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Elucidating the activation mechanism of botulinum neurotoxin a: role of α-clostripain and NTNH

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Botulinum neurotoxin A (BoNT/A) is one of the most toxic substances known. The bacterium *Clostridium botulinum* produces it as a 150-kDa polypeptide with relatively low potency. Surprisingly, the endogenous protease that transforms this polypeptide into an activated, full-potency toxin, consisting of a 100-kDa heavy chain connected to a 50-kDa light chain (LC) by a disulfide bond, is still unknown. This study aimed to identify the BoNT/A-activating protease. We screened cation-exchange chromatography fractions of *C. botulinum* A culture supernatant for activity using a toxin-simulating substrate comprising the LC and the translocation domain (HN). Proteomic analysis of the active fraction identified α-clostripain as a candidate BoNT/A-activating protease. Recombinant α-clostripain cleaved the simulating substrate between the toxin LC and HN. However, incubation of recombinant α-clostripain with recombinant inactivated BoNT/A (rBoNT/Ai) resulted in non-specific digestion of the toxin. Since similar non-specific digestion was observed also by *C. botulinum* A culture supernatant, we hypothesized that the toxin should be protected by an accessory protein to prevent non-specific cleavage. Indeed, incubation of rBoNT/Ai with α-clostripain or culture supernatant in the presence of recombinant NTNH (non-toxic non-hemagglutinin) resulted in specific cleavage of the toxin into 100- and 50-kDa fragments. Subsequently, we evaluated the activation of rBoNT/A by α-clostripain in a mouse model. Cleavage of rBoNT/A by α-clostripain in the presence of NTNH resulted in a 77-fold increase in toxicity, corresponding to toxin activation. To the best of our knowledge, these results elucidate the mechanism of BoNT/A activation for the first time.

Botulinum neurotoxins (BoNTs) are among the most potent toxins known, with an estimated human lethal dose of 1 ng/kg when delivered intravenously[1]. Produced by the anaerobic bacterium *Clostridium botulinum* and several other Clostridium species, BoNTs are the causative agents of botulism, a rare but potentially fatal neuroparalytic disease. There are at least seven serotypes of BoNTs (A–G), of which A, B, E, and rarely F, are the cause of botulism in humans[1,2]. Despite their extreme toxicity, BoNTs have found widespread use clinically and cosmetically due to their ability to induce temporary muscle paralysis by disrupting neurotransmission at peripheral cholinergic nerve terminals[3–5].

BoNTs are heterodimeric proteins composed of a ~ 100-kDa heavy chain (HC) and a ~ 50-kDa light chain (LC) linked by a disulfide bond[6]. The HC is divided into two functional domains: the C-terminal receptor binding domain (H_C), which mediates highly specific binding to presynaptic nerve terminals followed by spontaneous endocytosis, and the N-terminal translocation domain (H_N), which facilitates the release of the LC into the cytosol[6,7]. Inside the cytoplasm, the LC acts as a zinc-dependent metalloprotease, cleaving one of three soluble N-ethylmaleimide-sensitive factor attachment (SNARE) proteins involved in the fusion of acetylcholine-containing vesicles with the presynaptic membrane, thereby blocking the release of the neurotransmitter and causing muscle paralysis[8]. In their naturally occurring forms, BoNTs are part of large protein complexes that include non-toxic accessory proteins, such as NTNH (non-toxic non-hemagglutinin), HA-17, HA-33, and HA-70 for BoNT/A. The accessory proteins protect the toxin as it travels throughout the gastrointestinal system, and facilitate its absorption by and transport through the intestinal epithelium[9].

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Botulinum neurotoxins are expressed by the bacterium as a low-activity single polypeptide of 150-kDa. The toxins are post-translationally cleaved at a specific site between the LC and HC by a protease to generate the di-chain highly potent form[9,10]. This specific proteolytic cleavage is termed activation, and is associated with a two-orders of-magnitude increase in toxicity for BoNT/E[11], and likely has a similar effect for other BoNTs. The origin of the activating protease can be either endogenous to the bacterium or exogenous, depending on the *C. botulinum* species[8,12]. For *C. botulinum* A species, an endogenous activating protease is responsible for toxin activation[13]. Toxin expression is enhanced during the late exponential growth phase of the bacterium, and following cell autolysis, the toxin is excreted into the extracellular medium where activation takes place[14,15]. Although the BoNT/A activation process is of great importance, the identity of the BoNT/A activating protease and the activation mechanism remains still unknown[16]. Identification of the protease may facilitate the development of toxin-activation inhibitors that could be applied to foods prone to *C. botulinum* contamination. Additionally, the mechanism of BoNT/A activation can be harnessed to produce active recombinant BoNT/A for therapeutic and cosmetic applications.

In this study, we developed a specific activity assay to discover the BoNT/A-activating protease. By screening fractions of *C. botulinum* A culture supernatant for activity, in combination with a proteomic analysis, we identified α-clostripain as a candidate BoNT/A-activating protease. Interestingly, we found that the accessory protein NTNH was required during activation to prevent non-specific degradation of the toxin. These findings led us to suggest here a mechanism for BoNT/A activation.

Materials and methods

Ethic statement

All animal experiments were performed in accordance with Israeli law and were approved by the Ethics Committee for Animal Experiments at the Israel Institute for Biological Research (Protocol No. M-51-21).

Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Yeast extract, tryptone, and gelatin were obtained from Becton Dickinson and Company (Franklin Lakes, NJ, USA). *E. coli* strains and plasmids were purchased from Novagen (Madison, WI, USA). *C. botulinum* A was obtained from the IIBR collection. The BoNT/A sequence is identical to that of the Hall A strain (accession number P0DPI1). Synthetic genes with optimized codon usage were prepared by GenScript (Piscataway, NJ, USA).

Protein expression and purification

All recombinant proteins were expressed in *E. coli* BL21(DE3) harboring the relevant plasmid (Table 1S). The bacteria were grown in a volume of 2 L of terrific broth media for 40 h at 18 °C with agitation, and cells were harvested by centrifugation. For proteins harboring a his-tag (except rα-clostripain), cells were resuspended in 200 mL binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4), and disrupted by three passages in a cell homogenizer at 25 kPa (C series cell disruptor 1.1 KW, Constant Systems). The cell extract was centrifuged (20,000 × g, 40 min) and the supernatant was loaded onto a 5 mL HisTrap FF column (Cytiva) mounted on an AKTA Explorer FPLC system (Cytiva). The column was washed with 10 CV (column volume) of binding buffer and 10 CV of binding buffer containing 40 mM of imidazole. The protein was eluted from the column with elution buffer (20 mM of sodium phosphate, 0.5 M of NaCl, 500 mM of Imidazole, pH 7.4). For proteins harboring Strep-tag, cells were resuspended in 200 ml binding buffer (100 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, pH 8.0) and disrupted by homogenization. The cell extract was centrifuged (20,000 × g, 40 min), and the supernatant was loaded onto a StrepTrap XT 1 ml column (Cytiva) mounted on an AKTA Explorer FPLC system. The column was washed with 15 CV of binding buffer and the protein was eluted from the column with elution buffer (100 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 50 mM biotin, pH 8.0). As rα-clostripain could not be purified on a nickel column, probably due to removal of the tag by the protease, it was purified by cation exchange chromatography. Bacterial cell lysate in 20 mM sodium acetate buffer pH 5.0 was loaded onto a HiTrap SP HP column (Cytiva) mounted on an AKTA Explorer FPLC system. The column was washed with 15 CV of sodium acetate buffer pH 5.0 to remove unbound proteins, and the protein was eluted from the column with a gradient of elution buffer containing 20 mM sodium acetate, 1 M NaCl, pH 5.0.

C. botulinum A culture supernatant was prepared as follows. A starter culture was prepared by applying a heat shock to spores (80 °C, 20 min) and then inoculating them into 30 ml growth media (2% NZ amine, 2% yeast extract, 1% glucose). Following 24 h of incubation anaerobically at 35 °C, the starter was inoculated into 100 ml of growth media. After five days of incubation, the culture supernatant was separated by centrifugation (13,000 × g, 20 min, 4 °C), and stored at −70 °C.

Protease activity assays

The activity of the activating protease in culture supernatant and culture supernatant fractions was determined by incubating the substrate LCH_N (0.6 mg/mL) with the tested sample at 35 °C for 5 h. Thereafter, the enzymatic reactions were mixed with Novex™ Tris–glycine sample buffer (Thermo Fisher Scientific) supplemented with 5% ß-mercaptoethanol, heated (100 °C, 10 min) and loaded onto NuPAGE™ 10%, Bis–Tris SDS-PAGE (Thermo Fisher Scientific). The gels were stained with InstantBlue® Coomassie Protein Stain (abcam). A sample was considered positive for activating protease activity when the intensity of the 100-kDa band of intact substrate was reduced and two ~ 50-kDa bands corresponding to the LC and H_N appeared.

The activity of purified rα-clostripain was tested by incubating rBoNT/Ai (140 µg/mL) with rα-clostripain (20 µg/mL) for 4 h at 35 °C. The reactions contained 10 mM $CaCl_2$, 10 mM DTT, and 0.1 M acetate buffer pH 6.0. For reactions in which protection by rNTNH was evaluated, rBoNT/Ai and rNTNH (270 µg/mL) were

pre-incubated at room temperature for 30 min before the addition of the protease. The reaction products were analyzed by SDS-PAGE as described above.

Chromatographic fractionation of culture supernatant and proteomic analysis

Culture supernatant was heat-treated (60 °C, 30 min), and the resulting non-soluble proteins were removed by centrifugation (23,426 × g, 10 min, 4 °C). Nucleic acids were then removed by adding 0.1% protamine sulfate, stirring for 5 min, and centrifugation (23,426 × g, 15 min, 4 °C). The supernatant (30 mL) was then diluted five-fold with start buffer (20 mM sodium phosphate, pH 5.5) and loaded onto a 1 mL HiTrap SP HP column (Cytiva). Following loading, the column was washed with start buffer until the 260- and 280-nm absorbance returned to baseline. Then, column-bound proteins were released with an elution buffer (20 mM sodium phosphate, 1 M NaCl, pH 5.5) gradient: from 0 to 0.5 M NaCl over 20 column volumes and from 0.5 to 1 M NaCl over 10 column volumes. Throughout the elution, the eluent was collected in 1-mL fractions.

Proteomic analysis of selected fractions was performed at the de Botton Institute for Protein Profiling (Weizmann Institute, Rehovot, Israel). The samples were digested with trypsin using the S-trap method. The resulting peptides were analyzed using nanoflow liquid chromatography (nanoAcquity) in conjunction with high-resolution, high-mass accuracy mass spectrometry (Q Exactive HF). The samples were analyzed separately in discovery mode. The data were processed using Proteome Discoverer version 2.1 and searched against the *Clostridium botulinum* A strain ATCC 3502 protein database, to which a list of common laboratory contaminants was added. The search was performed with the SequestHT engine.

Determination of toxins LD_{50}

The median lethal dose (LD_{50}) of non-activated rBoNT/A and activated rBoNT/A was determined using the Spearman-Karber method[17]. Six concentrations of the toxin were prepared in gelatin buffer using a 1.5-fold serial dilution. A volume of 0.5 mL of each concentration was injected intraperitoneally into groups of CD-1 mice (n = 5, weighing 22–27 g) (Charles-River, UK) on the left side of the peritoneum. The injected mice were observed for 4 consecutive days, and mortality was recorded 96 h post-injection. The study is reported in accordance with ARRIVE guidelines (https://arriveguidelines.org).

Results

Development of an activity assay to discover BoNT/A-activating protease

To identify the activating protease of BoNT/A, we first developed an assay for the detection of its activity. As the natural activity of the protease is the cleavage of a single chain of BoNT/A into a di-chain form (Fig. 1a), the natural substrate for the activating protease would be a single-chain BoNT/A. To simulate this activity, we designed a recombinant non-toxic derivative to serve as the substrate, consisting of amino acids 1–871 of the neurotoxin (Fig. 1b). This derivative included the LC and the H_N domains of the toxin (LCH_N), between which

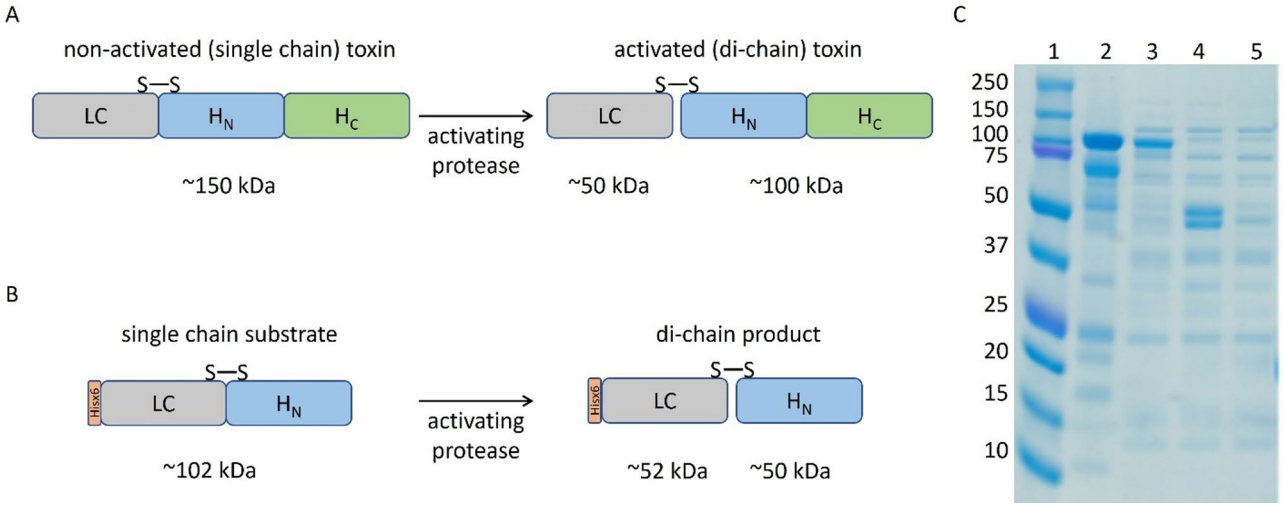

Fig. 1. Assay development for the BoNT/A-activating protease. (**A**) BoNT/A is expressed by *C. botulinum* A as a single polypeptide of ~ 150 kDa with low toxicity. The activating protease cleaves the single chain toxin at a specific site to produce the activated di-chain toxin, which consists of light (~ 50-kDa, LC) and heavy (~ 100-kDa, HC) chains connected by a disulfide bond. (**B**) Recombinant toxin-simulating substrate for the activating protease. The substrate is non-toxic and includes the catalytic (LC) and translocation (H_N) domains of BoNT/A, but lacks the receptor-binding domain (H_C). The substrate contains an N-terminal His-tag and two mutations in the catalytic domain (E224Q and H227Y) for further reduction of its toxicity. (**C**) SDS-PAGE analysis for evaluating the suitability of the assay to detect the activating protease. Lane 1–MW marker; lane 2–LCH_N; lane 3–LCH_N following incubation with *C. botulinum* A culture supernatant under non-reducing conditions; lane 4—LCH_N following incubation with *C. botulinum* A culture supernatant under reducing conditions; lane 5—*C. botulinum* A culture supernatant.

the cleavage site of the activating protease is located. To abolish possible toxicity of the derivative, we incorporated two mutations in the catalytic residues of the endopeptidase domain (E224Q and H227Y) as described by Shone et al[18]. The activity of the unknown protease on this substrate (102-kDa LCH_N) should result in the cleavage of the substrate into two polypeptide products connected by a disulfide bond. Following reducing SDS-PAGE analysis, the proteolysis products are expected to migrate as two bands of ~ 50- and ~ 52-kDa (Fig. 1b).

The suitability of LCH_N to serve as a substrate for the activating protease was evaluated by incubating a supernatant of *C. botulinum* A culture with the derivative and analyzing the products via reducing SDS-PAGE. As seen in Fig. 1c, the derivative migrated as a ~ 100-kDa band (lane 2). Following incubation with the culture supernatant, the 100-kDa band was replaced by two new bands of ~ 50-kDa (lane 4), indicating the substrate was cleaved by the unknown activating protease present in the culture supernatant. To validate that the protease cleaved the derivative at a specific site between the two connected cysteines, and not at an adjacent location that would result in two cleavage products of a similar molecular weight, the cleavage products were also analyzed by non-reducing SDS-PAGE (Fig. 1C, lane 3). The product migrated as a single 100-kDa band, similar to the un-cleaved LCH_N. This result shows that the recombinant derivative LCH_N includes a disulfide bond and that it was cleaved at a specific activation site by the unknown activating protease, present in the culture supernatant.

Fractionation of *C. botulinum* A supernatant and proteomic analysis

To identify the BoNT/A-activating protease, we performed chromatographic fractionation of the culture supernatant and applied the activity assay described above to detect the active fractions. The culture supernatant contains, in addition to the activating protease, many other *C. botulinum* proteins released during the bacterial lysis stage. This protein load may hamper the yield of the chromatographic fractionation step and add complexity to the proteomic analysis. Therefore, we evaluated the use of heat treatment as a means to reduce the protein load. Culture supernatant samples were incubated at several temperatures ranging from 50 to 90 °C for 30 min, and after removal of precipitated proteins by centrifugation, the activity was determined (Fig. 2a). Cleavage of the substrate was obtained for temperatures up to 60 °C. A protein content analysis showed that following heat treatment at 60 °C, the total protein concentration was reduced from 270 to 120 µg/ml, and SDS-PAGE analysis showed that the amount of protein bands also decreased after the heat treatment. Therefore, a heat treatment at 60 °C was found suitable as a preliminary step for reducing the total protein load without affecting the activating protease.

Next, we conducted cation exchange chromatography of a heat-treated *C. botulinum* culture supernatant. Following supernatant loading and washing of unbound proteins, the resin-bound proteins were eluted from the column with an increasing gradient of NaCl concentrations, during which the eluent was collected as fractions (Fig. 2b). The fractions were then analyzed for LCH_N cleavage activity (Fig. 2c). The complete digestion of the substrate into two ~ 50-kDa bands was observed between fractions A8 and B11, indicating these fractions were enriched with the activating protease. The protein profile of these fractions was then analyzed by SDS-PAGE (Fig. 2d). The profile of the fractions can be divided into two populations, one containing many protein bands (A8, A9, A10, and A11), and the other containing fewer protein bands (A12, B12, and B11). The common characteristic of all active fractions was the presence of a protein band at ~ 46-kDa and a protein band at < 25 kDa. To identify the activating protease, one active fraction from each population was subjected to proteomic analysis. In fraction A9 (many protein bands), a total of 94 proteins were identified, five of which were proteases. In fraction B12 (fewer protein bands), a total of 176 proteins were identified, of which 11 were proteases. In both fractions, α-clostripain was found to be the protease with the highest peptide spectrum matches (#PSM).

The most studied α-clostripain is from *Hathewaya histolytica* (formerly *Clostridium histolyticum)*. It is an extracellular cysteine protease that specifically cleaves proteins at the carboxyl peptide bond of arginine residues, and at a lower rate at lysine residues[19]. This heterodimeric enzyme is composed of heavy (~ 43-kDa) and light (~ 15-kDa) polypeptide chains associated by non-covalent interactions[20]. A BLAST search with the *C. botulinum* A α-clostripain sequence revealed the presence of homologous proteases in *C. botulinum* strains A, B, C, D, and F, but not in the non-proteolytic strain E. Interestingly, homologs of α-clostripain were found in the genomes of proteolytic B strains but not in the genome of the non-proteolytic *C. botulinum* Eklund 17B, which expresses the botulinum neurotoxin subtype B4. Taken together, we identified α-clostripain as a promising candidate for the BoNT/A-activating protease.

α-clostripain cleaves BoNT/A non-specifically

To test whether α-clostripain is the activating protease of BoNT/A we prepared a recombinant *C. botulinum* A α-clostripain (rα-clostripain) in *E. coli*. Analysis of the protein sequence with the SignalP 5.0 server (https://services.healthtech.dtu.dk/services/SignalP-5.0/)[21] showed that the protein has a Gram-positive signal peptide cleavage site between amino acids 30 and 31. Therefore, the recombinant protein was designed to include amino acids 31–527. To facilitate the purification of the protease, a 6xHis tag sequence was added to the N-terminal. However, attempts to purify the protein using a nickel column were unsuccessful. The protein did not bind to the column, and activity was found in the flow-through fractions. Additionally, the 6xHis tag could not be demonstrated by Western blot analysis. Examination of the N-terminal sequence of the recombinant protein identified a lysine residue at position 14 and an arginine residue at position 21. These residues likely served as a cleavage site for the α-clostripain, resulting in the removal of the tag. A similar observation was reported previously for another member of the C11 protease family[22]. To overcome this, the protein was purified by cation exchange chromatography. The purified recombinant α-clostripain preparation cleaved the LCH_N substrate into two ~ 50-kDa bands, similar to our results with the *C. botulinum* A culture supernatant (Fig. 3A).

Next, we tested the activity of recombinant α-clostripain on the full-length neurotoxin. For this purpose, we prepared a recombinant inactive BoNT/A (rBoNT/Ai) containing three mutations in the catalytic residues of the LC endopeptidase domain (E224Q, R363A, and Y366F)[16]. The activity of rα-clostripain on rBoNT/Ai was

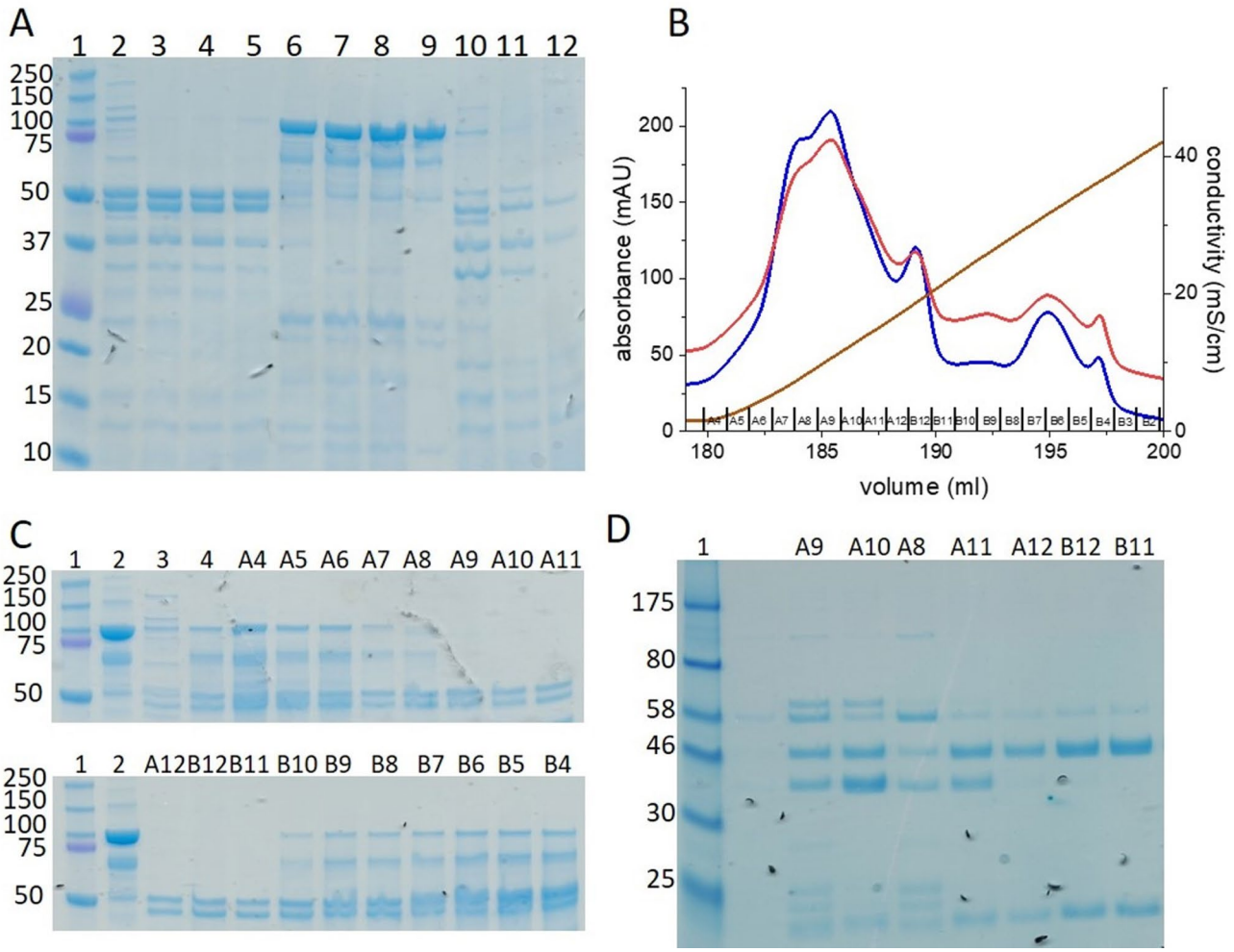

Fig. 2. Fractionation of *C. botulinum* A culture supernatant to identify the BoNT/A-activating protease. (**A**) Thermostability analysis of the activating protease. Culture supernatant was incubated for 30 min at various temperatures, followed by the activity assay. Lane 1 – MW marker; lanes 2–8–activity assay following incubation at 25 °C, 50 °C, 55 °C, 60 °C, 70 °C, 80 °C, and 90 °C, respectively; lane 9–LCHN; lanes 10–12 culture supernatant following 30-min incubation at 50 °C, 60 °C, and 70 °C, respectively. (**B**) Chromatogram of the fractionation of *C. botulinum* A culture supernatant by cation-exchange chromatography. Blue and red lines represent the absorbance at 280 and 260 nm, respectively; brown line represents conductivity. (**C**) Activity assay of the fractions. Lane 1–MW marker; lane 2–LCHN; lane 3–culture supernatant; lane 4–activity assay of culture supernatant; the remaining lanes show the activity assay results for the indicated fractions. (**D**) SDS-PAGE analysis of the fractions with the highest activating protease activity.

examined by incubating the proteins together and analyzing the products by SDS-PAGE. As a control, rBoNT/Ai was incubated with the culture supernatant of *C. botulinum* A. We expected that following incubation, rBoNT/Ai would appear on the gel as 100-kDa and 50-kDa bands, corresponding to the heavy and light chains of BoNT/A, respectively. Surprisingly, no typical bands were visible following the incubation of rBoNT/Ai with either rα-clostripain or the culture supernatant (Fig. 3b), indicating non-specific cleavage of the toxin.

NTNH is required for protecting BoNT/A from non-specific cleavage by α-clostripain

BoNT/A is found in nature in complex with several accessory proteins: NTNH, HA17, HA33, and HA70. Gu et al. demonstrated that NTNH shields BoNT/A from the effects of low pH and digestive proteases, which the toxin encounters as it travels along the digestive tract[16]. Therefore, we hypothesized that in addition to this role, NTNH also functions in protecting the toxin from non-specific cleavage by the activating protease. To test this hypothesis, we expressed and purified recombinant NTNH (rNTNH), pre-incubated it with BoNT/Ai to allow them to interact, and then added to this mixture rα-clostripain or culture supernatant. As demonstrated by an SDS-PAGE analysis (Fig. 4a), the addition of rNTNH resulted in the appearance of two bands at 100- and 50-kDa, corresponding to the heavy and light chains of activated BoNT, respectively. The HC and LC bands were present with both rα-clostripain and culture supernatant, but only in the presence of rNTNH (Fig. 4a, lanes 4 and 6 for rα-clostripain and culture supernatant, respectively). Therefore, we can infer that NTNH protects the toxin from non-specific cleavage and is required for toxin activation.

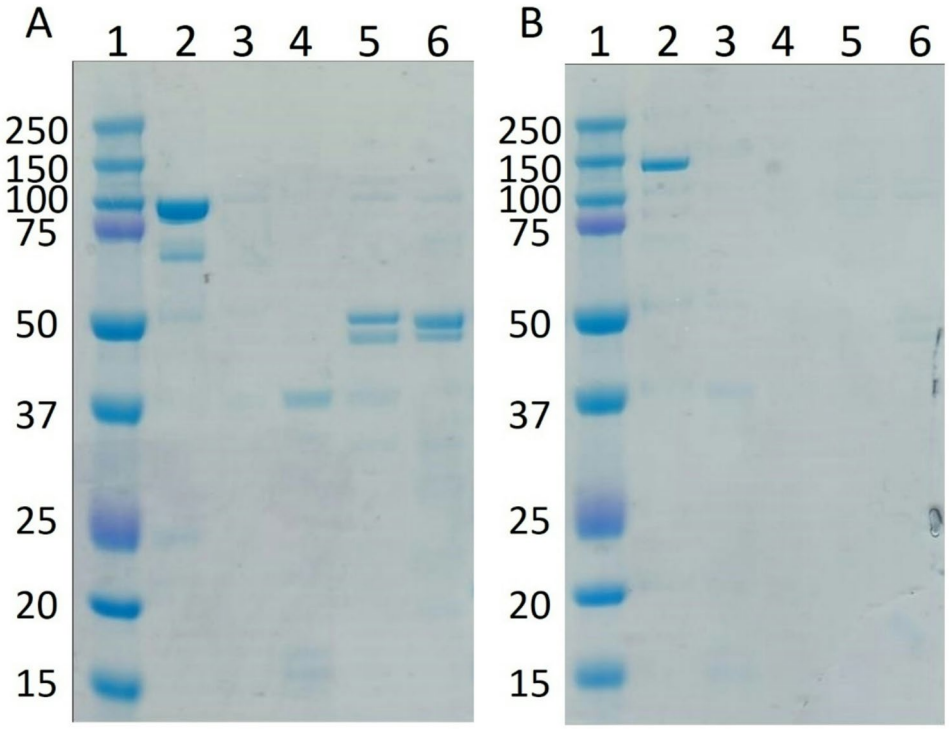

Fig. 3. Evaluation of the activity of α-clostripain on LCH_N and BoNT/Ai. (**A**) Activity assay with rα-clostripain and *C. botulinum* A culture supernatant. Lane 1–MW marker; lane 2–LCH_N; lane 3—*C. botulinum* A culture supernatant; lane 4–rα-clostripain; lane 5–activity assay of *C. botulinum* A culture supernatant; lane 6–activity assay of rα-clostripain. (**B**) Evaluation of the activity of rα-clostripain on rBoNT/Ai. Lane 1–MW marker; lane 2–rBoNT/Ai; lane 3–rα-clostripain; lane 4–rBoNT/Ai + rα-clostripain; lane 5—*C. botulinum* A culture supernatant; lane 6—rBoNT/Ai + *C. botulinum* A culture supernatant. rα-clostripain and *C. botulinum* A culture supernatant cleave rBoNT/Ai non-specifically.

Previous reports have shown that the interaction between NTNH and BoNT/A is pH-dependent[9,23]. The proteins associate at acidic pH and dissociate at physiologic pH. This pH-dependent association/dissociation-switch mechanism allows NTNH to protect the neurotoxin from the acidic environment of the digestive system and facilitate toxin release from the complex as it reaches the circulation. Consistent with this, when we incubated rBoNT/Ai with rNTNH and rα-clostripain or culture supernatant at pH 8.0, the toxin was non-specifically digested, indicating no protection was provided (Fig. 4b, lanes 5 and 7 for rα-clostripain and culture supernatant, respectively).

rBoNT/A cleavage by α-clostripain increases its toxicity by two orders of magnitude

Natural activation of BoNT/A by its endogenic activating protease is accompanied by a significant increase in its toxicity. Having demonstrated that rα-clostripain successfully cleaves rBoNT/Ai into a pattern consistent with activated BoNT/A in the presence of rNTNH, we next examined whether this specific cleavage translates into increased toxicity in a mouse model. To this end, the mutated catalytic residues in rBoNT/Ai were reversed to their natural amino acid sequence by site-directed mutagenesis, and the resulting rBoNT/A was expressed in *E. coli* and purified. For toxin activation, rBoNT/A was incubated with rα-clostripain in the presence of rNTNH. Subsequently, the activated recombinant toxin was separated in two steps: first, the rBoNT/A-rNTNH complex was separated from rα-clostripain by capturing the complex on a Strep-Tactin XT column at pH 6.0, allowing the rα-clostripain to be washed out; second, rNTNH was released with a dissociation buffer at pH 8.0 to break up the complex. Finally, the activated recombinant toxin was eluted from the column using an elution buffer.

The toxicity of both non-activated rBoNT/A and activated rBoNT/A was determined and compared in mice using the Karber method[17]. The specific activities of the preparations were found to be 1,920 pg and 25 pg for non-activated BoNT/A and activated BoNT/A per mouse intraperitoneal LD_{50}, respectively. This differential potency represents a 2-orders-of-magnitude increase in toxicity resulting from the toxin activation process.

Discussion

The activation of botulinum toxins by proteases was first proposed by Duff et al. in 1956[24]. They observed that type E strain cultures presented very low toxicity in mice (compared to types A, B, C and D) and that cultures contaminated with bacilli produced higher toxin E titers than pure cultures. This implied that the cultures produce a protoxin that can be activated by an exogenous protease[24]. Later, Bonventre and Kempe[25] found that while the toxicity of filtrates from older cultures of types A and B strains could not be increased, young cultures

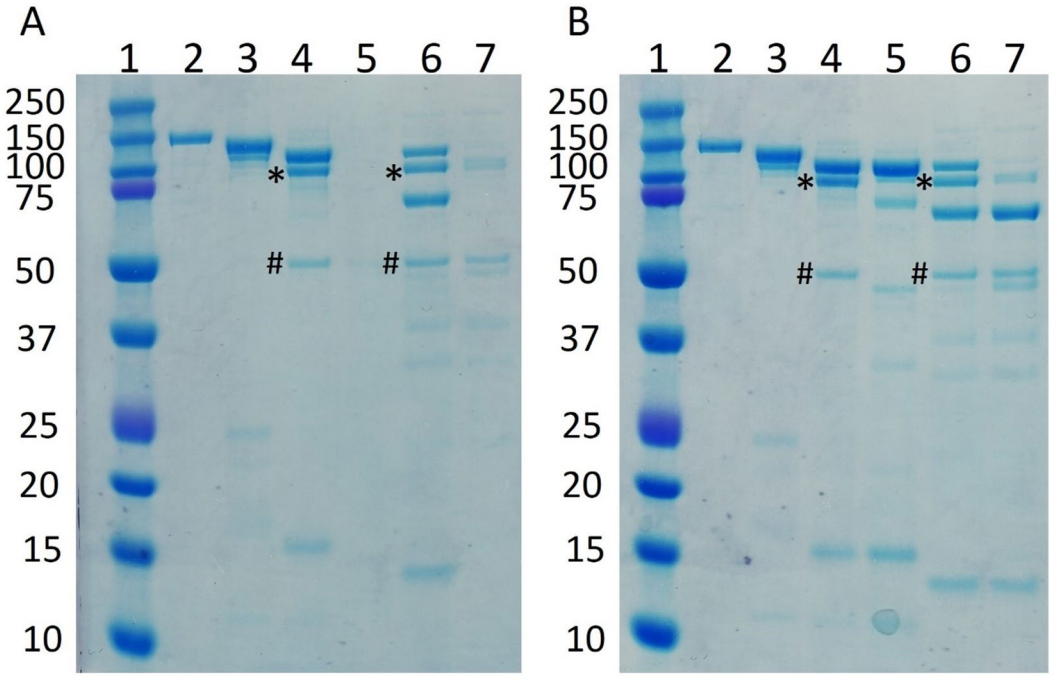

Fig. 4. Protection by NTNH is essential for specific BoNT/A activation by α-clostripain. (**A**) SDS-PAGE analysis for evaluating the activity of α-clostripain and *C. botulinum* A culture supernatant on rBoNT/Ai in the presence or absence of rNTNH. Lane 1–MW marker; Lane 2—rBoNT/Ai; Lane 3–rNTNH; Lane 4 – rBoNT/Ai + rNTNH + rα-clostripain; Lane 5–rBoNT/Ai + rα-clostripain; Lane 6—rBoNT/Ai + rNTNH + *C. botulinum* A culture supernatant; Lane 7—rBoNT/Ai + *C. botulinum* A culture supernatant. All reactions were performed at pH 6.0. (**B**) The protection of BoNT/A by NTNH is pH-dependent and influenced by the BoNT/A-NTNH interaction, which associate under acidic pH and dissociate under alkaline pH. Lane 1–MW marker; Lane 2–rBoNT/Ai; Lane 3–rNTNH; Lane 4—rBoNT/Ai + rNTNH + rα-clostripain at pH 6.0; Lane 5—rBoNT/Ai + rNTNH + rα-clostripain at pH 8.0; Lane 6—rBoNT/Ai + rNTNH + *C. botulinum* A culture supernatant at pH 6.0; Lane 7—rBoNT/Ai + rNTNH + *C. botulinum* A culture supernatant at pH 8.0. The 100-kDa heavy chain of rBoNT/Ai is marked with * and the 50-kDa light chain of rBoNT/A is marked with #.

could be made more toxic by trypsin treatment. This led them to postulate the presence of an endogenous activating protease in the culture. However, to date, the activation mechanism of BoNT/A and the identity of the activating protease have not yet been deciphered.

Previous studies have shed light on some aspects of the botulinum toxin A activation process. Suzuki et al. purified a toxin-nicking protease from the culture medium of *Clostridium botulinum* serotype C strain Stockholm and suggested it to be a clostripain-like protease[26]. In 1990, Dekleva and Dasgupta[27] used BAPNA (N-benzoyl-DL-arginine-p-nitroanilide), a synthetic substrate for trypsin, to isolate a protease from *C. botulinum* A culture supernatant. The isolated protease was not identified by name or sequence, but its properties were similar to α-clostripain. It migrated as two separate bands (48- and 15.5-kDa) in SDS-PAGE, exhibited thermostability, and required a thiol-reducing agent and Ca^{2+} for its activity. However, the authors reported that one hour incubation of BoNT/A with the isolated protease resulted in considerable degradation of the neurotoxin into various fragments. In another report by Dekleva and Dasgupta[28], it was observed that following 45 min of incubation with the isolated protease, the toxin was cleaved into two typical bands. However, no activation was obtained as the toxicity remained similar, indicating that important data remained unknown.

In our study, a comprehensive understanding of the activation mechanism of botulinum toxin A was achieved. We found that α-clostripain is the activating protease of BoNT/A, and revealed that NTNH is essential for toxin activation, as it protects the toxin from non-specific degradation by α-clostripain. Our findings clarify and further support the results of Dekleva and Dasgupta. In the absence of the accessory protein NTNH, the neurotoxin is prone to non-specific proteolysis and therefore degrades into various fragments following one-hour incubation. One possible explanation for the lack of increase in toxicity, even when a shorter incubation time was applied, could be non-specific degradation by the protease during the period between the halting of the reaction by cooling and when it was tested for toxicity.

Our results may also shed light on other previously reported observations. Patterson-Curtis and Johnson[29] found that in cultures of *C. botulinum* Okra B and Hall A, supplementing a minimal medium with a high arginine concentration (20 g/liter) significantly reduces the neurotoxin titer. Inzalaco et al[30] further investigated this phenomenon and discovered that when 2% arginine is added to the toxin production medium (TPM), the pH of the culture becomes more alkaline (pH 7.5–7.8) during the logarithmic growth phase, whereas in cultures grown in standard TPM, the pH decreases. Our finding that NTNH, which dissociates from the toxin under

alkaline pH, protects the toxin from non-specific cleavage by α-clostripain, may explain why the toxin becomes susceptible and undergoes nonspecific degradation in the alkaline environment generated in the presence of arginine in the medium.

rBoNT/Ai was completely degraded by the supernatant of *C. botulinum* culture and α-clostripain (Fig. 3B). This observation is surprising, since for the LCH_N substrate specific cleavage was obtained to yield intact LC and H_N. This result may suggest that the presence of the H_C domain in the intact holotoxin alters the overall structural dynamics, potentially leading to transient exposure of protease-sensitive sites that remain inaccessible or structurally masked in the LCH_N substrate and are shielded by NTNH. Nonetheless, incubation of rBoNT/Ai with lower concentrations of α-clostripain suggested that the protease initially cleaves the toxin into its di-chain form prior to its complete degradation (Fig. 1s).

Examining the 3D model of the predicted non-activated BoNT/A structure in complex with NTNH reveals how NTNH shields the toxin from non-specific digestion while allowing specific cleavage of the activation loop that connects the toxin's LC and HC (Fig. 5). The receptor-binding domain of the toxin is surrounded by a wide groove formed by NTNH, and thereby the access of proteases to susceptible sites on the domain is prevented. The translocation domain of BoNT/A is also in close contact with NTNH. Conversely, the activation loop is distant from NTNH, and thus readily available for proteolysis. Interestingly, the amino acid sequence of the activation loop contains several potential cleavage sites for α-clostripain, which cleaves proteins at the carboxyl peptide bond of arginine residues, and to a lesser extent, at lysine residues. The presence of multiple potential cleavage sites on the activation loop raises the question of whether it is a strategy intended to increase the chances of toxin activation, or whether, in practice, the cleavage occurs at only one site. This remains to be determined.

In this study, the mechanism of BoNT/A activation was elucidated. According to our findings, the activation depends on two factors: 1. The shielding of the neurotoxin by NTNH; and 2. The cleavage of the protected neurotoxin by α-clostripain at a specific site. During the exponential growth of the bacterium, it expresses α-clostripain as a pre-pro-protease and secretes it into the medium, where it undergoes autoactivation[27] (Fig. 6A). As the bacterial growth shifts from a logarithmic to a stationary phase, the expression of the toxin and its related genes increases[31,32], and the acidic pH allows the formation of the toxin complex with the accessory proteins

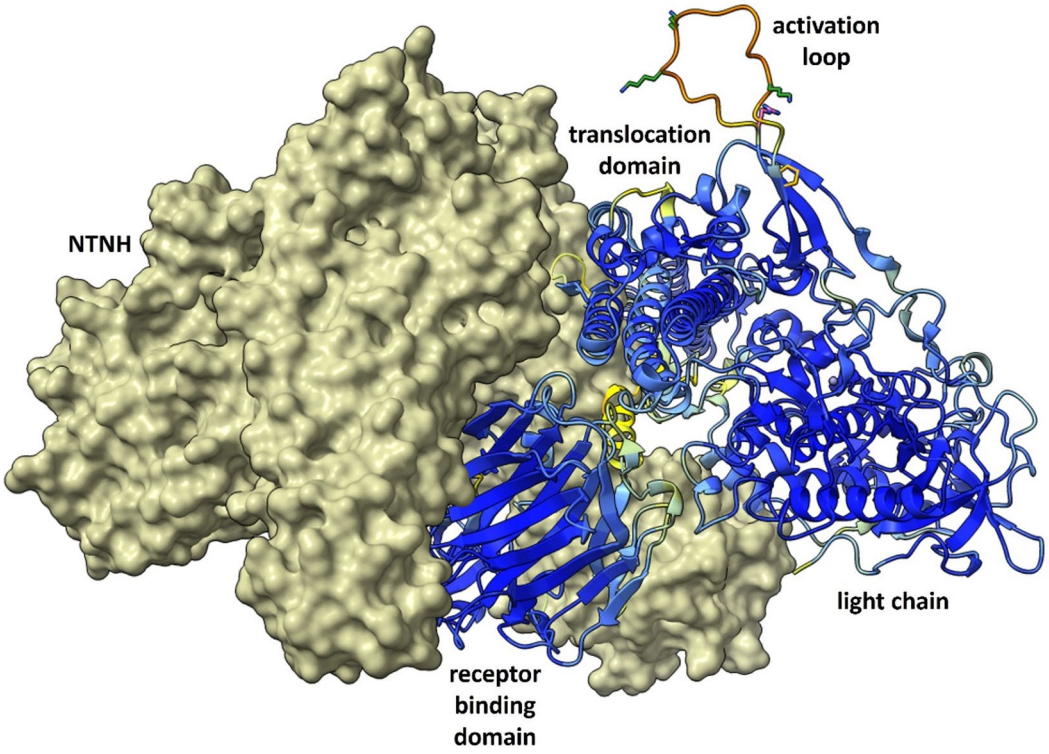

Fig. 5. Protection of BoNT/A by NTNH facilitates specific cleavage for toxin activation. NTNH shields the receptor-binding domain of the toxin while leaving the activation loop, which connects the toxin LC and HC, available for cleavage by proteases. The model was prepared by superposition of a predicted BoNT/A structure (Alphafold code AF-P0DPI1-F1-v4 (Jumper, Evans et al., 2021)) and the BoNT/Ai chain from the crystal structure of the BoNT/A-NTNH complex (PDB code 3v0a (Gu, Rumpel et al., 2012)). NTNH is colored beige and shown in surface display. BoNT/A is in cartoon representation and is colored according to Alphafold's per-residue model confidence score. Confidence levels are represented by a spectrum from blue to red, with blue representing highly confident regions, transitioning to yellow (medium confidence), and red (low confidence). Cys430 and Cys454, which connect the toxin LC and HC, are in stick representation and colored yellow. Arginine (magenta) and lysine (green) residues on the activation loop, which represent potential α-clostripain cleavage sites, are also shown in stick representation.

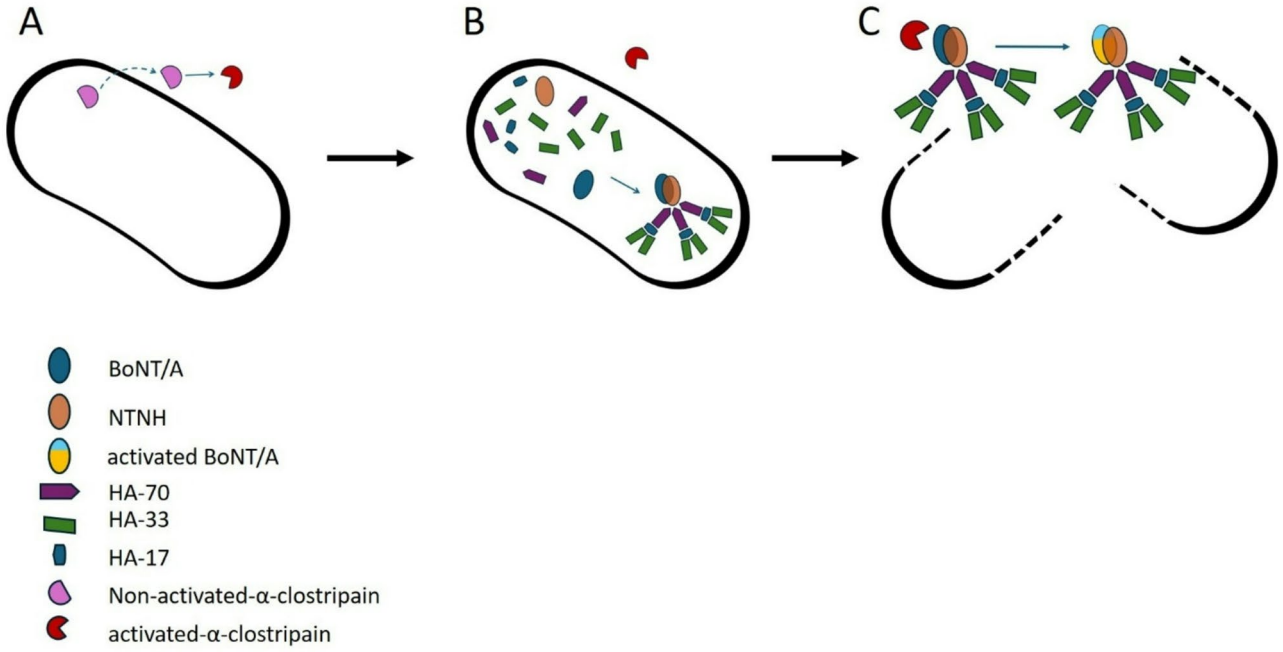

Fig. 6. Suggested mechanism for BoNT/A activation by α-clostripain. (**A**) During the logarithmic growth phase, the bacterium expresses α-clostripain as a pre-pro-protease and secretes it into the medium, where it undergoes autoactivation. (**B**) As the bacterial growth transitions from the logarithmic to the stationary phase, the expression of BoNT/A and its associated proteins increases, and the toxin complex is formed under acidic pH. (**C**) Following the cell lysis phase, the toxin complex is released into the medium, where α-clostripain cleaves protected BoNT/A specifically and activates it.

(Fig. 6B). The differential expression and extracellular activation of α-clostripain allow compartmentalization between active α-clostripain and the toxin, preventing non-specific cleavage and toxin inactivation inside the bacterial cytoplasm. During the lysis phase, the cell's contents are released into the medium, and the shielded toxin is specifically activated by α-clostripain (Fig. 6C).

Elucidating the BoNT activation mechanism has various implications applicable to several fields. Future discovery of edible inhibitors for α-clostripain can prevent toxin maturation in susceptible foods to prevent food poisoning. BoNT activation might also be prevented by the addition of chelators, such as citrate, that bind calcium ions required as co-factors for α-clostripain activity. Furthermore, inactivation of BoNTs may be achieved by shifting the pH from acidic to alkaline, thereby stripping the toxin of NTNH, thus making it vulnerable to proteolysis. External proteases, such as trypsin, may also be added to enhance degradation efficiency.

Additionally, our findings may have biotechnological implications. Pharmaceutical preparations of BoNT/A, such as BOTOX, are widely used as treatments for neurologic indications and for cosmetics. The commercial manufacturing of BoNT/A is based on its natural producer, *C. botulinum*. This process involves extended bacterial cultivation and multistep purification procedure, in which the toxin (either in complex or 'naked' state) is isolated from the entire cellular content released to the medium at the lysis phase. Although this process is well established, it remains relatively labor intensive and yields are inherently limited due to the low natural expression of the toxin. While this has not prevented commercial availability, alternative recombinant strategies offer potential advantages for streamlining production. By harnessing the natural activation mechanism of BoNT/A, it is now possible to generate a fully active recombinant toxin with a sequence identical to the native form, potentially enabling higher yields and simplified downstream processing. These approaches may support future scalability, diversification of products, and safer manufacturing under non-toxic host systems.

Data availability

The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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