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Expression and stability of the nontoxic component of the botulinum toxin complex

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ABSTRACT

Clostridium botulinum produces botulinum neurotoxin (BoNT) as a large toxin complex associated with nontoxic-nonhemagglutinin (NTNHA) and/or hemagglutinin components. In the present study, high-level expression of full-length (1197 amino acids) rNTNHA from *C. botulinum* serotype D strain 4947 (D-4947) was achieved in an *Escherichia coli* system. Spontaneous nicking of the rNTNHA at a specific site was observed during long-term incubation in the presence of protease inhibitors; this was also observed in natural NTNHA. The rNTNHA assembled with isolated D-4947 BoNT with molar ratio 1:1 to form a toxin complex. The reconstituted toxin complex exhibited dramatic resistance to proteolysis by pepsin or trypsin at high concentrations, despite the fact that the isolated BoNT and rNTNHA proteins were both easily degraded. We provide definitive evidence that NTNHA plays a crucial role in protecting BoNT, which is an oral toxin, from digestion by proteases common in the stomach and intestine.

Botulinum neurotoxin (BoNT; 150 kDa) is produced by the anaerobic bacterium *Clostridium botulinum* as a set of serologically distinct proteins, designated by the seven serotypes A through G, and is well known to be the most potent toxin in nature. Serotypes A, B, E and F are implicated in human botulism, whereas serotypes C and D are causative agents for animal and avian botulism. After oral ingestion of BoNT-contaminated food, BoNT passes through the gastrointestinal tract and is absorbed from intestinal epithelial cells into the bloodstream. BoNT then reaches the neuromuscular junctions and enters nerve cells via receptor-mediated endocytosis, where it cleaves specific sites on target proteins, inhibiting the release of neurotransmitters from peripheral cholinergic synapses through its zinc protease activity [1,2]. A series of these processes causes muscular paralysis in humans and animals leading to the botulism disease state.

The BoNT molecule in culture supernatants or in naturally contaminated foods ordinarily forms a non-covalent complex with other proteins, including a single nontoxic-nonhemagglutinin (NTNHA; 130 kDa) subunit and/or three hemagglutinin (HA) subcomponents (HA-70, HA-33 and HA-17). In serotypes A, B, C and D strains these proteins are encoded by two gene clusters: cluster 1 contains the *bont* and *ntnha* genes, and cluster 2 contains three HA genes, *ha-70*, *ha-33* and *ha-17* [3]. The toxin complex (TC) is found in three forms: the 750 kDa L-TC (a complex of BoNT, NTNHA, HA-70, HA-33 and HA-17), LL-TC (probably a dimer of L-TC), or the 280 kDa M-TC (a complex of BoNT and NTNHA). On the other hand, serotypes E [4] and F [5] are encoded by just two genes, *bont* and *ntnha*, and they therefore produce only the M-TC. The BoNTs and HAs that comprise the L-TCs, which are produced by all strains except serotype E and F strains, are first synthesized as single-chain polypeptides and are then converted to nicked forms by post-translational modifications by endogenous trypsin-like proteases [6,7].

The oral toxicities of the BoNT and TC forms were found to be radically different [8,9]; thus the nontoxic components of TCs are thought to play an important role in the onset of botulism caused by oral ingestion. TCs are exposed to acidic (pH 2) gastric juice containing pepsin in the stomach; they then enter the small intestine, where they encounter several more proteases. Despite these harsh conditions, BoNT and other nontoxic components can subsequently be detected in the blood and circulatory systems [10,11]. This implies that the overwhelming majority of botulism cases are due to oral poisoning, and NTNHAs and HAs seem to function as delivery vehicles for BoNT trafficking. Thus the NTNHAs must be able to bind to all TC forms. This complex formation is considered critical to elicit food poisoning because it shields the BoNT molecule from acidic conditions and proteases in the stomach [11], whereas HA subcomponents play an essential role in the effective absorption of TCs by the small intestine [12]. In this study,

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we demonstrate high-level expression of a single 130 kDa NTNHA polypeptide by an *Escherichia coli* system. This recombinant protein will allow us to understand the extraordinary stability of the BoNT complex against exposure to harsh conditions. In addition, X-ray crystallographic analyses using rNTNHA will be carried out in the near future and will yield further clues as to the basis for the structural integrity of the BoNT complex.

Materials and methods

Construction of the rNTNHA expression system. The serotype D strain 4947 (D-4947) *ntnha* gene (AB037920) sequence (3591 bp) was amplified by PCR using genomic DNA as a template with the primer set: forward 5′-caccatggatataaacgacgacgactt-3′ and reverse 5′-ctaacttttaatatccacaaatgtgcgcc-3′. The PCR product encoding the *ntnha* gene was cloned into the pET200-D/TOPO plasmid vector. The pET200-D/TOPO-*ntnha* plasmid was transformed into *E. coli* TOP10 cells. The transformants were selected on LB plates containing 100 μg/ml kanamycin. Plasmid DNA was extracted from *E. coli* cell cultures using a QIAprep Spin Miniprep Kit (Qiagen, Duesseldorf, Germany). Sequencing reactions were performed with vector-specific primers. Extension products were then dye-labeled with ABI PRISM Big Dye Terminator chemistry (Applied Biosystems, Carlsbad, CA) were analyzed using a 3130 DNA sequencer (Applied Biosystems).

Expression and purification of rNTNHA. The pET200-D/TOPO-*ntnha* construct (10 ng) was transformed into *E. coli* BL21 star (DE3) cells to produce rNTNHA. The *E. coli* BL21 cells were inoculated into 20 ml of LB broth containing 100 μg/ml kanamycin and grown overnight (16 h) at 37 °C with mild shaking. This culture was inoculated into 1000 ml of LB broth and incubated at 37 °C until the culture reached the mid-log phase (OD_{600} about 0.5). Isopropyl-D-thiogalactoside (IPTG) was added to a final concentration of 0.1 mM and the induction was allowed to proceed at 18 °C for 18 h. The cells were suspended in 80 ml of 50 mM phosphate buffer (pH 7.4) containing 0.3 M NaCl. The cell suspensions were then sonicated and centrifuged at 10,000*g* for 20 min at 4 °C. The supernatant was mixed with 5 ml of 50% Ni-Charged Resin suspension equilibrated with the same buffer. The resin mixture was poured into a glass column. After washing the column, the bound protein was eluted with equilibration buffer containing 300 mM imidazole. The eluted fraction containing rNTNHA was precipitated with 80% ammonium sulfate. The precipitate was dialyzed against 50 mM phosphate buffer (pH 6.0) containing 0.15 M NaCl and then applied to a Superdex 200 HR 10/30 (GE Healthcare Bio-Sciences, Piscataway, NJ) gel filtration column equilibrated with the same buffer. The eluted proteins were analyzed by SDS–PAGE.

Preparation of the D-4947 M-TC and isolation of BoNT. C. botulinum D-4947 was cultured using a dialysis method as described previously [13]. The BoNT complexes were purified from the supernatant by three consecutive chromatographic runs, on a SP-Toyopearl 650S (Tosoh, Japan) cation-exchange column, a HiLoad 16/60 Superdex 200 pg (GE Healthcare Bio-Sciences) gel filtration column and a Mono S HR 5/5 (GE Healthcare Bio-Sciences) cation-exchange column. The BoNT was prepared from L-TC by the method of Hasegawa et al. [14] using a HiLoad 16/60 Superdex 200 pg gel filtration column and a Mono Q HR 5/5 (GE Healthcare Bio-Sciences) anion-exchange column.

PAGE and N-terminal amino acid sequence analysis. SDS–PAGE was performed using a 13.6% polyacrylamide gel in the presence of 2-mercaptoethanol. The separated protein bands were stained with Coomassie Brilliant Blue R-250 (CBB). For N-terminal amino acid sequence analysis, the proteins separated by SDS–PAGE were transferred onto a polyvinylidene difluoride (PVDF) membrane. The amino acid sequence of each component was determined using an automated protein sequence analyzer (Model 492HT, Applied Biosystems).

Western blot analysis. The proteins separated by SDS–PAGE were electroblotted onto a PVDF membrane. The membrane was blocked in TBST (20 mM Tris–HCl buffer, pH 7.5, 150 mM NaCl and 0.1% Tween 20) with 3% skim milk and then probed with a rabbit anti-NTNHA antibody (1:300 dilution) in TBST with 3% skim milk at 4 °C overnight. Following three washes with TBST, the membrane was incubated with the anti-rabbit HRP-conjugated secondary antibody (1:1000 dilution) at room temperature for 1 h and washed again three times in TBST. After rinsing the membrane with the same solution, the bound antibody was visualized with a chemiluminescence detection system (GE Healthcare Bio-Sciences).

Reconstitution of M-TC using isolated BoNT and rNTNHA. Reconstitution of the M-TC was achieved by mixing isolated D-4947 BoNT and purified rNTNHA at a molar ratio of 1:1 in 50 mM phosphate buffer (pH 6.0) containing 0.15 M NaCl. After incubation at 25 °C for 2 days, the mixture was applied to a Superdex 200 HR 10/30 gel filtration column equilibrated with the same buffer. The reconstituted complex eluted as a separate peak and was subjected to SDS–PAGE analysis.

Long-term incubation of rNTNHA. The purified rNTNHA (250 μg/ml) was incubated at 25 °C in the dark, and 15 μl aliquots were removed at 2, 4, 7 and 12 days, added to a half volume of SDS–PAGE sample treatment buffer, and subjected to SDS–PAGE. The protease inhibitors 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM EDTA were added to some incubation mixtures. The rNTNHA was quantified by measuring the intensity of the CBB-stained SDS–PAGE band using Image J 1.38v software (http://rsb.info.nih.gov/ij).

Proteolysis with trypsin and pepsin. Solutions of rNTNHA, BoNT and the rNTNHA/BoNT complex (each 1 mg protein) were incubated at 37 °C with 100 μg of TPCK-trypsin (12,500 U/mg) in 50 mM phosphate buffer (pH 6.0) containing 0.15 M NaCl and with 10 μg of pepsin (3100 U/mg) in 50 mM acetate–HCl buffer (pH 2.7),

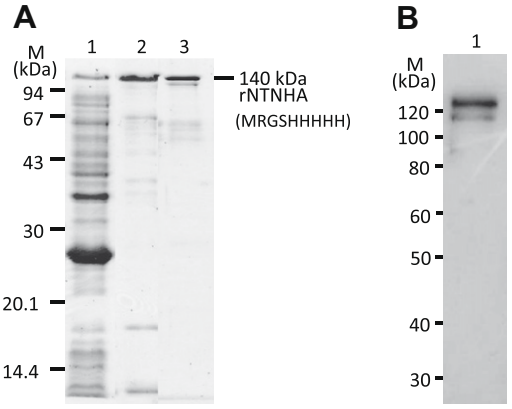

Fig. 1. SDS–PAGE and western blot analysis of purified rNTNHA expressed in the *E. coli* system. (A) Shows the CBB-stained SDS–PAGE gel. Lane 1, soluble fraction. Lane 2, rNTNHA after Ni-Charged Resin column chromatography. Lane 3, purified rNTNHA after Superdex 200 HR 10/30 gel filtration column chromatography. (B) Shows the protein detected using an anti-NTNHA antibody. Lane 1, purified rNTNHA after Superdex 200 HR 10/30 gel filtration column chromatography.

Table 1
Purification of recombinant NTNHA from the soluble fraction.

Purification Step	Total protein (mg)	Overall yield (%)
Soluble fraction	233	100
Nickel affinity chromatography	2	0.8
Gel filtration chromatography	1	0.4

each in a total volume of 1 ml. The reactions were terminated by removal of aliquots from the mixture at appropriate intervals, boiling and subjecting them to SDS–PAGE.

Results and discussion

Expression and purification of rNTNHA from an E. coli system

The NTNHA gene (3591 bp, 1197 amino acid residues) was amplified by PCR using genomic DNA as a template. The pET200-D/TOPO-*ntnha* plasmid was transformed into *E. coli* TOP10 cells. After selection of transformants, the pET200-D/TOPO-*ntnha* construct was transformed into a large-scale *E. coli* BL21 cell expression system to produce rNTNHA. The solubilized rNTNHA fraction was purified by Ni chelating resin affinity column chromatography. As shown in Fig. 1, eluted fraction contained an approximately 140 kDa protein, as indicated by SDS–PAGE. The calculated mass of rNTNHA, which contains 35 additional tag residues upstream from the N-terminus, is 142,570 Da. These fractions were further purified by gel filtration column chromatography, producing a single band on SDS–PAGE (Fig. 1A). The purified 140 kDa protein was determined to be rNTNHA by western blot analysis using an anti-NTNHA antibody (Fig. 1B). The N-terminal amino acid sequence of rNTNHA was determined to be MRGSHHHHH, which corresponded to the His-tagged N-terminus. Successive 1 L cultures typically yielded about 1 mg of rNTNHA (Table 1). Normally, D-4947 produces a relatively large amount of TCs (~10 mg) compared with other serotype strains. Indeed, in order to obtain 0.15 mg of isolated NTNHA from M-TC, it was necessary to process 1 L of culture supernatant by a routine dialysis tube method. By carrying out multiple chromatographic runs to isolate NTNHA from its toxin complex, this expression system can yield significant quantities of purified NTNHA.

Spontaneous nicking in the rNTNHA during long-term incubation

The NTNHAs of M-TCs produced by serotype A [15], C [16], and D [16–18] strains have always been found to be nicked at unique

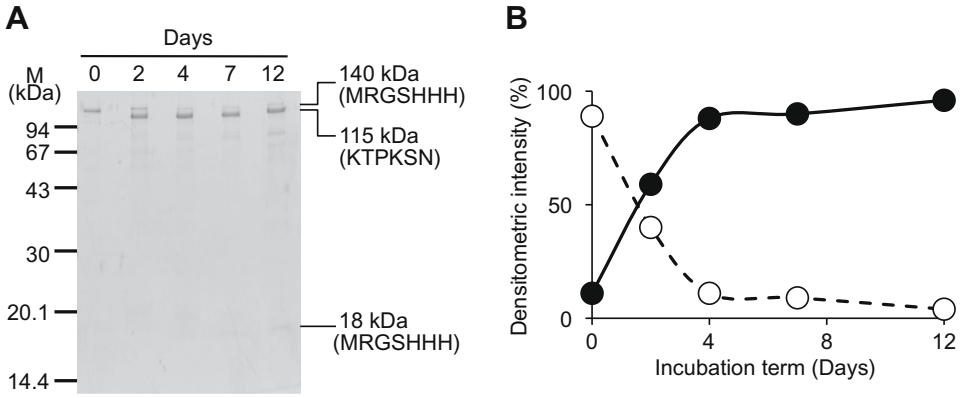

Fig. 2. Spontaneous processing of rNTNHA components to produce 18 and 115 kDa fragments during long-term incubation at 25 °C for 0, 2, 4, 7 and 12 days. (A) Shows SDS–PAGE profiles. (B) Shows densitometric intensity of 140 kDa (open circle with dotted line) and 115 kDa (closed circle with solid line) rNTNHA on each incubation day. The percentage is calculated as the ratio of the intensity of each generated fragment against the combined intensity from all fragments.

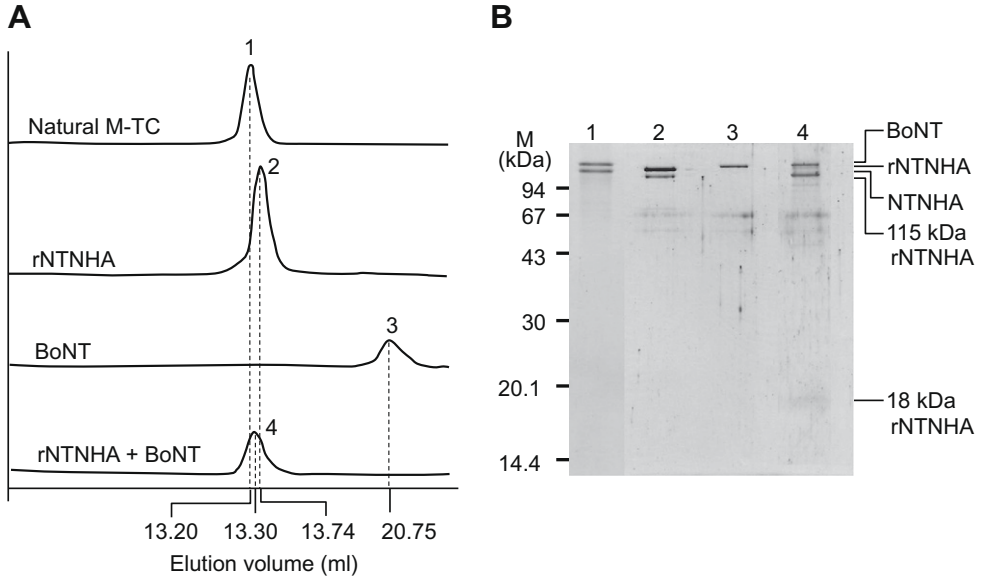

Fig. 3. Gel filtration profiles and SDS–PAGE banding patterns of reconstituted M-TC with BoNT and rNTNHA. (A) Shows gel filtration elution profiles of natural M-TC, rNTNHA, isolated BoNT and reconstituted M-TC corresponding to peaks 1–4, respectively, as indicated by the elution volumes. (B) shows the SDS–PAGE banding patterns of natural M-TC, with each peak indicated in (A).

sites. This leads to the appearance of two bands, 15 and 115 kDa, on SDS–PAGE, while the NTNHAs in the L-TCs remained unprocessed single-chain polypeptide. Previously we found that a unique strain, D-4947, produces both intact M- and L-TC without any nicking [16]. In addition, we inadvertently found that both isolated NTNHA and NTNHA in the M-TC spontaneously converted to the nicked forms [16]. In the present study, we investigated spontaneous nicking of rNTNHA. As shown in Fig. 2A and B, when the rNTNHA was incubated for long periods at 25 °C without addition of protease, SDS–PAGE analysis showed that disappearance of the 140 kDa band was accompanied by the appearance 18 and 115 kDa bands, and the latter bands increased with longer incubation times. The N-terminal amino acid sequences of the 18 and 115 kDa bands were determined to be MRGSHHH and KTPKSN, corresponding to the N-terminal amino acid sequence of rNTNHA and residues K^{127} to N^{132} of NTNHA, respectively (Fig. 2A).

Nicking of rNTNHA was considered to be due to contamination by a small amount of trypsin-like protease derived from the *E. coli* expression system. Accordingly, protease activity in the rNTNHA preparation was evaluated using *N*-alpha-benzoyl-DL-arginine-*p*-nitroanilide as a serine protease substrate. However, the purified rNTNHA preparation showed no any significant protease activity (data not shown). Additionally, rNTNHA was incubated for a long time in the presence of protease inhibitors (PMSF and EDTA) to exclude the possibility of contamination from trace amounts of proteases, but specific nicking of the rNTNHA still occurred in a manner similar to that previously observed for natural NTNHA [16,17] (data not shown). Several amino acid residues were previously found to be deleted in the nicking region of NTNHA produced by serotype C and D [16,19] strains. On the other hand, in the M-TC produced by serotype E and F strains, no NTNHA nicking has been observed. Interestingly, these proteins lack the specific nicking site in the N-terminal region (33 amino acid residues); this phenomenon was noted for both NTNHA molecules corresponding to serotypes A, C and D strains, based on alignment of the N-terminal regions of multiple NTNHA sequences [20].

Reconstitution of M-TC using rNTNHA and isolated BoNT

Since M-TC readily separates into BoNT and NTNHA under alkaline conditions (pH greater than 7.5), reconstitution was performed at pH 6.0. A mixture containing rNTNHA and D-4947 BoNT at a molar ratio 1:1 was incubated at pH 6.0 and subjected to gel filtration. As shown in Fig. 3, a single peak corresponding to the elution volume of the M-TC (estimated at 300 kDa) showed an identical banding pattern on SDS–PAGE as rNTNHA and BoNT. Other peaks contained components that could not assemble to form the M-TC, as determined by SDS–PAGE. The reconstituted M-TC reversibly dissociated into rNTNHA and BoNT at pH 8.8. However, during reconstitution experiments, intact rNTNHA in the reconstituted M-TC spontaneously converted to the nicked form, as indicated by 18 and 115 kDa bands on SDS–PAGE.

According to our previous reports [18,21] on assembly pathways of BoNT complexes, the observation that M- and L-TC were found in D-4947 culture could be explained by a model in which M-TC forms first by association of BoNT and NTNHA, and then the M-TC forms a L-TC by incorporating the remaining HA subcomponents, HA-70, HA-17 and HA-33. On the other hand, NTNHA nicking, whether spontaneous or induced by protease treatment, produced only M-TC as a dead-end product in the course of assembly.

Proteolysis of reconstituted M-TC, BoNT and rNTNHA by trypsin and pepsin

Most proteins are degraded into short peptides and amino acids in the stomach and small intestine during the process of digestion. In the present experiments, proteolysis was conducted under harsh conditions mimicking those found along the gastrointestinal tract, although the physiological protease concentrations depend on the feeding state of the organism. High concentrations of proteases, e.g., 30 U pepsin or 1250 U trypsin, were incubated with the rNTNHA/BoNT complex and with isolated BoNT and rNTNHA. As shown in Fig. 4A, the rNTNHA was digested into several fragments after incubation with pepsin at pH 2.7 for 60 min, whereas trypsin treatment for 360 min produced just nicking in rNTNHA, as indi-

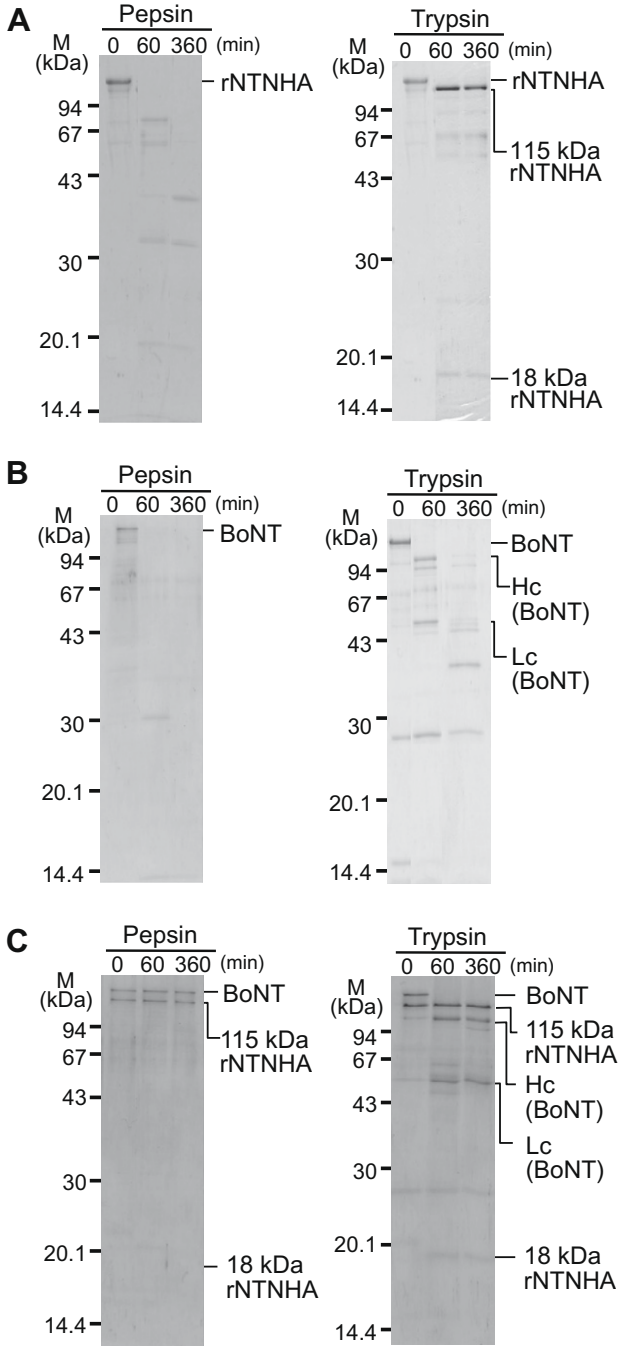

Fig. 4. SDS–PAGE of rNTNHA, BoNT and M-TC (BoNT/rNTNHA) after pepsin and trypsin treatment. Digestions were terminated by the removal of aliquots from the mixture at 0, 60 and 360 min as indicated, followed by boiling and SDS–PAGE of these aliquots. The proteolysis patterns of (A) rNTNHA, (B) BoNT and (C) M-TC with pepsin (each left panel) and trypsin (each right panel) are shown as indicated. The bands corresponding to each component and fragments generated after proteolysis are shown.

cated by 115 and 18 kDa fragments on SDS–PAGE. On the other hand, under the same conditions, isolated BoNT was highly sensitive to both trypsin and pepsin digestion, yielding no bands on SDS–PAGE after a 60 min incubation, as shown in Fig. 4B. This finding was supported by the fact that oral administration of purified BoNT to mice resulted in much lower toxicity than administration of BoNT complex forms [22]. Similarly, most reports on the botulinum TCs indicate that nontoxic proteins probably play a role in protecting the BoNT from harsh conditions [11].

In contrast, when the BoNT assembled with rNTNHA to form a complex, both proteins demonstrated an amazing resistance to proteolysis, as shown in Fig. 4C. When the M-TC was incubated with pepsin, BoNT showed no sign of proteolysis, while two bands derived from nicked rNTNHA were observed on SDS–PAGE. On the other hand, tryptic proteolysis of M-TC yielded specific bands corresponding to nicked rNTNHA and nicked BoNT. Reconstituted M-TC treated with each protease showed a single peak at the elution volume corresponding to the M-TC (data not shown), implying that the complex was maintained despite nicking of rNTNHA and BoNT molecules at specific sites. Limited trypsin digestion of BoNT (e.g., protein ratio 1:100) usually produced a nicked di-chain structure, consisting of the 50 kDa Light chain (Lc) and the 100 kDa Heavy chain (Hc) linked by a disulfide bond [23]. Since only the reconstituted M-TC exhibited strong tolerance against both trypsin and pepsin attacks, it is likely that NTNHA protein component can alter its conformation to assemble with BoNT, forming an oral toxin that protects BoNT from harsh conditions, such as low pH in the digestive tract. The three-dimensional structure of NTNHA has not been determined, so the structural features of this remarkable complex are not yet known. X-ray crystallographic analysis of NTNHA could indicate the assembly mechanism that produces the unique M-TC form, as well as the molecular mechanism for spontaneous processing of the NTNHA. This is the subject of current investigations.

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