomic DNA as a template. The primers were: forward 5′-CACCATGTCTCAACAAATG-3′ and reverse 5′-TTATAATCTTGTTATAATCATTGTGC-3′. The PCR product encoding the ha-33 gene was cloned into the pET200/D-TOPO plasmid vector, and the recombinant plasmid transformed into Escherichia coli TOP 10. Transformants were selected on LB plates with 50 µg/ml kanamycin, and plasmid DNA was extracted using a QIAPrep Spin Miniprep kit (Qiagen, Dusseldorf, Germany). Sequencing was performed with the aid of vector-specific primers. Extension products were dye-labelled using an ABI PRISM Big Dye Terminator kit (Applied Biosystems, Carlsbad, CA) and sequenced on a Model 3130 DNA platform (Applied Biosystems). The pET200/D-TOPO-ha-33 construct (10 ng) was next transformed into E. coli BL21-star (DE-3) cells for production of recombinant HA-33 (rHA-33). E. coli was inoculated into 10 ml of LB broth with 100 µg/ml kanamycin and grown overnight (18 h) at 37°C, with gentle shaking. This culture was inoculated into 400 ml of LB broth and incubated at 37°C until mid-log phase was
attained (the OD$_{600}$ was about 0.5). Isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 0.1 mM and induction proceeded at 18°C for a further 18 h. Harvested cells were suspended in 40 ml of 50 mM phosphate buffer (pH 7.4) with 0.3 M NaCl, sonicated, and centrifuged at 10,000 g for 20 min at 4°C. The supernatant was mixed with 5 ml of a 50% (w/v) Ni-charged Resin (Bio-Rad, Hercules, CA) suspension equilibrated with the same buffer and the mixture was poured into a glass column. After washing, the bound protein was eluted in equilibration buffer with 300 mM imidazole. rHA-33 was precipitated by addition of ammonium sulfate at 80% saturation. The precipitate was dialyzed against 50 mM phosphate buffer (pH 6.0) with 0.15 M NaCl and finally subjected to gel filtration on a Superdex 200 HR 16/60 (GE Healthcare) column equilibrated with the same buffer.

2.2 Crystallization and X-ray data collection

Crystals were grown at 20°C by the hanging-drop vapor-diffusion method (Adachi et al. 2003). A protein solution was concentrated to minimal volume using Amicon Ultra-0.5 filtration (molecular weight cut-off: 10 kDa; Millipore, Concord Road Billerica, MA) and diluted to 5.0 mg/ml in 50 mM acetate buffer (pH 5.0) in the absence of any other salt. This solution was filtered through an Ultrafree-MC (0.22 μm in pore diameter; Millipore, Concord Road Billerica, MA). Initial crystallization screening was performed using the Crystal Screen (Hampton Research, Aliso Viejo, CA). Each reservoir solution (500 μl) was transferred to a VDX plate (Hampton Research), crystallization drops were added, and the wells individually sealed with coverslips. Crystals were flash-cooled in a stream of gaseous nitrogen at -173.15°C in the presence of a cryoprotectant [30% (w/v) polyethylene glycol 400, in 0.1 M Tris
buffer (pH 8.5)]. Diffraction data were collected using beamline AR-NW12A, fitted with an ADSC quantum 210r CCD detector (Photon Factory, Tsukuba, Japan), and processed using HKL2000 software (Otwinowski and Minor 1997).

3. Results and discussion

The serotype C HA-33 protein of C-St was earlier crystallized and the structure determined (Nakamura et al. 2007). However, the extent of amino acid sequence identity between the HA-33 of C-Yoichi and C-St is only 73.4%, and falls to 46.1% in the C-terminal halves of the proteins (Sagane et al. 2001). The HA-33/C-Yoichi recognizes galactose-terminated sugar chains on the cell surface, whereas most serotype C strains recognize sialic acid-terminated chains. Thus, the HA-33/C-Yoichi is atypical. The HA-33/A recognizes principally galactose moieties, as does HA-33/C-Yoichi. However the amino acid sequences of serotypes A and C-Yoichi are only 35.8% identical. Therefore, it may be that the HA-33/C is novel. We therefore cloned and crystallized the HA-33/C-Yoichi, and obtained preliminary structural data.

As shown in Fig. 1, highly purified rHA-33 ran as a single band on SDS-PAGE, and had a molecular mass of 37 kDa, as expected from addition of the estimated molecular mass of HA-33 and that of the N-terminal His-Tag. One early report suggested that the HA-33/C-Yoichi was cleaved in the C-terminal region, in an unknown manner, increasing mobility on SDS-PAGE (Sagane et al. 2001). In the present study, we found no evidence of such cleavage, and concluded that recombinant protein produced in E. coli was not in fact cleaved. We crystallized the protein.

When incubated in drops consisting of 3.0 µl of protein solution and 2.0 µl of a crystallization reservoir solution with 8% (w/v) polyethylene glycol 8000 and 5% (w/v)
polyethylene glycol 400, in 0.1 M Tris buffer at pH 8.5, two types of crystals appeared within 3 days of incubation and grew to maximum dimensions of 0.05 x 0.05 x 0.1 mm [crystal (a) of Fig. 2] and 0.01 x 0.01 x 0.2 mm [crystal (b) of Fig. 2]. Diffraction data were obtained from the later crystals under cryo-conditions; a full set of intensity data was collected at a resolution of 2.2 Å (Fig. 3). The other type of crystal did not yield diffraction data. Data collection statistics and crystal data are summarized in Table 1. In this experiment more exposure time than as usual was needed because of beamline trouble. Two molecules formed an asymmetric unit, as was also true of crystals of HA-33/C-St (Inoue et al. 2003). Determination of the complete structure of the unique HA-33/C-Yoichi may help us to understand how L-TC is absorbed from the intestine. It will be useful to understand how the novel HA subunit of the TC of C. botulinum binds to the cell.
Table 1 Data collection statistics.

Values in parentheses represent those of the shell of highest resolution.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crystal to film distance (mm)</td>
<td>212.4</td>
</tr>
<tr>
<td>Exposure time (sec)</td>
<td>5</td>
</tr>
<tr>
<td>Oscillation angle (degree)</td>
<td>1</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>1.000</td>
</tr>
<tr>
<td>Temperature (K)</td>
<td>100</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>50 – 2.2 (2.24 – 2.2)</td>
</tr>
<tr>
<td>No. of unique reflections</td>
<td>48,719</td>
</tr>
<tr>
<td>Redundancy</td>
<td>2.9 (2.8)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>100 (99.8)</td>
</tr>
<tr>
<td>$R_{merge}$ †</td>
<td>0.085 (0.461)</td>
</tr>
<tr>
<td>$\langle I/\sigma(I) \rangle$</td>
<td>15.5 (2.8)</td>
</tr>
<tr>
<td>Space group</td>
<td>$R3$</td>
</tr>
<tr>
<td>Unit-cell parameters</td>
<td></td>
</tr>
<tr>
<td>$a = b$, $c$ (Å)</td>
<td>142.52, 126.79</td>
</tr>
<tr>
<td>$\alpha = \beta$, $\gamma$ (°)</td>
<td>90, 120</td>
</tr>
</tbody>
</table>

$R_{merge} = \Sigma_{hkl} \Sigma_{i} | I(hkl) - \langle I(hkl) \rangle | / \Sigma_{hkl} \Sigma_{i} I_i(hkl)$, where $\langle I(hkl) \rangle$ is the mean intensity of multiple observations of symmetry-related reflections.
Fig. 1. SDS–PAGE of purified rHA-33. The sizes of molecular weight markers, in kDa, are marked on the left. Lane 1, molecular weight standard; lane 2, purified rHA-33.
Fig. 2. Two types of rHA-33 crystals grown using the hanging-drop vapor-diffusion method. The average dimensions of the crystals were 0.05 x 0.05 x 0.1 mm (a) and 0.01 x 0.01 x 0.2 mm (b). The later crystals were used to obtain X-ray diffraction data. Scale bar: 0.1 mm.
Fig. 3. An X-ray diffraction image from a rHA-33 crystal. The edge of the detector corresponds to a resolution of 2.2 Å.
Summary

In this chapter the author performed crystallization and X-ray analysis of HA-33/C-Yoichi that recognizes galactose unlike major serotype C strains. The C-Yoichi ha-33 gene (AB061780) sequence (857 bp) was amplified by PCR and was cloned into Escherichia coli TOP 10. The gene was transformed into E. coli BL21 Star (DE-3) cells, and E. coli culture supernatant was applied for column chromatography for the production of recombinant HA-33 (rHA-33). Crystallization of rHA-33 was attempted under 286 patterns with various conditions including temperature, protein concentration, precipitation drug and buffer in reservoir solution by hanging drop method. When incubated in drops comprised of 3.0 µl protein solution and 2.0 µl crystallization reservoir solution consisting of 8% (w/v) polyethylene glycol 8000 in 0.1 M Tris buffer pH 8.5, crystals appeared within 3 d of incubation and grew to maximum dimensions of 0.01 x 0.01 x 0.2 mm. Thus the author succeeded in producing a crystal of C-Yoichi HA-33, which is available for X-ray analysis, for the first time. X-ray analysis of the crystal with beamline AR-NW12A fitted with an ADSC Quantum 210r CCD detector (Photon Factory, Tsukuba, Japan) demonstrated that a resolution was 2.2 Å, and the crystal belonged to space group R3 with Unit-cell parameters of a = b = 142.52 Å and c = 126.79 Å.
Chapter III

Determination of 3D structure and prediction of sugar-binding sites of HA-33 from *Clostridium botulinum* serotype C strain Yoichi
Chapter III-1

The solution structures of HA-33 from

*Clostridium botulinum* serotype D strain 4947
1. Introduction

Botulinum toxin complex (TC), which is produced by the anaerobic gram-positive bacterium *Clostridium botulinum*, is the causative agent of food-borne botulism. The TC is composed of the botulinum neurotoxin (BoNT; 150 kDa) and auxiliary nontoxic proteins. *C. botulinum* is classified into seven serotypes, A–G, based on the antigenicity of the BoNT. Human botulism is caused predominantly by serotypes A, B, E and F while animal and avian botulism is due to the serotypes C and D (Montecucco and Schiavo 1994; Li and Singh 1999). Orally ingested TC passes through the digestive tract and enters the blood stream via the intestinal wall. Once in the blood stream BoNT dissociates from the TC then reaches and penetrates into the nerve cells of the neuromuscular junction, where it cleaves a specific site on the protein involved in neurotransmitter release resulting in paralysis of the muscle. The auxiliary nontoxic proteins of the TC include nontoxic nonhemagglutinin (NTNHA; 130 kDa) and three types of hemagglutinin (HA) components with molecular masses of 70, 33 and 17 kDa (HA-70, HA-33 and HA-17, respectively). The TC assembly includes association of a single BoNT and a single NTNHA to form M-TC, which is amazingly stable in the acidic and proteolytic conditions of the digestive tract (Miyata *et al.* 2009). Further binding of three HA-70 proteins onto the M-TC yields M-TC/HA-70. Finally, attachment of three HA-33/HA-17 trimers, each of which are comprised of a single HA-17 and two HA-33 proteins, to M-TC/HA-70 via binding of HA-70 and HA-17 results in forming the 14-mer mature L-TC. In addition, there are intermediate L-TC species having fewer HA-33/HA-17 trimers in the culture supernatant of *C. botulinum* (Mutoh *et al.* 2003).

Generally, the physical barrier presented by the intestinal wall prevents the
entrance of macromolecules such as proteins into the body. All the TCs, however, produced by D-4947, including pure BoNT, M-TC, M-TC/HA-70 and L-TC, can be transported across a rat intestinal epithelial cell (IEC-6) monolayer (Ito et al. 2011). Additionally, transport of the toxins is enhanced depending on the number of HA-33/HA-17 trimers. Similar to the TC species, the HA-33/HA-17 trimer isolated from the L-TC/D-4947 can also be transported across the IEC-6 cell layer (Niwa et al. 2010). Therefore, the HA-33/HA-17 trimer plays an important role in the effective transport of toxin across the intestinal wall.

Hasegawa et al. (2007) demonstrated the crystal structure of D-4947 HA-33/HA-17 trimer (HA-33/HA-17 trimer/D-4947). Although the analysis of crystal structure of protein provides information as to a spatial arrangement of amino acids of each domain, the structure in crystal may not be regarded as native appearance because protein complex functions in a solution in biological conditions. In fact, the interaction of HA-33 and carbohydrate on cell surface occurs in the intestinal fluid. Thus, analysis of crystal structure and solution structure of HA-33 may provide important information as to the difference of sugar recognition between reference strain and C-Yoichi.

In the Chapter III, 3D structure of HA-33/C-Yoichi was analyzed to clarify the molecular basis of galactose-recognition. In the first section the author attempted to clarify and compare the crystal and solution structures of HA-33. At first, the solution structure of reference strain HA-33/D-4947 was determined using small-angle X-ray scattering (SAXS) analysis, and was compared with crystal structure that has already been established. Furthermore, the solution structure of the HA-33/HA-17 trimer was investigated in the absence and presence of the sugars N-acetylneuraminic acid, glucose and galactose to know HA-33/HA-17 trimer shows any conformational change by
binding to sugars.

2. Material and methods

2.1. Production and purification of botulinum TC

*C. botulinum* D-4947 was cultured using a dialysis method as described previously (Miyata *et al.* 2012). The TC in the culture supernatant was precipitated with 60% saturation of ammonium sulfate. The resultant precipitate was dissolved and dialyzed against 50 mM acetate buffer (pH 4.0) with 0.2 M NaCl and applied to a TOYOPEARL SP-650S (Tosoh) cation-exchange column (1.6 x 40 cm) equilibrated with dialysis buffer. Bound protein was eluted with a linear gradient of NaCl (0.2–0.8 M). The peak fraction containing the L-TC, judged by SDS- and native-PAGE, was collected, concentrated and further purified with a HiLoad 16/60 Superdex 200 pg (GE Healthcare) equilibrated with 50 mM acetate buffer (pH 5.0) with 0.15 M NaCl. The peak fraction containing the L-TC was precipitated with 80% saturation of ammonium sulfate.

2.2. Isolation of the HA-33/HA-17 trimer from the L-TC

Isolation of the HA-33/HA-17 trimer from the L-TC was performed as previously reported (Kouguchi *et al.* 2002). The concentrated L-TC, in a 250 mg precipitate pellet, 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. Treated sample was applied to a HiLoad Superdex 200 pg 16/60 gel-filtration column equilibrated with the Gdn buffer. The fraction containing HA-33/HA-17 trimer was collected, diluted to 0.1 absorbance at 280 nm with Gdn buffer, and then dialyzed against 20 mM Tris-HCl (pH
7.8) at 4°C for 15 h to remove the guanidine hydrochloride.

2.3. PAGE analysis

SDS–PAGE was performed as described by Laemmli (Laemmli 1970) using a 13.6% polyacrylamide gel in the presence of 2-mercaptoethanol. Native PAGE was carried out using the method of Davis (1964) at pH 8.8 using a 5-12.5% polyacrylamide linear gradient gel. The separated peptides were stained with Coomassie Brilliant Blue R-250.

2.4. Small-angle X-ray scattering analysis

Small-angle X-ray scattering (SAXS) measurements of the HA-33/HA-17 trimer in 20 mM Tris–HCl (pH 7.8) were performed on a Rigaku BioSAXS-1000 using 10 - 20µl of protein solution. A total of eight datasets were collected after 120 min exposure (15 min per data set). Raw data were analyzed using the SAXSLab software package (Rigaku, Tokyo, Japan). SAXS curves were generated after subtracting the scattering due to the solvent in the protein solution, using the program PRIMUS from the ATSAS package.

3. Results

HA-33/HA-17 trimer was purified from L-TC produced by C. botulinum D-4947 in the presence of guanidine hydrochloride. The purified HA-33/HA-17 trimer displayed two bands in SDS–PAGE with molecular masses of 33 and 17 kDa (Fig. 1B). The crystal structure of the D-4947 HA-33/HA-17 trimer was previously determined and revealed an isosceles triangle-like structure in which one end of each of two
ellipsoidal HA-33 molecules is attached to a sphere-shaped HA-17 molecule (Fig. 1C) (Hasegawa et al. 2007). After removal of the guanidine hydrochloride, the HA-33/HA-17 trimer was subjected to SAXS analysis and the dummy atom model (DAM) was generated (Fig. 2). The SAXS-derived DAM image also indicated that the HA-33/HA-17 trimer forms an isosceles triangle-like structure but kinked at the middle of the triangle. Further, the SAXS DAM image is slightly larger than that seen in the crystallographic image as shown in the right panel of Fig. 2. Therefore the junction between the single HA-17 and dual HA-33 molecules would be flexible allowing iterative hinge-like movement of the trimer from flat to a kinked triangle as shown in the middle panel of Fig. 2.

Fig. 3 shows the SAXS DAM images of the HA-33/HA-17 trimer in the presence of 1 mM of Neu5Ac, glucose and galactose. All the images in the presence of sugars displayed similar appearance, with a more elongated shape than that seen in the absence of the sugars. As shown in the rightmost panel of Fig. 3, a single HA-17 and two HA-33 molecules were superimposed into the SAXS DAM image manually, so that all molecules were fitted into the image. The second panel from the right of Fig. 3 contains the best-fit model of the HA-33/HA-17 trimer in the presence of the sugars. In the absence of the sugars, the dual HA-33 molecules are attached onto the single HA-17 molecule in juxtaposition (Fig.1C and 2). In contrast, the rearranged model in the presence of the sugars indicates that the two HA-33 molecules are not juxtaposed, instead they are attached to HA-17 at a drastically increased angle of ~120°.
4. Discussion

In a previous report, Sagane et al., (2012) revealed the crystallographic and SAXS images of the NTNHA protein, another nontoxic component of the botulinum TC. By comparing the images, a part of the SAXS image of the NTNHA displayed a more extended appearance than that seen in the crystallographic image. The discrepancy observed in the crystallographic and SAXS images implied a high flexibility of the C-terminal region of the NTNHA molecule. SAXS analysis therefore would be useful to clarify the structural dynamics of protein in solution. In this study we revealed a sugar-induced structural shift of the HA-33/HA-17 trimer in solution. Sugar binding proteins sometimes demonstrate a conformational change induced by the presence of sugars, such as D-Glucose/D-Galactose binding protein (Borrok et al. 2007) and lactose permease (Nie and Kaback 2010).

Previously, Nakamura et al. 2008 identified the two sugar-binding sites of the serotype C HA1 (our HA-33) designated as site I and II. Site I is involved in binding with the sugars Neu5Ac, N-acetylgalactosamine, and galactose, whereas site II binds only with galactose. Additionally, X-ray analysis of the HA-33/HA-17 trimer crystal indicated that there is a region with low electron density in the HA-17 molecule (Hasegawa et al. 2007), implying its structural flexibility. Therefore, binding of the sugars to these sites may induce a structural shift. However, the glucose-binding site in the HA-33 molecule has not been identified. Another possibility to explain the difference observed in the trimer structure in the presence and absence of sugars, is that one of the HA-33 molecules could transfer to an alternate binding site on HA-17 in the presence of sugars. RADAR (http://www.ebi.ac.uk/Tools/pfa/radar) (Heger and Holm 2000), a program used to detect repeats in protein sequences, revealed two pairs of
repeat-sequences, DYG-FIYLSSSNNSLWNPI (72–90) and DYAWTIYDNNNITDQPI (109–126), and NGNYKIKSLFSDSLYLTY (10–27) and NGFRFSNV AEPNKYLAY (54–70), in the HA-17 molecule. The crystal structure of the HA-33/HA-17 trimer indicated that a set of Asp123, Pro125 and Leu127 residues in HA-17 is responsible for the interaction with one of the two HA-33 molecules, whereas the set of Asn106, Thr108, and Pro130 is involved in the binding to the other HA-33 molecule. Most of the residues in the first set are included in Asp109–Ile126, which is one-half of the first pair of internal repeat sequences. Therefore the site in the other half of the pair, Asp72–Ile90, of HA-17 would be a candidate for the binding site for the HA-33, which detached from the original position of the HA-17 molecule.

In this study, we found a sugar-induced structural shift of the HA-33/HA-17 trimer based on SAXS analysis. However, the physiological meaning of the structural shift remains unclear. Transport of botulinum TC through the intestinal epithelial cell layer is enhanced by the HA-33/HA-17 arm of the TC in a sialic-acid dependent manner (Ito et al. 2011). The structural shift of the HA-33/HA-17 arm of the TC may be induced by the binding of HA-33 to the sugar chain on the epithelial cell surface. This could result in enhancement of penetration by the TC across the cell layer. On the other hand, in the food-poisoning process the toxins transit the digestive tract with dietary macromolecules including polysaccharides. During transit, the polysaccharides would be degraded into monosaccharides, and such monosaccharides may affect transport of the botulinum toxin across the intestinal wall via the sugar-induced structural shift of the HA-33/HA-17 arms on the TC. In future studies, the effect of sugars on the transport of the TC across the intestinal epithelial cell layer should be examined to clarify the physiological meaning of the structural shift of the HA-33/HA-17 trimer.
Fig. 1. Assembly pathway of the botulinum toxin complex (TC) and characterization of the HA-33/HA-17 trimer used in this study. A. Assembly pathway of the botulinum TC. Binding of BoNT and NTNHA yields M-TC. Attachment of three HA-70 components to the M-TC forms M-TC/HA-70, finally three HA-33/HA-17 trimers consisting of a single HA-17 and two HA-33 proteins are conjugated to the M-TC/HA-70, resulting in the mature L-TC. B. SDS-PAGE banding pattern of purified HA-33/HA-17 trimer. After purification from L-TC, HA-33/HA-17 trimer was analyzed by SDS-PAGE using a 15 % polyacrylamide gel. The proteins in the gel were stained with Coomassie Brilliant Blue. The molecular masses of the protein standards (lane 1) are labeled on the left in kDa. The HA-33/HA-17 complex (lane 2) displayed two bands with molecular masses of 33 and 17 kDa. C. Surface representation of the D-4947 HA-33/HA-17 trimer revealed in a previous study (Hasegawa et al. 2007). The HA-33 and HA-17 molecules are indicated in blue and light blue, respectively. The left image is rotated 90˚ clockwise around the y-axis compared to the right image.
Fig. 2. Solution structure of HA-33/HA-17 trimer revealed by SAXS analysis.
Solution structure of the HA-33/HA-17 trimer is represented by the dummy atom model (DAM) (left). A model of the solution structure of the HA-33/HA-17 trimer (center) is illustrated based on the superimposition of the SAXS image and surface representation image of the crystal (right). Dotted line and arrow indicate the original position of the dual HA-33 molecules revealed by the crystallographic image (see Fig. 1B) and iterative movement of the molecules presumed by the SAXS DAM image, respectively. The HA-33 molecules in the trimer were displaced so that the crystal structure fits into the SAXS image.
Fig. 3. Solution structure of HA-33/HA17 trimer in the presence of various sugars.

SAXS images of HA-33/HA-17 trimer in the presence of Neu5Ac, glucose and galactose are represented in DAM. A model of the solution structure of the trimer in the presence of the sugars (second panel from right) is illustrated based on the superimposition of the SAXS image and surface representation image of the crystal (rightmost panels). The HA-33 molecules in the trimer were displaced so that the crystal structure fits into the SAXS image as shown in the rightmost panels.
Summary

In the Chapter III-1, the author determined the solution structure of HA-33/D-4947, which has been purified from culture supernatant of D-4947, and compared it with crystal structure. The HA-33/HA-17 trimer was subjected to SAXS analysis and the dummy atom model (DAM) was generated. The SAXS-derived DAM image indicated that the HA-33/HA-17 trimer forms an isosceles triangle-like structure but kinked at the middle of the triangle. Further, the SAXS DAM image is slightly larger than that seen in the crystallographic image. Therefore the junction between the single HA-17 and dual HA-33 molecules would be flexible allowing interactive hinge-like movement of the trimer from flat to a kinked triangle. In the absence of the sugars, the dual HA-33 molecules are attached onto the single HA-17 molecule in juxtaposition. In contrast, the rearranged model in the presence of the sugars indicates that the two HA-33 molecules are not juxtaposed, instead they are attached to HA-17 at a drastically increased angle of ~120°.
Chapter III-2

The crystal and solution structures of HA-33 from Clostridium botulinum serotype C strain Yoichi
1. Introduction

It has been shown that L-TC/C and D preferentially recognizes sialic acid on the cell surface via HA-33 molecule. On the other hand, unique HA-33 proteins produced by C-Yoichi preferentially recognizes galactose, rather than sialic acid. In the previous section, the three-dimensional structures for the sialic acid-binding HA-33 and D-4947 HA-33/HA-17 trimer, a reference strains, were determined. The results indicated that three-dimensional structure of HA-33/HA-17 trimer/D-4947 in solution was somewhat larger than that in crystal.

In this section, the author determined the three-dimensional structure of the galactose-binding HA-33 and HA-33/HA-17 trimer produced by the strain C-Yoichi. The results indicate the drastic conformational divergence between the sialic acid and galactose-binding HA-33/HA-17 trimers.

2. Material and methods

2.1. Production and purification of botulinum L-TC and isolation of HA-33/HA-17 trimer

*C. botulinum* C-Yoichi was cultured using a dialysis method reported previously (Miyata *et al.* 2012). The L-TC in the culture supernatants was precipitate with 60% saturation of ammonium sulfate. The precipitate was dialyzed against 50 mM acetate buffer (pH 7.4) with 0.2 M NaCl and applied to a TOYOPEARL SP-650S (Tosoh) equilibrated with dialysis buffer. Bound protein was eluted with a liner gradient of NaCl (0.2 - 0.8 M). The elution fraction containing the L-TC, judged by SDS- and Native PAGE, was collected, concentrated with 80% saturation of ammonium sulfate and further purified with a HiLoad 16/60 Superdex 200 pg (GE Healthcare) equilibrated
with 50 mM acetate buffer (pH 5.0) containing 0.15 M NaCl, The elution fraction containing the L-TC was precipitated with 80% saturation of ammonium sulfate.

Isolation of the HA-33/HA-17 trimer from the L-TC was performed as previously reported (Matsuo et al. 2011). The purified L-TC (250 mg) was incubated in 20 mM Tris–HCl (pH 7.8) with 4.0 M guanidine hydrochloride (Gdn buffer) at 21°C for 4 h. Treated sample was applied to a HiLoad Superdex 200 pg 16/60 gel-filtration column equilibrated with the Gdn buffer. The fraction containing HA-33/HA-17 trimer was collected, diluted to 0.05 absorbance at 280 nm with Gdn buffer and then dialyzed against 20 mM Tris–HCl (pH 7.8) at 4°C and then applied to a Mono S HR 5/5 (GE Healthcare) with elution by a liner gradient of NaCl (0 - 0.5 M).

2.2. Production and purification of recombinant HA-33

Production and purification of the recombinant HA-33 (rHA-33: C-Yoichi) were performed using a method reported previously (Hayashi et al. 2014). E. coli BL21 cells harboring a pET200/D-TOPO-ha33 vector, in which the ha-33 gene is ligated downstream of the 6x His-tag coding sequence, were cultured in 10 ml of LB broth containing 100 µg/ml kanamycin at 37°C overnight with mild shaking. This culture was inoculated into 400 ml LB broth and incubated at 37°C until mid-log phase was attained (the OD 600 was about 0.5). IPTG was added to a final concentration of 0.1 mM and induction proceeded at 37°C for a further 18 h. Harvested cells were suspended in 40 ml 50 mM phosphate buffer (pH 7.4) with 0.3 M NaCl, sonicated and centrifuged at 10,000g for 20 min at 4°C . The supernatant was mixed with 5 ml of 50% (w/v) Ni-charged Resin (Bio-Rad) suspension equilibrated with the same buffer and the mixture was poured into a glass column. After washing, the bound protein was eluted in
equilibration buffer with 300 mM imidazole. rHA-33 was precipitated by the addition of ammonium sulfate at 80% saturation. The precipitate was dialyzed against 50 mM phosphate buffer pH 6.0 with 0.15 M NaCl and finally subjected to gel filtration on a Superdex 200 HR 16/60 column (GE Healthcare) equilibrated with the same buffer. The purity of the eluted proteins was judged by SDS–PAGE.

2.3. Structural determination and crystallographic refinement of rHA-33

Crystals of the C-Yoichi HA-33 were obtained using the hanging drop vapor diffusion method as described previously (Hayashi et al. 2014). X-ray diffraction data of the C-Yoichi HA-33 was integrated and scaled using MOSFLM (Leslie and Powell 2007) and SCALA (Evans 2006), respectively, in the CCP4 suite (Winn et al. 2011). The initial structure of C-Yoichi HA-33 was solved by molecular replacement using the program MOLREP (Vagin and Tepljakov 1997) in the CCP4 suite using the coordinates of D-4947 HA-33/HA-17 trimer (PDB code:2E4M) as a target model. Refinements for structures were carried out first with the program CNS (Brunger et al. 1998), with the program REFMAC5 (Murshudov et al. 1997) in the CCP4 suite. Figures were prepared with the Pymol.

2.4. Small-angle X-ray scattering analysis

Small-angle X-ray scattering (SAXS) measurements of the HA-33/HA-17 trimer (10 mg/ml) in 20 mM Tris–HCl (pH 7.8) were performed on a Rigaku BioSAXS-1000 using 20 µl of protein solution. A total of eight datasets were collected after 120 min exposure (15 min per data set). Raw data were analyzed using the SAXS Lab software package (Rigaku, Tokyo, Japan). SAXS curves were generated after subtracting the
scattering due to the solvent in the protein solution, using the program PRIMUS from the ATSAS package.

3. Results and discussion

The crystal structures of HA-33/C and D preferentially binding to the sialic acid have been published in the HA-33 protein from the strain C-St (Inoue et al. 2003) and HA-33/HA-17 trimer/D-4947 (Hasegawa et al. 2007). In this section the author determined the structure in crystal and solution of the HA-33/C-Yoichi to compare the three-dimensional structure of the sialic acid- and galactose-binding HA-33/C and D.

The HA-33/C-Yoichi was produced as a recombinant protein in the E. coli, and then the crystal was obtained using the hanging drop vapor diffusion method as described in the chapter I. Diffraction data obtained in this report was employed from the determination of X-ray crystal structure of the rHA-33 at 2.2 angstrom resolution by molecular replacement using the HA-33 structure in HA-33/HA-17 trimer/D-4947 (Hasegawa et al. 2007). The final model was refined to an R-factor of 19.6 % and a free R-factor of 22.4 %. The final model (center panel of Fig. 1) includes two protein molecules in the asymmetric unit, as was also true for the crystals of C-St HA-33 (left panel of Fig. 1). As shown in right panel of the Fig. 1, the crystal structure shows that the C-terminal half domain (HA-33_v region) of the C-Yoichi protein warps to outside slightly than that of C-St protein, but drastic structural change in the crystals due to the amino acid replacements was not observed.

In the previous section, the author revealed the conformational flexibility of the HA-33/HA-17 trimer in the solution based on SAXS analyses. Thus the junction between the HA-33 and HA-17 molecules would be flexible allowing the hinge-like
movement of the trimer. A similar conformational flexibility has also been demonstrated in botulinum NTNHA protein (Sagane et al. 2012). Here, the author clarified the solution structure of HA-33/HA-17 trimer/C-Yoichi by SAXS analysis as shown in Fig. 2. The SAXS image of HA-33/HA-17 trimer/C-Yoichi (upper panels in Fig. 2) displayed more elongated shape than that seen in the D-4947 trimer (lower panels in Fig. 2). In the Fig. 2, the crystal structures of the two HA-33 molecules and a single HA-17 molecule were superposed into the SAXS image from the C-Yoichi and D-4947 molecules, so that all molecules were fitted into the image. In the D-4947 molecule, both of two HA-33 molecules are attached onto the single HA-17 molecule in juxtaposition. In the C-Yoichi trimer, the HA-33 molecules are not juxtaposed on the HA-17.

In conclusion, the present study revealed that the solution structure of C-Yoichi HA-33/HA-17 trimer is extremely different from that of major C and D trimer. A schematic model for comparison of 3D structure of HA-33/C-Yoichi and HA-33/C and D in crystal and solution is shown in Fig. 3.
Fig. 1. Crystal Structure of HA-33/C-St and C-Yoichi. The final model (center panel) includes two protein molecules in the asymmetric unit, as was also true of crystals of C-St HA-33 (left panel). As shown in right panel, the crystal structure shows that the C-terminal half domain (HA-33<sub>3V</sub> region) of the C-Yoichi protein warps to outside slightly than that of C-St protein, but drastic structural change in the crystals due to the amino acid replacements was not observed.
Fig. 2. Superimposed of the crystal and solution structure of HA-33/HA-17 trimer/C-Yoichi and D-4947. The SAXS image of HA-33/HA-17 trimer/C-Yoichi (upper panels) displayed more elongated shape than that seen in the D-4947 trimer (lower panels). The crystal structures of the two HA-33 molecules and a single HA-17 molecule were superposed into the SAXS image from the C-Yoichi and D-4947 molecules, so that all molecules were fitted into the image.
Fig. 3. A schematic model for comparison of 3D structure of HA-33/C-Yoichi and HA-33/C and D in crystal and solution
Summary

In this section, the author determined 3D structure of the galactose-binding HA-33 and HA-33/HA-17 trimer produced by the strain C-Yoichi in crystal and solution to compare it with sialic acid-binding HA-33 from major serotype C and D strains. X-ray analysis with the crystal obtained in the Chapter I showed that C-Yoichi HA-33 includes two protein molecules in the asymmetric unit; the C-terminal half domain the C-Yoichi protein warps to outside slightly than that of C-St protein, but drastic structural change in the crystals was not observed. When the solution structure was determined with SAXS, HA-33/HA-17 trimer/C-Yoichi displayed more elongated shape than that seen in the D-4947 trimer; In the D-4947 molecule, both of two HA-33 molecules are attached onto the single HA-17 molecule in juxtaposition whereas the HA-33 molecules are not juxtaposed on the HA-17 in C-Yoichi trimer. Thus the present study clearly revealed that the solution structure of HA-33/HA-17 trimer/C-Yoichi is extremely different from that of major C and D trimer.
Chapter III-3

Prediction of sugar-binding sites of HA-33 from

*Clostridium botulinum* serotype C strain Yoichi
1. Introduction

In the Chapter III-2, the author compared the crystal and solution structures of HA-33/HA-17 trimer/C-Yoichi and C-St or D-4947, a reference strain. The results showed that the angle of two HA-33 molecules in HA-33/HA-17 trimer/C-Yoichi was larger than that of reference strains and the difference of the angle was obvious in solution than in crystal. It has been shown that, HA-33/A and B preferentially recognizes galactose, whereas type C and D predominantly bind to sialic acid. However, HA-33/C-Yoichi dominantly recognizes galactose moiety like HA-33/A and B. These facts raise the possibility that the structure of galactose-recognition region of HA-33/C-Yoichi is analogous to that of HA-33/A and B. Additionally, another possibility may be considered that HA-33/C-Yoichi has a particular structure for galactose-recognition region distinct from HA-33/A and B.

In this section the author attempted to determine the specific site responsible for galactose-recognition of HA-33/C-Yoichi by comparing three-dimensional structures of HA-33 with *in silico* study.

2. Material and methods

2.1 The phylogenetic tree of C-Yoichi and the other type HA-33

Amino acid sequences of the HA-33 proteins from serotype A, B, C, D and G strains were gathered from the NCBI protein database and aligned (ClustalW). To consider a homology of C-Yoichi and the other type HA-33, the phylogenetic tree was made by UPGMA based on amino-acid sequence.
2.2 Comparison of the 3D structure of HA-33

3D structure of HA-33 molecules were prepared and compared by using PyMOL (DeLano Scientific).

2.3 Sugar recognition site prediction of C-Yoichi HA-33

Prediction of sugar-recognition site of C-Yoichi HA-33 was performed with a eF-seek (http://ef-site.hgc.jp/eF-seek/top.do).

3. Results and discussion

At first the author attempted to classify the HA-33 based on the molecular evolution and to determine the motif in HA-33/C-Yoichi that is responsible for the sugar-recognition. Fig.1 shows a phylogenetic tree of HA-33 created by applying the amino acid sequence of HA-33 to an unweighted pair group method with arithmetic mean. HA-33 was divided into three groups: serotypes A and B (A/B), serotypes C and D (C/D), and serotype G. As mentioned in former chapters, serotypes A/B and serotypes C/D have been shown to recognize galactose and sialic acid, respectively. Thus it is considered that sugar-recognizing property of HA-33 molecule is associated with its evolution. On the other hand, HA-33/C-Yoichi was involved in not serotypes A/B group but serotypes C/D group in this classification although it recognizes galactose similarly to serotypes A/B. Thus HA-33/C-Yoichi recognizes galactose regardless of low homology to serotypes A/B, suggesting that the galactose-recognizing motif in HA-33/C-Yoichi is distinct from that in HA-33 of serotypes A/B.

Fig. 2 shows schematic models for the three dimensional structure of HA-33/ serotype A strain 62A (A-62A) and C-St. It has been shown that HA-33/A-62A has a
sugar recognition motif, Site A, in its $\beta$-trefoil domain in which Asp263, Gly266, Gln276, Phe278, His281 and Asn285 contribute to interact with galactose. Although HA-33/C-Yoichi recognizes galactose similarly to HA-33/A-62A, it lacks the region corresponding to Site A, suggesting again that galactose-recognizing characteristics of HA-33/C-Yoichi is distinct from that of HA-33/A-62A. It has also been shown that HA-33/C-St has two motifs (Site Ic and IIc) for sugar recognition in its $\beta$-trefoil domain of C-terminal region. Site Ic involving Asn167, Leu168, Trp176, Phe179 and Arg183 predominantly recognizes sialic acid, and W176 is responsible for the holding of an aromatic ring of sugar by stacking interaction. Site IIc involving Asp256D, Asn259, Asp271D, His274, Asn278 and Gln279 recognizes only sialic acid. In HA-33/C-Yoichi, the region corresponding to Site IIc is lacked but that to Site Ic existed; In the region corresponding to Site Ic, three amino acids were replaced but Trp176, that is prerequisite to hold aromatic ring in sugar, was preserved as Trp175. Thus the region corresponding to Site Ic might contribute to recognize galactose in HA-33/C-Yoichi (Fig.3). To obtain further insight, a sugar recognition site was predicted by eF-Seek, a database for ligand binding sites. When the sequence of amino acids of HA-33/C-Yoichi was applied to eF-Seek with a hemolysin of *Vibrio vulnificus* as a template motif, a galactose-recognition site was predicted to exist in $\beta$-trefoil domain of C-terminal region (Fig. 4). This region corresponded to Site Ic of HA-33/C-St, and eight amino acid residues (Asp164, Lys165, Asn166, Gln167, Trp175, Trp178 and Gln183) were predicted to contribute to bind galactose. As mentioned above, Trp175 is thought to hold aromatic ring in sugar.

Collectively, it was presumed that galactose-recognizing mechanism of C-Yoichi HA-33 is different from that of HA-33/A and that a variation of amino acids in the
region corresponding to site Ic contributes to unique characteristics of sugar recognition in HA-33/C-Yoichi.
Fig. 1. Molecular dendrogram of C-Yoichi and the other type HA-33. To consider a homology of C-Yoichi and the other type HA-33, the phylogenetic tree was made by UPGMA based on amino-acid sequence.
Fig. 2. Crystal structure of HA-33/A and C and close up views of the sugar-binding sites of HA-33. Structure of HA-33/A-62A (upper panel) and C-St (lower panel) as represented by a ribbon diagram. Close up view of site 1c bound sialic acid. Sugar-binding residues is represented using a stick model. close up view of site IIc and Site A bound galctose.
Fig. 3. Superimposed illustrations of the area near the sugar binding sites of HA-33/ C-St and C-Yoichi. Close-up view of the $\beta$-trefoil domain for C-terminal of HA-33/C-St superimposed on the same region of HA-33/C-Yoichi with a Galactose molecule model. The putative sugar binding residues as observed in type C HA-33/C-St (green) and their counterparts in HA-33/C-Yoichi (blue). Residue labels are indicated for HA-33/C-Yoichi; those from HA-33/C-St structure are in parentheses.
Fig. 4. Superimposed illustrations of the area near the expected sugar binding sites of HA-33/C-Yoichi and *Vibrio vulnificus* Hemolysin/Cytolysi. Close-up view of the β-trefoil domain for C-terminal of HA-33/C-Yoichi superimposed on the same region of *ibrio vulnificus* Hemolysin/Cytolysin with a Galctose molecule model. The putative sugar binding residues as observed in HA-33/C-Yoichi (blue) and their counterparts in *ibrio vulnificus* Hemolysin/Cytolysin (purple). Residue labels are indicated for HA-33/C-Yoichi; those from the *ibrio vulnificus* Hemolysin/Cytolysin structure are in parent.
Summary

In this section the specific site responsible for the carbohydrate recognition of HA-33/C-Yoichi was determined based on the arrangement of amino acids in 3D structure. Comparison of 3D structure of HA-33/C-Yoichi, serotype A strain 62A (A-62A) and C-St demonstrated that HA-33/C-Yoichi lacks the region corresponding to Site A that recognizes galactose in HA-33/A-62A. In β-trefoil domain of C-terminal region, HA-33/C-Yoichi has a region corresponding to Site Ic that recognizes galactose in C-St; in the region corresponding to Site Ic, three amino acids were replaced but 176W, that is prerequisite to hold aromatic ring in sugar, was preserved as W175. When the sequence of amino acids of HA-33/C-Yoichi was applied to eF-Seek, a database for ligand-binding site, a galactose-recognition site was predicted to exist in β-trefoil domain of C-terminal region corresponded to Site Ic of HA-33/C-St. These results suggest that galactose-recognizing mechanism of HA-33/C-Yoichi is different from that of HA-33/A and that a variation of amino acids in the region corresponding to site Ic contributes to unique characteristics of sugar recognition in HA-33/C-Yoichi.
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ACKNOWLEDGEMENTS

The author wishes to express many sincere thanks to Professor Koichi Niwa, Tokyo University of Agriculture, for experiment with cultured cells, his kind advice, valuable discussion and continuous warm encouragement during the course this study.

The author wishes to express many sincere thanks to Professor Toshihiro Watanabe, Tokyo University of Agriculture, for the kind guidance and valuable advice during the course of this study.

The author wishes to express many thanks to Professor Yoshimasa Sagane, Tokyo University of Agriculture, for his kind advice, valuable discussion and continuous warm encouragement during the course of this study.

The author wishes to express many thanks to Professor Junichi Nakagawa, Tokyo University of Agriculture, for his kind advice, valuable discussion and continuous warm encouragement during the course of this study.

The author wishes to express many thanks to Professor Yoichi Niimura, Tokyo University of Agriculture, for his kind advice, valuable discussion and continuous warm encouragement during the course of this study.

The author wishes to express many sincere thanks to Professor Tohru Ohyama, Tokyo University of Agriculture, for his guidance and valuable advice during the course this study.

The author wishes to express many thanks to Mr. Syunsuke Yajima for Structural determination.

The author is indebted to Dr. Shingo Mutoh, Keita Miyata, Ken Inui and Shin-Ichiroya Miyashita for their friendliness and cooperativeness during this study.

The author wishes to express many thanks to Mr. Tomonori Akiyama for Structural
The author wishes to express many thanks to Mr. Satoshi Kano, Mr. Takehiro Kawane, Miss Sayuri Kurihara and Mr. Hotaka Atarashi for their friendliness, encouragement and cooperativeness during the course of this study.

Special thanks are due to Miss Kana Hirano, Mr. Takayuki Koimori, Mr. Ryota Asano, Mr. Takayuki Umezawa, Miss Momoe Tomizawa, Miss Nagisa Umeda, Mr. Syun Takahashi, Mr. Takuya Naka, Miss Shinoka Kanda, Miss Yuki Kanagwa, Miss Saki Hasegawa, Miss Saki Ogawa, Miss Ikumi Shinji, Miss Miya Kim, Miss Takahashi Yui, Miss Ibuki Sawaki, Miss Misaki Nagato, and Mr. Takahide Kasuga for their friendliness and cooperativeness during this study.

Finally, I would like to acknowledge the affectionate support of my family.
ABSTRACT

*Clostridium botulinum* produces a botulinum neurotoxin (BoNT) that is the causative agent of human and animal botulism. After the absorption from intestine, BoNT enters nerve cells and cleaves specific sites on target proteins. This results in the inhibition of acetylcholine release and flaccid muscle paralysis. BoNT is serologically classified into seven serotypes A through G. Serotype A, B, E and F are mainly responsible for human botulism, whereas serotypes C and D causes in animal and avian botulism. In culture supernatant and naturally contaminated foods, BoNT molecule is associated with auxiliary proteins including nontoxic nonhemagglutinin (NTNHA) and hemagglutinin (HA) subcomponents having molecular mass of 70, 33 and 17 kDa (HA-70, HA-33 and HA-17), yielding botulinum toxin complex (TC). Although the 3D structure of L-TC has long been obscure, a recent study by Hasegawa *et al.* clarified that L-TC has an ellipsoidal-shaped structure with three extended “arms” of the HA-33/HAlA-17 (2:1) trimer.

HA-33/HAlA-17 trimer is thought to contribute to the effective transport of the toxin across the intestinal epithelia. It has been shown that HA-33 binds to intestinal cells via sugar chains on the cell surface, and that HA-33 of serotype A and B (HA-33/A and /B) predominantly recognizes galactose whereas HA-33 of serotype C and D (HA-33/C and /D) preferentially binds to sialic acid moiety but not galactose moiety on the cell surface.

Sagane *et al.* (2001) demonstrated that HA-33 of serotype C strain Yoichi (C-Yoichi), showing poor hemagglutination activity, has a deletion of 31 amino acid residues from the C-terminus and that the HA-33/C-Yoichi showed only 46% amino acid identity in the C-terminal half region compared with a reference serotype C strain.
A recent study reported that L-TC/C-Yoichi showed full cell-binding activity when the cells were pretreated with neuraminidase, and the binding was completely inhibited in the presence of galactose or lactose (Matsuo et al., 2011). Thus HA-33/C-Yoichi shows unique sugar-recognizing property that binds to the galactose like HA-33/A and /B, whereas other serotype C and D HA-33 preferentially binds to sialic acid.

Although it is likely that the unusual recognition of the galactose moiety of HA-33/C-Yoichi could be due to mutations and/or a truncation of the protein, the mechanism by which this protein recognizes galactose unlike other serotype C strains is still unknown. In this study, the author focused on the structure of HA-33/C-Yoichi and investigated its unique sugar recognition mechanism. In this thesis, the author compared the binding of HA-33/C-Yoichi and HA-33 from serotype C strain Stockholm (C-St) that recognizes sialic acid, to several types of epithelial cells to confirm the functional difference (Chapter I). Further, the author attempted crystallization of HA-33/C-Yoichi to perform X-ray analysis (Chapter II). Finally, the author determined the three-dimensional (3D) structures of HA-33/C-Yoichi and HA-33 from other C and D strains in a crystal and solution, and made prediction for the specific site responsible for the sugar recognition of HA-33/C-Yoichi based on 3D structure and in silico analysis (Chapter III).

Chapter I. Host-cell and sugar-binding specificity of the HA-33 from Clostridium botulinum serotype C strain Yoichi

In this chapter the author investigated the binding of HA-33/HA-17 trimer to epithelial cells to clarify the function of HA-33/C-Yoichi in intoxication, using rat intestinal cell line (IEC-6), bovine aortic endothelial cells (BAEC) and human colon
carcinoma (Caco-2). The binding of C-Yoichi HA-33/HA-17 trimer to cells was smaller or larger than that of C-St in IEC-6 or Caco-2, respectively. HA-33/HA-17 trimer of both strains bound to BAEC to the same extent, implying that HA-33 of two strains recognizes cells with different mechanisms. Pre-treatment of the cells by neuraminidase inhibited the binding of C-St trimer toward the cells, whereas dramatically increased cell binding of the C-Yoichi trimer. This finding suggests that the HA-33/C-Yoichi, similar to HA-33/A and /B, binds to the cells via sugar chain having galactose on its terminus, regardless of animal species and tissular origin of host-cell.

Chapter II. Crystallization and preliminary X-ray analysis of a HA-33 from

*Clostridium botulinum* serotype C strain Yoichi

In this chapter the author performed crystallization and X-ray analysis of HA-33/C-Yoichi that recognizes galactose unlike major serotype C strains. The C-Yoichi *ha*-33 gene was amplified by PCR and was cloned into *Escherichia coli* TOP 10 to produce the recombinant HA-33 (rHA-33). Crystallization of rHA-33 was attempted under 286 patterns of various conditions including temperature, protein concentration, precipitation drug and buffer in reservoir solution by the hanging drop method. When incubated under the condition of 5.0 mg/ml protein, 8% (w/v) polyethylene glycol 8000, 0.1 M Tris buffer of pH 8.5 at 4°C, crystals appeared within 3 d of incubation and grew to maximum dimensions of 0.01 × 0.01 × 0.2 mm. Thus the author succeeded in producing a crystal of C-Yoichi HA-33, which is available for X-ray analysis, for the first time. X-ray analysis of the crystal demonstrated that a resolution was 2.2 Å and that the crystal belonged to space group *R*3 with Unit-cell parameters of \(a = b = 142.52\) Å and \(c = 126.79\) Å.
Chapter III. Determination of 3D structure and prediction of the sugar-binding sites of HA-33 from Clostridium botulinum serotype C strain Yoichi

In this chapter the author determined the 3D structures of HA-33/C-Yoichi in the crystal and solution and compared with those of other HA-33/C and D. Crystallographic analysis revealed that HA-33/C-Yoichi is a V-shaped dimer and that each HA-33 molecule consists of N- and C-terminal β-trefoil domains that are connected by α-helix site. The angle made by upper C-terminal domains was slightly greater in HA-33/C-Yoichi than that in HA-33/C-St. The solution structures of HA-33/HA-17 trimers of C-Yoichi and D-4947 were determined with small-angled X-ray scattering (SAXS). When the structures of D-4947 HA-33/HA-17 trimer in crystal and solution were superimposed, the structure in solution roughly corresponded to that in crystal. On the other hand, the solution structure of C-Yoichi trimer was largely different from crystal structure. Thus solution structure of C-Yoichi HA-33/HA-17 trimer was not symmetrical, and the angle made by two HA-33 molecules was larger than that in crystallographic structure. The results clearly demonstrated that 3D structure of C-Yoichi HA-33/HA-17 trimer was different from those from majority of the serotype C and D in solution. It is likely that the unique sugar recognition of HA-33/C-Yoichi can be attributed to its distinct 3D structure.

Additionally, the specific site responsible for the sugar recognition of HA-33/C-Yoichi was predicted based on the 3D structure and in silico analysis. Considering the sugar-biding specificity, it was presumed that the sugar-binding site in the HA-33/C-Yoichi would be similar to that in the HA-33/A that recognizes galactose. In the HA-33/A, the galactose-binding site is located in the C-terminal region of the protein. Comparison of 3D structure of HA-33/C-Yoichi and /A demonstrated that
HA-33/C-Yoichi however lacks the region corresponding to the site that recognizes galactose in HA-33/A. Thus the galactose-recognizing characteristic of HA-33/C-Yoichi is distinct from that of HA-33/A. The HA-33/C protein that recognizes the sialic acid contains two sugar-binding sites Ic and IIc. On the other hand, in HA-33/C-Yoichi, the region corresponding to Site IIc is lacked but that to Site Ic existed. The Site Ic in the HA-33/C and /D that bind to the sialic acid is involved in the recognition of sialic-acid moiety. However, in the region corresponding to Site Ic of HA-33/C-Yoichi, even though the Trp176, that is prerequisite to hold aromatic ring in sugar, was preserved; three of five amino acid residues responsible for the sugar recognition were replaced. It was presumed that this mutation would bring the shift in sugar-recognition manner (sialic acid $\rightarrow$ galactose) to the HA-33 molecule. Sugar-binding site search using the ligand binding site database eF-seek indicated that a structure similar to galactose-recognition site in the *Vibrio vulnificus* hemolysin exists in the Site Ic region in the C-Yoichi HA-33. These results suggest that the galactose-recognizing manner of HA-33/C-Yoichi was evolved from sialic acid-recognizing HA-33 of the serotype C and D, not from galactose-recognizing HA-33 of the serotype A and B.

In this thesis, the author demonstrated that the C-Yoichi HA-33 recognizes galactose regardless of cell types, dissimilar to majority of HA-33/C and /D. The author succeeded in crystallization and X-ray analysis of C-Yoichi HA-33 protein. Further the author demonstrated that 3D structure of C-Yoichi HA-33/HA-17 trimer was different from that of other serotype C and D strains in solution. Furthermore, a galactose-recognition site was predicted to exist in the HA-33/C-Yoichi protein. The results in this thesis would contribute a knowledge showing the protein evolution
occurring in the relationship between toxic protein and the host animal. The results of this study indicate new insights in the pathogenesis of botulism in humans and livestock. It is believed to contribute significantly to the establishment of prevention and treatment of botulism.
ABSTRACT (in Japanese)

ボツリヌス菌（Clostridium botulinum）は、ヒトや動物のボツリヌス症の原因となるボツリヌス神経毒素（botulinum neurotoxin; BoNT）を産生する。腸管から吸収された BoNT は、神経細胞に侵入し、神経伝達物質の放出に関与するタンパク質を特異的に切断することで神経伝達を阻害し、筋肉の麻痺を引き起こす。BoNT は抗原性の違いにより A から G 型の 7 種に分類され、A、B、E および F 型は主にヒトの、C および D 型はウシや家禽のボツリヌス症の原因となる。BoNT は培養液や汚染食品中では単独で存在せず、非毒非血球凝集素（nontoxic nonhemagglutinin; NTNHA）や分子量 70、33 および 17 kDa の 3 種の血球凝集素（hemagglutinin; HA-70, HA-33 および HA-17）などの無毒タンパク質と会合した巨大な毒素複合体（large toxin complex; L-TC）を形成する。L-TC の詳細な構造は長い間不明であったが、近年、Hasegawa ら（2007）によって、立体構造が明らかにされ、L-TC が 1 分子の BoNT、1 分子の NTNHA、3 分子の HA-70、6 分子の HA-33 および 3 分子の HA-17 から構成される 14 量体であり、3 本の HA-33/HA-17 （2:1）複合体が「腕」のように L-TC の表面に突き出していることが明らかにされた。

HA-33/HA-17 複合体は、L-TC の腸管上皮からの効率的な吸収に寄与していると考えられている。すなわち、HA-33 が細胞表面の糖鎖を認識して小腸上皮細胞と結合し、腸管から体内への侵入を促進する。しかし、認識する糖の特異性は血清型によって異なり、A および B 型 HA-33 はガラクトースを認識するのに対し、C および D 型 HA-33 はシアル酸を認識する。

先に Sagane らは、C 型菌 Yoichi 株（C-Yoichi）の産生する HA-33 において、その C 末端側 31 アミノ酸残基が欠落していること、そしてその C 末端側 1/2 の領域の同様性が他の C 型 HA-33 と比較し、46%と顕著に低いことを示した。さら
に、Matsuoら（2011）は、C-Yoichi L-TCが、ノイラミニターゼ処理した細胞にのみ結合し、さらにその結合がガラクトースによって完全に阻害されることを明らかにした。すなわち、他の C および D 型 HA-33 がシアル酸を認識するのに 対し、C-Yoichi HA-33 は、A および B 型のようにガラクトースを認識する特殊な糖鎖認識特異性を持つことが示唆された。
C-Yoichi HA-33 の C 末端側の変異および欠落が糖鎖の認識特異性に何らかの影響を与えていていることが推測される。しかし、C-Yoichi HA-33 が、なぜ他の C およ び D 型 HA-33 と異なる糖鎖認識機構を示すのかについては、不明のままであっ た。本論文では C-Yoichi HA-33 の特異な糖認識機構を明らかにするため、ガ ラクトース認識型である C-Yoichi HA-33 とシアル酸認識型である C 型菌 Stockholm 株（C-St）由来 HA-33 の各種細胞への結合活性を比較し、それぞれの機能の差異を明らかにした（第 1 章）。次に、C-Yoichi HA-33 の立体構造の特異 性を明らかにするため、C-Yoichi HA-33 の結晶化および X 線回折を分析し（第 2 章）、さらに他の C および D 型由来のシアル酸認識型 HA-33 の立体構造との比 較を行った。また、立体構造およびコンピュータ解析をもとに C-Yoichi HA-33 分子内の糖認識部位を予測し、糖鎖への結合メカニズムを推測した（第 3 章）。

第 1 章 ボツリヌス C 型菌 Yoichi 株由来 HA-33 が結合する細胞種および糖鎖の特異性

本章では、細胞結合における C-Yoichi HA-33 の機能を明らかにするため、 HA-33/HA-17 複合体の各種細胞（ラット小腸上皮細胞 IEC-6、ウシ大動脈内皮細胞 BAEC およびヒト大腸癌由来細胞 Caco-2）への結合活性を比較した。その結果、IEC-6 では C-Yoichi HA-33 の方が、Caco-2 では C-St HA-33 の方が、より高 い結合活性を示した。また、BAEC では、結合量はいずれも同程度であった。こ
ことから、C-Yoichi および C-St 由来 HA-33 は、それぞれ異なる機構によって細胞に結合していることが示唆された。一方、ノイラミニターゼにより細胞表面のシアル酸を除去すると、細胞種に関係なく C-St HA-33 の結合量は減少し、C-Yoichi HA-33 の結合量は上昇した。以上の結果から、C-Yoichi HA-33 は、A および B 型 HA-33 と同様に、細胞の動物種あるいは組織種の違いにかかわらずガラクトースを認識していることが示唆された。

第 2 章 ボツリヌス C 型菌 Yoichi 株由来 HA-33 の結晶化および X 線分析

C-Yoichi HA-33 の糖鎖認識特性は、その構造に起因するものと推測される。したがって、本章では、HA-33 の立体構造解明するため、C-Yoichi HA-33 の結晶化および X 線回折分析を行った。C-Yoichi 株 ha-33 遺伝子を PCR 法によって増幅し、大腸菌 TOP10 株に導入し、組換え HA-33 （rHA-33）を得た。結晶化に適した条件を明らかにするため、温度、タンパク質濃度、沈殿剤および緩衝液の組み合わせをもとに 286 種類の条件で結晶化を試みた。その結果、ハンギングドロップ蒸気拡散法で、タンパク質濃度 5 mg/ml、温度 4℃、リザーバー溶液の組成が 8% (w/v) PEG 8,000、0.1 M Tris hydrochloride、pH 8.5 の条件で 0.01 × 0.01 × 0.2 mm の大きさの柱状結晶を得ることができた。これにより、著者は X 線解析に供することのできる C-Yoichi HA-33 の結晶を作成することに世界で初めて成功した。この結晶を、X 線回折実験に供したところ、2.2Å の分解能が得られ、C-Yoichi rHA-33 の結晶は、空間群 R3 に属し、格子定数は a = b = 142.52 Å、c = 126.79 Å であることを明らかにした。
第3章 ボツリヌスC型菌Yoichi株由来HA-33の立体構造および糖認識部位
予測

本章では、C-Yoichi HA-33の結晶構造および溶液構造を解析し、他のCおよびD型HA-33の構造と比較した。結晶構造解析の結果から、C-Yoichi HA-33はV字型の2量体として存在しており、それぞれのHA-33分子はN末端側とC末端側の2つのβ-trefoilドメインから構成されていた。2量体のHA-33分子のC末端側領域が形成する角度は、C-Yoichi HA-33の方がC-St HA-33より若干外側に広がっていたが、顕著な違いは認められなかった。さらに溶液中のHA-33/HA-17複合体の立体構造を比較するため、C-YoichiおよびD-4947 HA-33/HA-17複合体を小角X線散乱法(SAXS)により解析した。その結果、D-4947 HA-33/HA-17複合体(シアル酸結合型)の溶液構造は、結晶構造とその形状はほぼ一致していた。しかし、C-Yoichi HA-33/HA-17の溶液中の構造は結晶構造とは大きく異なっており、溶液中では、HA-17を中心として2分子のHA-33が左右に大きく開いていることが示唆された。以上の結果から、C-Yoichi HA-33/HA-17複合体の溶液中での立体構造は、他のCおよびD型HA-33/HA-17複合体とは大きく異なっていることが示された。

さらに、立体構造の比較およびコンピュータ解析からC-Yoichi HA-33の糖認識部位を予測した。糖鎖への結合特異性から、C-Yoichi HA-33の糖鎖認識部位は、ガラクトースを認識するA型HA-33と類似しているものと予測した。A型HA-33は、その分子内のC末端領域にガラクトース結合部位をもつ。しかし、C-YoichiおよびA型HA-33の立体構造を比較したところ、A型のガラクトース結合部位に相当する領域は、C-Yoichi HA-33タンパク質から欠落した領域に存在していた。したがって、予想とは異なり、C-Yoichi HA-33のガラクトース結合部位はA型HA-33のそれとは関連性が低いことが示された。一方、シアル酸を認識するC
型 HA-33 タンパク質には、2 つの糖鎖認識部位（Site Ic および IIc）が存在する。C-Yoichi HA-33 タンパク質では、Site IIc に相当する領域が欠落していたが、Site Ic に相当する部位は存在していた。シアル酸認識型の C および D 型 HA-33 分子内において Site Ic はシアル酸の認識に関与する。一方、C-Yoichi HA-33 分子中の Site Ic に相当する領域は、糖認識に関与すると予測される 5 アミノ酸残基のうち、糖の芳香環との相互作用に関与すると考えられる Trp173 は保存されていたものの、3 残基が変異していた。この変異が、C-Yoichi HA-33 に糖認識機構の変化（シアル酸→ガラクトース）をもたらしたものと推測された。さらに、リガンド結合部位データベース検索 eF-seek により糖認識部位を予測したところ、*Vibrio vulnificus* 由来 hemolysin 分子内のガラクトース結合部位に類似した構造が、C-Yoichi HA-33 分子内の Site Ic に存在することが示された。以上の結果から、C-Yoichi HA-33 のガラクトース認識部位は、ガラクトース認識型の A および B 型 HA-33 分子からではなく、シアル酸認識型の C および D 型 HA-33 から進化して形成されたものであることが示唆された。

**本論文において、著者は、他の C および D 型 HA-33 とは異なり、C-Yoichi HA-33 が細胞の違いにかかわらず、シアル酸ではなく、ガラクトースを認識することを示した。また、C-Yoichi HA-33 タンパク質の結晶化に初めて成功した。さらに、C-Yoichi HA-33/HA-17 複合体の溶液構造が、他の C および D 型由来の複合体とは大きく異なっていることを明らかにし、他の型の HA-33 分子や糖鎖結合タンパク質の立体構造との比較から、C-Yoichi HA-33 分子内のガラクトース認識部位を予測することに成功した。本研究の成果は、毒素タンパク質と宿主生物の間の相互作用によって進行するタンパク質の分子進化に関わる重要な知見である。また、ヒトや家畜のボツリヌス中毒の発症機構に新たな知見を加えるものである。**
る。現在でも家畜に対するポツリヌス中毒の発生は、世界中に散見されており、
本研究の成果は、その予防法や治療法の確立に大きく貢献するものと考えられ
る。