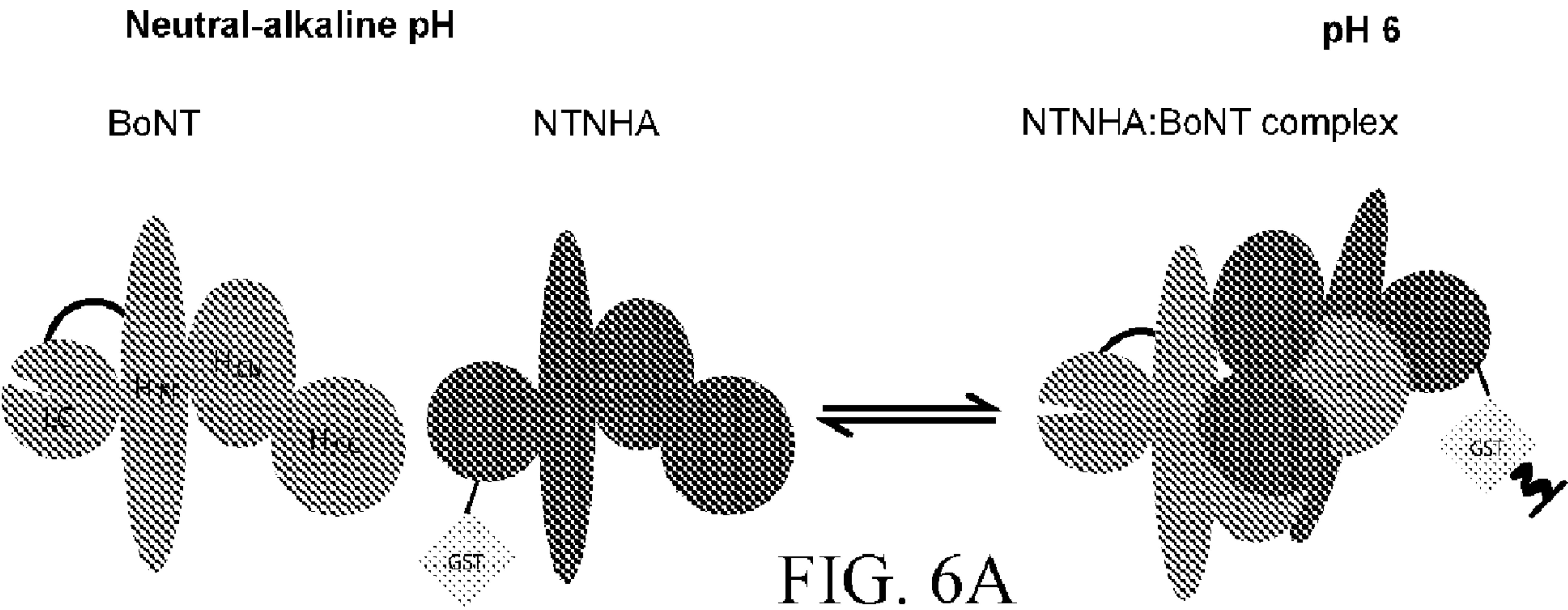




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(57) **Abrégé/Abstract:**
Disclosed herein are methods for the isolation and purification of a botulinum neurotoxin (BoNT) protein, or a polypeptide comprising a receptor binding domain of BoNT, from a solution. The method comprises contacting the solution containing the protein or polypeptide to a matrix which has attached thereto a non-toxic non-hemagglutinin (NTNHA) under conditions appropriate for binding, washing the matrix to thereby remove unbound materials, and eluting the protein or polypeptide with a solution that dissociates the bound protein from the NTNHA. Conditions appropriate for binding are a pH of less than 7.5 (e.g, 6). Conditions appropriate for dissociation are a pH greater than or equal to 7.5 (e.g., 8). Compositions specific to the methods are also disclosed.

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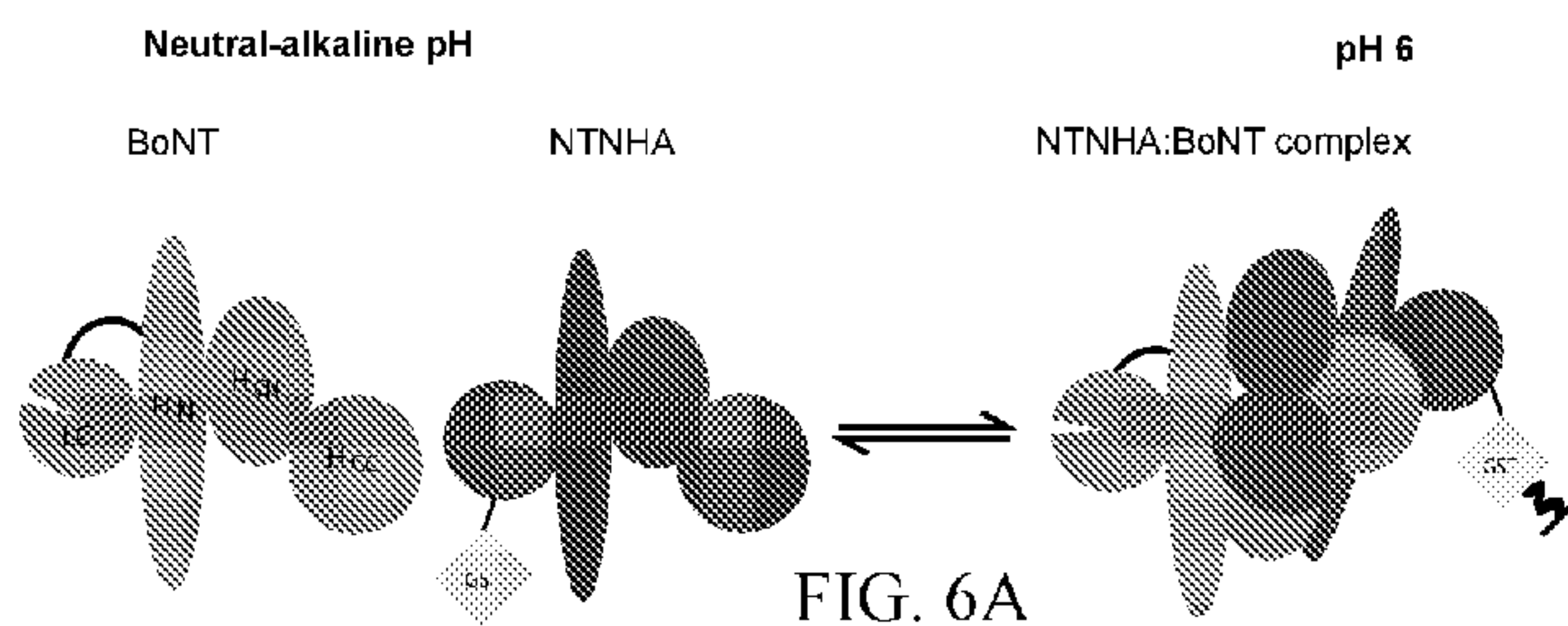
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(54) Title: METHOD FOR PURIFICATION AND ACTIVATION OF BOTULINUM NEUROTOXIN



(57) Abstract: Disclosed herein are methods for the isolation and purification of a botulinum neurotoxin (BoNT) protein, or a polypeptide comprising a receptor binding domain of BoNT, from a solution. The method comprises contacting the solution containing the protein or polypeptide to a matrix which has attached thereto a non-toxic non-hemagglutinin (NTNHA) under conditions appropriate for binding, washing the matrix to thereby remove unbound materials, and eluting the protein or polypeptide with a solution that dissociates the bound protein from the NTNHA. Conditions appropriate for binding are a pH of less than 7.5 (e.g., 6). Conditions appropriate for dissociation are a pH greater than or equal to 7.5 (e.g., 8). Compositions specific to the methods are also disclosed.

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METHOD FOR PURIFICATION AND ACTIVATION OF BOTULINUM NEUROTOXIN

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 62/336,958, filed on May 16, 2016, the content of which is hereby incorporated by reference herein in its entirety.

SEQUENCE LISTING

[0002] The present specification makes reference to a Sequence Listing (submitted electronically as a .txt file named “0342941-0584_SL.TXT” on May 16, 2017). The .txt file was generated on May 16, 2017, and is 96,153 bytes in size. The entire contents of the Sequence Listing are hereby incorporated by reference.

FIELD OF THE INVENTION

[0003] The present invention relates to the field of therapeutic use of neurotoxins.

BACKGROUND OF THE INVENTION

[0004] Botulinum neurotoxins (BoNTs) are the most toxic substances known to humans. Seven serotypes of BoNTs (A–G) have been identified; with many subtypes within each serotype. BoNTs are ~150 kDa proteins produced by different strains of the bacterium *Clostridium botulinum* (Montal 2010). These toxins cause botulism in animals, a severe neurological disease manifested in extreme flaccid paralysis and possible death. The molecular basis of this toxicity lies in the ability of the BoNTs to bind and enter motor neurons and release their enzymatic domain into the cytosol, which cleaves cellular machinery responsible for

synaptic vesicle fusion at neuromuscular junctions (NMJs) and inhibits neurotransmission by blocking acetylcholine release.

[0005] The neuro-inhibitory function of BoNTs was explored as a treatment strategy for many muscular disorders ranging from strabismus to managing multiple dystonias (Masuyer et al. 2014), not to mention the steep increase in cosmetic uses of BoNTs (A) to induce flaccid paralysis in facial muscles to smooth wrinkles. The market for BoNTs is approaching 2 billion dollars and still grows at a fast pace.

[0006] Several challenges in BoNTs productions exist currently. BoNTs need to be produced in bacteria and isolated from bacterial lysates. The current therapeutic BoNTs are still produced and isolated utilizing old methodologies similar to those originating over 50 years ago when the first batch of lab-prepared BoNT/A was described (Bonventre & Kempe 1959; Pickett 2014). These methods typically involve lengthy incubation/fermentation of the natural bacterial strains that produce these toxins (spore-forming clostridium strains) and many subsequent labor-intensive chromatography steps. Aside from the engineering and containment challenges, these processes may also compromise the final yield, efficacy, and reproducibility of BoNT preparations.

[0007] Expressing BoNTs recombinantly from common host systems used for protein production in industry, such as *E.coli* and insect cells has been explored in recent years. An affinity tag, such as His-6 (SEQ ID NO: 1) or GST, is usually fused to BoNTs to facilitate purification via affinity purification. Although isolation of recombinant BoNT with affinity tags simplifies the purification steps, it introduces new problems. The tag may adversely affect biological activity of the toxin and/or have undesired antigenicity. As a result, the tag must be removed after purification, which involves additional enzymatic treatment and purification steps. Furthermore, there are often additional residues left attached to the toxin from the cleaved tag, creating a non-native N- or C-termini which may affect activity or promote immunological consequences in a patient.

[0008] Isolation of natural forms of BoNTs is greatly preferred but remains a labor- and time-intensive process.

[0009] Purified BoNTs must further be activated through limited proteolysis prior to use. Recombinant BoNTs are usually activated post-purification by incubation with an endoproteinase, such as trypsin. Such activation can cause non-specific degradation, and requires an additional purification step to remove the activation endoproteinase, both of which compromise toxin activity and yield.

SUMMARY OF THE INVENTION

[0010] As will be apparent to those skilled in the art reading the present disclosure, the present invention encompasses the recognition of a problem with compositions and methods for production, purification, and/or activation of botulinum neurotoxins (BoNTs) or portions or fragments thereof. Among other things, the present invention identifies challenges in providing materials and procedures that facilitate production, purification, and/or activation of BoNTs with desired characteristics (*e.g.*, relatively uncompromised biological activity; limited introduction of undesired antigenicity; limited contaminants such as undesired endoproteinases and/or degradation products; and high quality, potency, and/or reproducibility of the desired BoNT), while reducing limitations of prior approaches (*e.g.*, limited efficiency of production, time-consuming and/or laborious steps, and/or harsh conditions).

[0011] One aspect of the invention relates to a molecule comprising a non-toxic non-hemagglutinin (NTNHA) polypeptide covalently linked to a heterologous affinity moiety. In one embodiment, the NTNHA and affinity moiety are expressed as a fusion protein. In one embodiment of the compositions disclosed herein, the affinity moiety is located at a position selected from the group consisting of the N-terminus of NTNHA amino acid sequence, the C-terminus of NTNHA amino acid sequence, and internal to the NTNHA amino acid sequence. In one embodiment of the compositions disclosed herein, the affinity moiety effectively binds a binding target under conditions of about pH 6 to about pH 8. In one embodiment of the compositions disclosed herein, the affinity moiety is selected from the group consisting of glutathione -S-transferase (GST), C-myc tag, Chitin-binding domain, Streptavidin binding protein (SBP), Cellulose-binding domain, Calmodulin-binding peptide, S-tag, Strep-tag II, FLA, Protein A, Protein G, histidine affinity tag (HAT), Poly-His, and maltose binding protein (MBP).

In one embodiment of the compositions disclosed herein, the NTNHA is from serotype A, B, C1, D, E, F, or G. In one embodiment of the compositions disclosed herein, the NTNHA is from serotype B. In one embodiment of the compositions disclosed herein, the molecule is in a complex with a compatible Botulinum neurotoxin (BoNT) or a polypeptide comprising a receptor binding domain thereof. In one embodiment of the compositions disclosed herein, the BoNT or the polypeptide comprises a modified receptor binding domain of Clostridial botulinum serotype B (B-H_c). In one embodiment of the compositions disclosed herein, the molecule is further bound to a binding target through the affinity moiety. In one embodiment of the compositions disclosed herein, the binding target is stably attached to a matrix.

[0012] Another aspect of the invention relates to an aqueous solution comprising one of the molecules described herein.

[0013] Another aspect of the invention relates to a nucleic acid that encodes one of the functional NTNHA and affinity moiety fusion protein described herein.

[0014] Another aspect of the invention relates to an expression vector comprising the nucleic acid that encodes one of the functional NTNHA and affinity moiety fusion protein described herein.

[0015] Another aspect of the invention relates to a host cell that comprises and expresses the nucleic acid that encodes one of the functional NTNHA and affinity moiety fusion protein described herein. In one embodiment, the host cell further expresses a compatible Botulinum neurotoxin (BoNT). In one embodiment of the host cells described herein, the BoNT comprises a modified receptor binding domain of Clostridial botulinum serotype B (B-H_c). In one embodiment of the host cells described herein, the host cell is prokaryotic or eukaryotic.

In one embodiment of the host cells described herein, the host cell is a bacterial cell, a yeast cell, a mammalian cell, an insect cell, a plant cell, or an amphibian cell.

[0016] Another aspect of the invention relates to a method of purifying Botulinum neurotoxin (BoNT) comprising contacting the BoNT to a compatible non-toxic non-hemagglutinin (NTNHA), under conditions appropriate for binding of the NTNHA to the BoNT to thereby form a NTNHA-BoNT complex. In one embodiment, the BoNT is in solution, and

the NTNHA is attached to a matrix, whereby the solution is contacted to the matrix to thereby contact the BoNT to the NTNHA. In one embodiment of the methods described herein, the method further comprises washing the matrix to thereby remove unbound materials, and eluting the BoNT from the matrix by contacting the matrix with an aqueous solution that dissociates the BoNT from the NTNHA-BoNT complex. In one alternate embodiment of the methods described herein, following contacting of the BoNT solution to the NTNHA matrix, the method further comprises washing the matrix to thereby remove unbound materials, contacting the matrix with a protease under conditions that preserve the NTNHA-BoNT complex and are appropriate for cleavage of the BoNT within the NTNHA-BoNT complex, washing the matrix to thereby remove the protease and unbound materials, and eluting the BoNT from the matrix by contacting the matrix with an aqueous solution that dissociates the BoNT from the NTNHA-BoNT complex. In one embodiment of the methods described herein, the NTNHA is covalently linked to an affinity moiety, the matrix is linked to a binding target of the affinity moiety, and the NTNHA is non-covalently bound to the matrix through interactions of the affinity moiety and the binding target. In one embodiment of the methods described herein, the NTNHA is covalently linked to the matrix. In one embodiment of the methods described herein, the BoNT comprises a modified receptor binding domain of Clostridial botulinum serotype B (B-H_c). In one embodiment of the methods described herein, the aqueous solution that dissociates the BoNT from the NTNHA-BoNT complex has a pH of ≥ 7.5 . In one embodiment of the methods described herein, the solution comprising the BoNT is a cleared cell extract from BoNT expressing cells. In one embodiment of the methods described herein, the cleared cell extract further comprises 1 mM of phenylmethylsulfonyl fluoride (PMSF). In one embodiment of the methods described herein, conditions appropriate for binding comprise contacting the BoNT in the context of a binding buffer which has a physiological ionic strength and a pH of < 7.5 . In one embodiment of the methods described herein, washing is with a wash buffer that is of physiological ionic strength with a pH of < 7.5 . In one embodiment of the methods described herein, the binding buffer and/or wash buffer is between 100-200 mM KCl or NaCl. In one embodiment of the methods described herein, the binding buffer and/or wash buffer has a pH of about 6. In one embodiment of the methods described herein, the binding buffer and/or wash buffer comprises 50 mM MES, 150 mM NaCl, pH 6. In one embodiment of the methods

described herein, the aqueous solution that dissociates the BoNT from the NTNHA-BoNT complex is an elution buffer of about 50 mM Tris, 150 mM NaCl. In one embodiment of the methods described herein, the aqueous solution is an elution buffer of about pH 8. In one embodiment of the methods described herein, the affinity moiety is selected from the group consisting of glutathione -S-transferase (GST), C-myc tag, Chitin-binding domain, Streptavidin binding protein (SBP), Cellulose-binding domain, Calmodulin-binding peptide, S-tag, Strep-tag II, FLA, Protein A, Protein G, histidine affinity tag (HAT), Poly-His, and maltose binding protein (MBP).

[0017] In one embodiment of the methods described herein, the affinity moiety is GST, and the binding target is glutathione.

[0018] In one embodiment of the methods described herein, the NTNHA is present at a molar ratio between about 1:1 and about 10:1 to the BoNT, for example about 2:1, 3:1, 4:1 or 5:1 to the BoNT. In one embodiment of the methods described herein the BoNT and the NTNHA are co-expressed in the same host cell, for example *E. coli*. In one embodiment of the methods described herein the BoNT and the NTNHA are expressed in different host cells. In one embodiment of the methods described herein the BoNT is produced in a recombinant manner in a heterologous host cell such as *E. coli*. In one embodiment of the methods described herein the BoNT is produced in its native Clostridial cell. In one embodiment of the methods described herein the NTNHA is produced in a recombinant manner in a heterologous host cell such as *E. coli*. In one embodiment of the methods described herein the NTNHA is produced in its native Clostridial cell.

[0019] In one embodiment of the methods described herein, the protease is selected from trypsin, pepsin, Lys-C endoproteinase, Lys-N endoproteinase, . arginyl endopeptidase, plasmin, omptin and a clostridial protease as described in EP2524963. In a preferred embodiment, the protease is trypsin or Lys-C endoproteinase. In one embodiment, the protease is a protease that cleaves a BoNT non-native (i.e. exogenous) cleavage site. In such clostridial toxins, the native protease cleavage site (also known as the activation site) is modified or replaced with a protease cleavage site that is not native to that clostridial toxin. Non-native proteases that may be employed include Enterokinase (DDDDK↓ (SEQ ID NO: 2)), Factor Xa (IEGR↓ (SEQ ID NO:

3)/ IDGR↓(SEQ ID NO: 4)), TEV(Tobacco Etch virus) (ENLYFQ↓G (SEQ ID NO: 5)), Thrombin (LVPR↓GS (SEQ ID NO: 6)) and PreScission (LEVLFQ↓GP (SEQ ID NO: 7)).

[0020] In one embodiment of the methods described herein, the protease is added at a molar ratio of from about 1:2 to about 1:1000 to the NTNHA, preferably from about 1:5 to about 1:100 to the NTNHA, for example about 1:10, 1:20, 1:30, 1:40 or 1:50. In one embodiment of the methods described herein, the protease is added at a molar ratio from about 1:2 to about 1:1000 to the BoNT, preferably from about 1:5 to about 1:100 to the BoNT, for example about 1:10, 1:20, 1:30, 1:40 or 1:50. Appropriate conditions for the specific protease used will be determined by the skilled practitioner. The length of time for exposure to the protease will also vary with the protease, the concentration used, and the temperature. In one embodiment of the methods described herein, the protease is contacted to the matrix at a temperature from about 2°C to about 40°C, preferably from about 4°C to about 37°C, for example 4°C, 16°C, 20°C or 37°C. In one embodiment of the methods described herein, the protease is contacted to the matrix at room temperature (about 20-22°C). In one embodiment of the methods described herein, the protease is contacted to the matrix from about 10 minutes to about 18 hours, preferably from about 30 minutes to about 5 hours for example about 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours or 5 hours. In one embodiment of the methods described herein, the protease is contacted to the matrix at a pH of about 5.5 to about 8.5, preferably from about 6 to 8, for example at a pH of about 6, 7 or 8. In one embodiment, the protease is selected from the proteases: trypsin and Lys-C endoproteinase, and is contacted to the matrix at room temperature for about 30 minutes to 2 hours at a pH between 6 and 7.

[0021] In one embodiment of the methods described herein, the protease is added at a molar ratio of about 1:10 to the NTNHA. In one embodiment of the methods described herein, the protease is contacted to the matrix at room temperature. In one embodiment of the methods described herein, the protease is contacted to the matrix for about 30 minutes to 12 hours.

[0022] Another aspect of the invention relates to a method of purifying Botulinum neurotoxin (BoNT) comprising contacting a cleared cell extract comprising the BoNT to a glutathione coated matrix which has attached thereto a compatible non-toxic non-hemagglutinin (NTNHA) fused to glutathione-S-transferase, in a binding buffer with a pH of about 6 to thereby form a NTNHA-BoNT complex, washing the matrix with a wash buffer with a pH of about 6 to

thereby remove unbound materials, contacting the matrix with a protease in a buffer with a pH of about 6 to thereby cleave the BoNT within the NTNHA-BoNT complex, washing the matrix with a wash buffer with a pH of about 6 to thereby remove the protease and unbound materials, and eluting the BoNT from the matrix by contacting the matrix with an elution buffer that has a pH of ≥ 7.5 to thereby dissociate the BoNT from the NTNHA-BoNT complex. In one embodiment of the methods described herein, the BoNT comprises a modified receptor binding domain of Clostridial botulinum serotype B (B-H_c). In one embodiment of the methods described herein, the binding buffer and/or wash buffer comprises 50 mM MES, 150 mM NaCl. In one embodiment of the methods described herein, the binding buffer further comprises 1 mM phenylmethylsulfonyl fluoride (PMSF). In one embodiment of the methods described herein, the elution buffer comprises 50 mM Tris, 150 mM NaCl, and has a pH of about 8. In one embodiment of the methods described herein, the glutathione coated matrix is glutathione-linked agarose beads. In one embodiment of the methods described herein, the glutathione coated matrix is a column. In one embodiment of the methods described herein, the glutathione coated matrix has about 5 mg/ml bound NTNHA. In one embodiment of the methods described herein, the protease is trypsin or Lys-C endoproteinase.

[0023] Another aspect of the invention relates to a method of purifying a polypeptide comprising a receptor binding domain (Hc polypeptide) of Botulinum neurotoxin, comprising the steps contacting a solution comprising the Hc polypeptide to a matrix which has attached thereto compatible non-toxic non-hemagglutinin (NTNHA), under conditions appropriate for binding of the NTNHA to the Hc-polypeptide to thereby form a NTNHA-Hc polypeptide complex, washing the matrix to thereby remove unbound materials, and eluting the Hc polypeptide from the matrix by contacting the matrix with an aqueous solution that dissociates the Hc polypeptide from the NTNHA-Hc polypeptide complex. In one embodiment of the methods described herein, the receptor binding domain of the Hc polypeptide is a modified receptor binding domain of Clostridial botulinum serotype B (B-H_c). In one embodiment of the methods described herein, the Hc polypeptide is a Botulinum neurotoxin (BoNT) polypeptide. In one embodiment of the methods described herein, the Hc polypeptide is a chimeric Botulinum neurotoxin (BoNT) polypeptide.

[0024] Another aspect of the invention relates to the use of a molecule described herein in a method or purifying a Botulinum neurotoxin (BoNT) polypeptide.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] This patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing (s) will be provided by the Office upon request and payment of the necessary fee.

[0026] FIG. 1A and FIG. 1B is an illustration of an embodiment of a purification principle and protocol for BoNTs as described herein. FIG. 1A) Schematic illustration of pH-dependent bi-molecular complexation of BoNTs and NTNHA. LC: light chain, HN: translocation domain, HCN, HCC: N-terminal and C-terminal segments of the receptor binding domain, respectively. NTNHA has the same domain contents as BoNTs, it is shown as a GST (Glutathione-S-transferase) fused protein immobilized on Glutathione-Agarose resin. FIG. 1B) A flow chart describing a comprehensive purification, activation, and elution protocol of BoNTs using their natural binding partner NTNHA.

[0027] FIG. 2A and FIG. 2B are images of gel fractionated proteins. The experimental results indicate the successful purification of BoNT/B using NTNHA/B as a model complex for BoNTs. FIG. 2A) A monoclonal antibody against BoNT/B is used to monitor the presence of BoNT/B along each purification step described in FIG. 1B (except that samples here were not treated with trypsin). FIG. 2B) An SDS-PAGE gel of selected samples stained with Coomassie shows the purity of BoNT/B purified as described in panel A. A major band (~150 kDa) corresponding to BoNT/B is observed in Elution fraction.

[0028] FIG. 3A and FIG. 3B are images of two sets of gel fractionated proteins. The experimental results indicate that BoNT/B is efficiently activated in NTNHA/B•BoNT/B complexes. FIG. 3A) Representative immunoblot of NTNHA-bound BoNT/B activation by trypsin, which separates BoNT/B into two fragments (100 kDa and 50 kDa, respectively). The two fragments of BoNT/B remain attached with each other by a single disulfide bond. They separate from each other when DTT is added to reduce disulfide bond. FIG. 3B) Coomassie

stained Elution fraction shows the toxin bands corresponding to the cleaved toxin fragments (at 100 and 50 kDa, respectively). The 150 kDa band is the portion of full-length toxin that remains to be cleaved.

[0029] FIG. 4 is an image of gel fractionated proteins. The experimental results establish successful purification of chimeric BoNT/A1B toxin using NTNHA/B. A polyclonal antibody against BoNT/A was used to track the purification steps of a chimeric toxin BoNT/A1B, which is made of BoNT/A1 light chain and translocation domain, with the receptor binding domain from BoNT/B. Full-length BoNT/A1B (the 150 kDa band in the Elution fraction) was successfully purified and eluted using NTNHA/B. We note that the prominent band at 100 kDa is a degradation product of this chimeric toxin, likely cut by endogenous proteases in *E.Coli*.

[0030] FIGs. 5A-5I (SEQ ID NO.s 22-30) is a list of the amino acid sequences of various serotypes of NTNHA, and variants thereof.

[0031] FIGs. 6A-6C is an illustration of an embodiment of a purification principle and protocol for BoNTs, as described herein. FIG. 6A) Schematic illustration of a pH-dependent bi-molecular complexation of BoNTs and NTNHA. LC: light chain, H_N: translocation domain, H_{CN}, H_{CC}: N-terminal and C-terminal segments of the receptor binding domain, respectively. NTNHA has the same domain contents as BoNTs and is shown as a GST (Glutathione-s-transferase) fused protein that may be immobilized on Glutathione-Agarose resin. Interaction between BoNT and NTNHA under slightly acidic conditions (for example, ~pH 6), can be disrupted by manipulating the buffer conditions toward a neutral-alkaline pH. FIG. 6B) BoNT isolation and activation protocol. A flow diagram describing a strategy for purification, activation, and elution of tagged and untagged BoNTs from crude lysates using NTNHA. FIG. 6C) SDS-PAGE analysis of a typical isolation of an inactive BoNT (BoNT/B_(RY)) from clarified *E.coli* lysate using GST-NTNHA/B immobilized on Glutathione agarose beads. Binding and wash steps were performed at pH 6 and eluting by exchanging the buffer to pH 8.

[0032] FIG. 7A and FIG. 7B show isolated BoNT/B using immobilized NTNHA is pure and binds its canonical neuronal receptor. FIG. 7A) SDS-PAGE analysis (left) shows three elution fractions that are pooled and concentrated (lane 5). A monoclonal antibody against BoNT/B to detect the toxin in all steps (WB, right). The eluted fractions contain non-activated

BoNT/B as the major band at ~150kDa corresponding to a single-chain BoNT/B_(RY) toxin. FIG. 7B) Anisotropy-detected binding: the eluted full-length toxin shows similar affinity to a FITC-tagged fragment of its canonical synaptic vesicle receptor Synaptotagmin 1 (Syt 1) as its recombinant H_C domain; BoNT/A H_C does not bind Syt. Error bars represent mean + SEM of 3 samples.

[0033] FIGs. 8A-8C show complexed BoNT is efficiently activated yet protected from non-specific cleavage. FIG. 8A) Trypsin-mediated activation (cleavage) of BoNT/B_(RY) is visualized on an 8% SDS-PAGE. Time-course cleavage of the single chain (SC) toxin results in two fragments: Heavy chain (HC) and Light Chain (LC) linked by a single disulfide bond. FIG. 8B) WB analysis shows that activation of BoNT/B while complexed with NTNHA/B protects it from non-specific trypsinization while allowing for efficient washing and removal of endoproteinase. FIG. 8C) Lys-C endoproteinase can also be used as a specific activator to produce active, dichain toxins using this method.

[0034] FIG. 9 shows isolation of chimeric BoNT/A1B1 toxin using NTNHA/B. A polyclonal antibody against BoNT/A is used to track the purification of a chimeric toxin made of BoNT/A (LC_(RY), H_N) fused to BoNT/B H_C domain. Eluted fractions contain non-activated BoNT/A1B1 protein at ~150 kDa. The prominent band at ~70 kDa is likely a fragment of NTNHA/B that is recognized by the polyclonal antibody.

DETAILED DESCRIPTION OF CERTAIN EMBODIMENTS THE INVENTION

[0035] Botulinum neurotoxins (BoNTs) are highly potent protein toxins produced by spore-bearing *Clostridium botulinum*. In the last few decades, these deadly agents have been found useful in treating numerous neuromuscular disorders and in aesthetic applications by blocking neurotransmitter release in the injected muscles. Now established therapeutic agents, BoNTs are widely produced in large scales by several manufacturers around the world. Available data suggest manufacturing procedures rely on decades-old methodologies that utilize sporulating strains, and toxin isolation is achieved by many laborious and inefficient bulk

purification steps. An improved method for the direct purification and activation of therapeutic BoNTs is needed.

[0036] Botulinum neurotoxins (BoNTs) are the most toxic substances known to man. Seven serotypes of BoNTs (A–G) proteins have been identified as ~150 kDa products of different strains of the bacterium *Clostridium botulinum* (Montal 2010). These toxins cause botulism in animals, a severe neuromuscular disease manifested in extreme flaccid paralysis. The molecular basis of this toxicity lies the toxins' ability to potently bind receptors on motor neurons at the neuromuscular junction (NMJ), internalize by endocytosis, and traverse the endosomal membrane to release their enzymatic chain into the cytosol. The released protease then cleaves the cellular machinery (SNARE proteins) responsible for synaptic vesicle fusion at the NMJ, thus inhibiting neurotransmission by blocking acetylcholine release (Blasi et al. 1993; Borden Lacy et al. 1998; Rossetto et al. 2014).

[0037] Botulinum neurotoxins (BoNTs) can also be used as tools to locally control muscle activities, especially uncontrolled activities or abnormalities due to muscular spasticity (Masuyer et al. 2014). This neuro-inhibitory function of BoNTs was explored as a treatment strategy for many muscular disorders, including strabismus and managing multiple dystonias and lower urinary tract dysfunctions (LUTD) (Jankovic & Brin 1991; Truong & Jost 2006; Visco et al. 2012; Jiang et al. 2015). As a therapeutic and/or cosmetic agent, BoNTs can be used to paralyze facial muscles for the purpose of smoothing wrinkles (Hexsel et al. 2011). Additional applications of the toxins aim to alleviate depression and prophylactic treatment of migraines (Finzi & Rosenthal 2014; Jackson et al. 2012). The clinical uses of the toxin have garnered much public interest (Sifferlin 2017).

[0038] Botulinum neurotoxins (BoNTs) can be isolated from a growth of spore-forming clostridium strain and subsequently purified to a final product (Pickett 2014). The available data on BoNTs production processes and isolation suggests that producers utilize methodologies of culture and growth conditions in native strains similar to those originating decades ago (Pickett & Perrow 2009; Snipe & Sommer 1928; Duff, Wright, et al. 1957; Duff, Klerer, et al. 1957; Bonventre & Kempe 1959; Schantz & Johnson 1992; Pickett 2014). Such methods are limited by the efficiency in which the native clostridium strain can produce the toxin and typically involve lengthy fermentation periods of the natural source of the toxin (spore-producing

clostridium strains) followed by laborious toxin-isolation procedures often under harsh conditions involving several acid/alcohol precipitations, crystallizations and/or multiple chromatographic steps (DasGupta & Boroff 1967; Tse et al. 1982; Schantz & Johnson 1992; Malizio et al. 2000).

[0039] Producing tagged BoNTs recombinantly is feasible with the inclusion of an affinity tag (e.g. His_{6X} or GST-fusion) to aid in toxin purification using affinity chromatographies. Such approaches, however, have disadvantages, for example in use of BoNTs as therapeutic biologics. For instance, the affinity tags may adversely affect biological activity of the toxin and/or have undesired antigenicity. Removal of a tag after purification also requires additional enzymatic and purification steps while producing non-native N- or C-termini in the final product. Further, recombinant BoNTs need to be activated post-purification by an endoproteinase to obtain functional and potent dichain toxins. This proteolytic step leads to non-specific degradations which necessitates additional purification steps to remove endoproteinases and or degradation products. Aside from engineering and containment challenges for toxin production from spore-forming strains and subsequent purifications (Malizio et al. 2000; Pickett 2014), these recombinant approaches can compromise most properties in the final product, ranging from quality and potency to efficient reproducibility. A new strategy to safely and efficiently isolate active therapeutic BoNTs would be beneficial for large-scale production and facile isolation of BoNTs.

[0040] Studies on the biochemical properties and cellular mechanisms by which clostridial neurotoxins gain entry to the neuronal cytosol have provided some understanding of the structural, molecular, and mechanistic functions of clostridial neurotoxins (Blasi et al. 1993; Borden Lacy et al. 1998; Dong et al. 2006; Rossetto et al. 2014). Foodborne botulisms require passage of intact toxins and other products of the bacterium through a host's gastrointestinal tract. The molecular and structural basis of this ability to avoid degradation remained a mystery until recently, when larger complexes called "progenitor toxin complexes" (PTCs) were characterized to constitute the whole toxic agent encountered by a target organism. In addition to the proteolytically active toxin, these multi-protein complexes are typically comprised of a serotype-specific non-toxic non-hemagglutinin (NTNHA) protein and three hemagglutinin proteins (HAs) (Lee et al. 2014). Previously considered to aid toxin functions (Schantz &

Johnson 1992), PTCs are now known to physically shield and protect BoNTs from the harsh gastrointestinal environment to safely reach its destinations: first to epithelial barriers and subsequently to NMJs where it can be internalized into the cytosol via synaptic vesicle recycling mechanisms. In a structural study (Gu et al. 2012), Gu and coworkers indicated in atomic details a minimally effective PTC (m-PTC) in a non-covalent complex of BoNT/A:NTNHA/A. The co-crystal structure of the toxin:NTNHA complex indicated a pH-dependent complex formation. BoNT/A and NTNHA/A were reported to be able to form a tight complex with nanomolar-level affinity under slightly acidic conditions (~pH 6). However, such complex formation was said not to occur at neutral-alkaline pH.

[0041] Disclosed herein are compositions and methods relating to the purification of BoNT which utilize the natural affinity of the BoNT molecule for the non-toxic non-hemagglutinin (NTNHA) protein. BoNT naturally forms a dimer complex with the NTNHA chaperone protein and is protected from protease and acidic degradation in the gastrointestinal tract. The binding is reversible and is dependent on pH, binding at $\text{pH} < 7$, and dissociating at $\text{pH} > 7.4$. The NTNHA protein is added to a mixture containing the BoNT at a pH that promotes binding. The BoNT:NTNHA complex is isolated from other components of the mixture by immobilization of the NTNHA within the complex. Following washing, the BoNT is then released from the complex by raising the pH to promote dissociation. Since this method does not rely on an affinity modification of the BoNT, un-tagged forms of the toxin can be purified.

[0042] The purification methods described herein also make possible activation of the BoNT while in the BoNT:NTNHA complex. Following activation, the BoNT can be released from the complex thereby generating a purified, activated form of the toxin.

[0043] Aspects of the invention relate to a method of purifying a BoNT. Typically the BoNT is in the context of an aqueous solution containing contaminating components, such as a cellular extract. The method comprises combining the solution with the NTNHA molecule under conditions appropriate for binding of the BoNT to the NTNHA. Practically, this can involve combining the NTNHA molecule with the aqueous solution (e.g., cellular extract or a cleared cellular extract). The BoNT can be isolated by virtue of the NTNHA molecule. Generally this is accomplished by immobilization of the NTNHA to a matrix. Unbound materials are removed from the complex, for example, by washing the matrix (e.g, using a wash buffer amount of 3-4

volumes of the matrix). Following washing, the BoNT is released from the complex, for example, by elution from the matrix bound NTNHA, to produce a purified polypeptide.

[0044] The BoNT can be activated prior to release from the complex by digestion with a protease. This can be accomplished by contacting the matrix bound complex with a protease under conditions appropriate for cleavage of the BoNT that do not otherwise disrupt the complex (e.g., that preserve the required pH). The protease is eliminated along with other unbound materials, by washing the matrix (e.g., with a wash buffer). The activated, purified BoNT can then be eluted by contacting the matrix with an aqueous solution that dissociates the BoNT from the NTNHA complex (e.g., with an elution buffer). In some embodiments, activation of the polypeptide is not required or desired.

[0045] The NTNHA used in the method must be compatible with the BoNT. The term compatible, when used in reference to the NTNHA and the BoNT, refers to the molecules being able to form a tight and stable complex with one another. In one embodiment, the BoNT and the NTNHA are components of the same naturally occurring BoNT serotype protein complex. This occurs when the BoNT and NTNHA coding sequences are from the same operon. As the term “serotype” is used herein to describe the NTNHA molecule, being “from a serotype” refers to an NTNHA molecule derived from an operon which encodes a specific serotype of BoNT. Compatible may also refer to a BoNT or chimeric polypeptide having a region (e.g., the Hc region) that is compatible with the NTNHA. In one embodiment, the NTNHA and the Hc region of the BoNT are both derived from the same naturally occurring BoNT serotype complex.

[0046] Immobilization of the NTNHA to the matrix can occur prior to or following binding of the BoNT. In one embodiment, the NTNHA is attached to a matrix and a solution comprising the BoNT is added to the matrix to thereby contact the Hc polypeptide to the NTNHA and promote complex formation. In one embodiment, the NTNHA and BoNT are in a complex prior to attachment of the NTNHA to the matrix.

[0047] In one embodiment, an affinity moiety is introduced onto the NTNHA protein (e.g., by expression as a fusion protein), and the tagged protein is used to bind and isolate the BoNT under conditions that promote the BoNT:NTNHA binding. The BoNT:NTNHA complex is isolated by affinity purification of the NTNHA within the complex.

[0048] Binding buffers, incubation buffers, wash buffers, and protease digestion buffers, will promote conditions appropriate for formation and preservation of the Hc-NTNHA complex. This includes, without limitation, having a pH that promotes complex formation. Typically this will be a pH of less than 7.5, for example less than 6. In one embodiment, the buffer pH is from 2-8. In one embodiment, the buffer pH is from 5-7. In one embodiment the pH is about 5, about 6, or about 7. The binding buffers, incubation buffers and wash buffers may all be highly similar or the same. The buffers may further contain additional components other than those specified herein. In one embodiment, the buffer further contains a stabilizing agent for the BoNT polypeptide (e.g., serum albumin, polysaccharide, trehalose, or surfactant). The pH of the buffers can be optimized for the various components therein within the specified ranges. The skilled practitioner will appreciate that buffer pH should preserve the overall protein structure, avoiding a pH that approaches the PI of the protein which may precipitate the protein.

[0049] The buffers will preferably have physiological ionic strength (e.g., within the range of 100-200 mM KCl or NaCl). A variety of salts are available to create the required ionic strength. Salt concentrations that are too high may disrupt the interactions due to polar/ionic interference. In one embodiment, the salt concentration is 400 mM or less. Conditions of low salt are also expected to work sufficiently. In one embodiment, the salt concentration is 150 mM. In one embodiment, the buffer comprises 50mM MES, 150 mM NaCl, and has pH 6. In one embodiment, the buffer in which binding occurs (binding buffer) further comprises one or more protease inhibitors (e.g., phenylmethylsulfonyl fluoride (PMSF)). In one embodiment, the binding buffer comprises PMSF at a concentration of from about 0.1 to 1 mM. In one embodiment, the PMSF is about 1 mM.

[0050] Washing can be performed, for example, using a wash buffer. A typical amount for washing is 3-4 volumes of the matrix.

[0051] The BoNT molecule contains several domains and binds to the NTNHA molecule through its receptor binding domain (otherwise referred to as the Hc domain). As such, the herein described methods are applicable to the purification of any polypeptide comprising a receptor binding domain (Hc polypeptide) of Botulinum neurotoxin (e.g, full length BoNT or fragment thereof comprising the Hc polypeptide, or a chimeric polypeptide comprising the Hc domain).

[0052] In one embodiment of the methods described herein, the NTNHA is present at a molar ratio between about 1:1 and about 10:1 to the BoNT or the receptor binding domain thereof, for example about 2:1, 3:1, 4:1 or 5:1 to the BoNT or receptor binding domain thereof.

[0053] Activation of bound BoNT or a fragment thereof, is achieved by contacting the BoNT:NTNHA complex (e.g., when bound to the matrix) with an appropriate protease. In one embodiment, the protease cleaves a protein after a lysine residue. In one embodiment, the protease is, without limitation, trypsin, pepsin, Lys-C endoprotease, Lys-N endoprotease, arginyl endopeptidase, plasmin, omptin, or the clostridial protease as described in EP2524963. Preferred conditions will result in no substantial degradation of the NTNHA, of any associated affinity moieties, or of their binding target. Conditions appropriate for cleavage include the appropriate concentration of protease, and the appropriate conditions for activity of the protease (e.g., temperature, incubation time, buffer components, etc.). Such conditions can be achieved by use of an appropriate protease digestion buffer. The amount of the protease used can be determined by the amount of the NTNHA molecule or by the amount of the BoNT molecule. In one embodiment, the protease is present at a molar ratio of from about 1:2 to about 1:1000 to the NTNHA molecule. In one embodiment, the protease is present at a molar ratio of from about 1:5 to about 1:100 to the NTNHA molecule, for example, about 1:10, 1:20, 1:30, 1:40, or 1:50. In one embodiment of the methods described herein, the protease is added at a molar ratio from about 1:2 to about 1:1000 to the BoNT (e.g., from about 1:5 to about 1:100 to the BoNT), or about 1:10, 1:20, 1:30, 1:40 or 1:50.

[0054] Appropriate conditions for the specific protease used will be determined by the skilled practitioner. The length of time for exposure to the protease will also vary with the protease, the concentration used, and the temperature. In one embodiment, the protease is contacted at a temperature between 2 °C and 40 °C, preferably between 4 °C and 37 °C, (e.g., 4 °C, 16 °C, 20 °C, or 37 °C). In one embodiment, the protease is contacted at room temperature (about 20-22 °C).

[0055] In one embodiment, the protease is contacted for about 10 minutes to about 18 hours, preferably between 30 minutes and 5 hours (e.g., about 30 minutes, 1 hour, 2, 3, 4 or 5 hours). In one embodiment, the protease is contacted for about 4 hours. In one embodiment the protease is Lys-C endoprotease and the incubation time is about 30 minutes.

[0056] In one embodiment, the protease is contacted to the matrix at a pH of about 5.5 to about 8.5. In one embodiment, the protease is contacted to the matrix at a pH of about 6 to about 8, (e.g., about 6, 7 or 8).

[0057] In one embodiment, the protease is selected from the proteases trypsin and Lys-C endoproteinase, and is contacted to the matrix at room temperature for about 30 minutes to 2 hours at a pH between 6 and 7.

[0058] Elution of the BoNT from the BoNT-NTNHA complex is accomplished using an aqueous solution with a pH that promotes dissociation of the complex (referred to herein as an elution buffer). Preferably, the elution buffer disrupts the BoNT-NTNHA complex by being of the appropriate pH, while otherwise substantially preserving the integrity of the Hc polypeptide and substantially preserving the immobilization of the NTNHA (e.g., preserving binding of the NTNHA to a matrix). The elution buffer will further preferably have physiological ionic strength. A variety of buffers available are appropriate for use (e.g., Tris, MOPS, HEPES, phosphate buffer, etc). In one embodiment, the elution buffer is the same as the binding and/or wash buffer, differing only in pH. In one embodiment, the elution buffer is about 50 mM Tris, 150 mM NaCl with an appropriate pH discussed herein (e.g., pH 8).

[0059] The elution buffer used (e.g., those described herein) can be from about pH 7 to about pH 11. In one embodiment, the pH is 7.5 or greater. In one embodiment, the pH is about 8. The elution buffer may further contain additional components other than those specified herein. The pH of the elution buffer can be optimized for the various components therein.

[0060] Typically the BoNT is purified from a cellular extract. In one embodiment, the cellular extract is a cleared cellular extract. The term “cleared cellular extract” refers to the extract being substantially free of all particulate matter such as when removed by centrifugation and/or filtration.

[0061] The BoNT and the NTNHA may be co-expressed in the same host cell, for example *E. coli*. The method may utilize the NTNHA expressed therein with the BoNT. Alternatively, the BoNT and the NTNHA can be expressed in different host cells. The respective cell extracts can be used to produce/isolate the respective proteins. The BoNT can be produced in a recombinant manner in a heterologous host cell such as *E. coli*, or produced in its

native Clostridial cell. The NTNHA can be produced in a recombinant manner in a heterologous host cell such a *E. coli*, or in its native Clostridial cell.

[0062] “Purification”, or “purified”, as used herein, refers to a BoNT or fragment thereof that is "substantially pure", with respect to other components of a preparation (e.g., other polypeptides). It can refer to a BoNT or fragment that is at least about 50%, 60%, 70%, or 75%, preferably at least about 85%, more preferably at least about 90%, and most preferably at least about 95% pure, with respect to other components. Recast, the terms "substantially pure" or "essentially purified", with regard to a BoNT or fragment, refers to a preparation that contains fewer than about 20%, more preferably fewer than about 15%, 10%, 8%, 7%, most preferably fewer than about 5%, 4%, 3%, 2%, 1%, or less than 1%, of one or more other components (e.g., other polypeptides or cellular components).

[0063] Other aspects of the invention relate to the components used in the methods described herein. One aspect of the invention relates to the NTNHA polypeptide used to bind the BoNT. The NTNHA polypeptide can be full length NTNHA, or a functional fragment thereof. A functional fragment of NTNHA is considered to retain the binding property to the compatible BoNT Hc domain, and protect the BoNT from degradation, while allowing activation. The NTNHA polypeptide may further comprise additional heterologous amino acids. As the term is used herein, heterologous refers to a molecule of a different origin. For example, a heterologous affinity moiety differs from any internal affinity moieties naturally present in the NTNHA molecule.

[0064] Heterologous sequences may be covalently linked to the NTNHA (e.g., by expression as a fusion protein or by post-translational modification of the NTNHA molecule). In one embodiment, the additional heterologous amino acid sequences is a heterologous affinity moiety.

[0065] Heterologous amino acid sequences can be present on the N-terminus, C-terminus, or internally. Such sequences when present should be designed to preserve the interaction of the NTNHA with the BoNT Hc domain. In one embodiment, the heterologous sequence is an affinity moiety and there is no intervening sequence between the affinity moiety

and the NTNHA sequence. In one embodiment, the heterologous amino acids are located at the N-terminus of the NTNHA.

[0066] In one embodiment, the heterologous amino acids lack a functional protein cleavage site such as those that typically are used to remove an affinity tag from a fusion protein. In one embodiment, the invention excludes an NTNHA polypeptide comprising a myc-tag fused to the N-terminus, e.g., NTNHA-A1 (Gu et al., Science 335: 977-981 (2012)).

[0067] In one aspect of the invention, the NTNHA polypeptide is stably attached to a matrix. Stable attachment refers to attachment that is not disrupted by the conditions of the various buffers described herein. Attachment to the matrix can be via covalent or non-covalent interactions. In one embodiment, attachment to the matrix is through the interaction of a heterologous affinity moiety on the NTNHA polypeptide with a corresponding binding moiety on the matrix (e.g., a GST affinity moiety on the NTNHA with glutathione present on the matrix).

[0068] In one embodiment the NTNHA polypeptide in the various forms described herein (e.g., linked to an affinity moiety and/or stably attached to a matrix) is further in a complex with a compatible BoNT or a polypeptide comprising a receptor binding domain (Hc) thereof. In one embodiment, the BoNT or Hc is a native protein. In one embodiment, the BoNT or Hc is a genetically modified receptor binding domain (e.g., with increased binding for a specific receptor).

[0069] In one embodiment, the NTNHA polypeptide comprising the affinity moiety is further bound to a binding target through binding of the affinity moiety. The binding target may further be stably attached to a matrix.

[0070] Another aspect of the invention relates to an aqueous solution which contains the NTNHA polypeptide described herein. The NTNHA polypeptide within the solution can be of any form described herein, such as linked to an affinity moiety, stably attached to a matrix, and/or bound to a binding target through an affinity moiety, any of which may be further bound to a compatible BoNT.

[0071] Nucleic acid sequences that encode the NTNHA and affinity moiety fusion protein described herein are also encompassed by the invention. The nucleic acid sequences encoding the proteins can be optimized for *E. coli* expression. In one embodiment, the nucleic

acid sequences are in the context of a vector (e.g. an expression vector). Vectors should be compatible with the host cells in which the nucleic acids are intended to be propagated and/or expressed.

NTNHA

[0072] NTNHA is a 140 kDa protein synthesized by *Clostridium botulinum*. NTNHA genes occur within operons that encode a particular serotype BoNT protein. BoNT and the NTNHA produced from the same operon are components of the same naturally occurring BoNT protein complex, and form a tight, stable complex with one another. NTNHA binds the BoNT with a K_d of approximately 30.8 nM, at a 1:1 stoichiometry (Shenyan et al., Science 335: 977-981 (2012)). Preferably the NTNHA is derived from the same *Clostridium botulinum* strain which produces that serotype (and subtype) of BoNT or Hc fragment being purified (A, A1, A2, A3, A4-A, A4-B, types B, C, C1, D, E, F or G). Some overlap of binding between serotypes can be expected. The amino acid sequences of different NTNHA proteins are available to the skilled practitioner, as are the encoding nucleic acid sequences, such as NTNHA proteins derived from operons encoding BoNT serotypes: A1 (YP_001253341.1), A2 (WP_012704905), B (WP_003404192.1), C1 (YP_398515.1), D (BAA75083.1), E (WP_003409842), F (YP_001390122.1), and G (CAA61228.1). In one embodiment, the invention excludes use of the NTNHA/A (NTNHA/A1) molecule and the encoding nucleic acids.

BoNT

[0073] Different serotypes of botulinum neurotoxins are known in the art (A-G), and many subtypes also exist (A1, A2, A3, A4-A, A4-B). Methods described herein can be used to purify native BoNT (produced by clostridial bacteria), or a recombinant protein. Recombinant BoNT can be produced in any other type of host such as other prokaryotic cells, eukaryotic cells, tissues or organisms.

[0074] Mutated variants of BoNT (e.g., resulting from amino acid substitutions, insertions, or deletions) can also be isolated. In one embodiment, the variant has increased toxicity (e.g., by having increased binding to the cellular receptors). Such mutated variants can comprise a “modified receptor binding domain” or “modified H_C”. A modified H_C, as the term is used herein, has one or more non-naturally occurring substitution mutations that enhance the binding of the *C. botulinum* neurotoxin molecule in which it is comprised, to a receptor for *C. botulinum* neurotoxin located on the surface of a target cell. Such a molecule is typically generated through genetic recombination technology. The modified H_C has a binding activity for the receptor for *C. botulinum* neurotoxin that is stronger than its wild type counterpart. Examples of modified receptor binding domains are disclosed in U.S. Application 2015/166972, the contents of which are incorporated herein by reference. The invention is further useful for isolating any molecule possessing or retaining the biological activity of the botulinum toxin, such as a fusion (or chimeric) protein, truncated protein, protein fragment, or a mutated variant of botulinum toxin such as a protein having one or more amino acids added, deleted or replaced.

[0075] In one embodiment, the BoNT isolated by the methods described herein has toxic activity. The activity of the BoNT can be determined by measuring the proteolytic activity on the appropriate substrate. Botulinum toxin types A and E toxins cleave the protein SNAP-25. Botulinum toxin types B, D, F and G cleave vesicle-associated membrane protein (VAMP, called synaptobrevin). Botulinum toxin type C1 cleaves both SNAP25 and also the protein syntaxin. Assays that can be used to determine this activity are known in the art such as described in WO 95/33850, the contents of which are incorporated by reference herein.

Affinity Moieties

[0076] The NTNHA can be attached to an affinity moiety. The affinity moiety specifically binds a binding target under the conditions of the methods described herein (e.g., from about pH 6 to about pH 8). A variety of affinity moieties are known in the art and available for use in the invention. An affinity moiety can be a member of a specific binding pair, such as an epitope that is specifically recognized by an antibody. When an epitope is used as the affinity moiety, the antibody is used as the binding target. Many such affinity moiety:antibody

combinations are known in the art and commercially available. Examples include, without limitation, c-myc (Roth et al, (1991) J. Cell Biol. 115:587-596), myc (EQKLISEEDL (SEQ ID NO: 8); Evan G I, et al. (1985) Mol. Cell Biol. 5:3610-3616; Munro S. and Pelham H R B, (1987) Cell 48:899-907; Borjigin J. and Nathans J., (1994) 269:14715-14727; Smith D J, (1997) BioTechniques 23:116-120) FLAG.RTM.(U.S. Pat. Nos. 4,703,004; 4,851,341 and 5,011,912), HA, derived from the influenza hemagglutinin protein (Wilson I A, et al., (1984) Cell, 37:767; Field J. et al. Mol. Cell Biol. (1988) 8:2159-2165; Xu Y, et al. (2000) Mol Cell Biol. 20:2138-2146), IRS (RYIRS (SEQ ID NO: 9); Liang T C et al. (1996) 329:208-214; Luo W et. al. (1996) Arch. Biochem. Biophys. 329:215-220), AU1 and AU5 ((DTYRYI (SEQ ID NO: 10) and TDFLYK (SEQ ID NO: 11)); Lim P S et al. (1990) J. Infect. Dis. 162:1263-1269; Goldstein D J et al. (1992) 190:889-893; Koralnik I J et al. (1993) J. Virol. 67:2360-2366), glu-glu (a 9 amino acid epitope from polyoma virus medium T antigen (EEEEYMPME (SEQ ID NO: 12)); Grussenmeyer, T. et al. (1985) PNAS. USA 82:7952-7954; Rubinfeld. B. et al. (1991) Cell 65:1033-1042), KT3 (an 11 amino acid epitope from the SV40 large T antigen (KPPTPPPEPET (SEQ ID NO: 13)); MacArthur H. and Walter G. (1984) J, Virol. 52:483-491; Martin G A et al. (1990) 63:843-849; Di Paolo G et al. (1997) 272:5175-5182), T7 (an 11 amino acid leader peptide from T7 major capsid protein (MASMTGGQQMG (SEQ ID NO: 14))), S-TAG, HSV (an 11 amino acid peptide from herpes simplex virus glycoprotein D (QPELAPEDPEDC (SEQ ID NO: 15))), VSV-G (an 11 amino acid epitope from the carboxy terminus of vesicular stomatitis virus glycoprotein, (YTDIEMNRLGK (SEQ ID NO: 16)); Kreis T. (1986) EMBO J. 5:931-941; Turner J R et al (1996) 271:7738-7744), Anti-Xpress (8 amino acid epitope, (DLYDDDK (SEQ ID NO: 17))), and VS (14 amino acid epitope from paramoxyvirus SV5, (GKPIPNPLLGLDST (SEQ ID NO: 18))).

[0077] Another epitope commonly used as an affinity moiety is the FLAG.RTM. This sequence typically consists of DYKDDDDK (SEQ ID NO: 19), but any combination of 3 to 6 aspartic or glutamic acid residues is also considered a FLAG.RTM. sequence. The FLAG.RTM. affinity tag has effectively been used in various expression systems for the purification of recombinant fusion proteins (Brizzard et al. (1994) BioTechniques 16:730-735; Lee et al. (1994) Nature 372:739-746; Xu et al. (1993) Development 117:1223-1237; Dent et al. (1995) Mol. Cell Biol. 15:4125-4135; Ritchie et al. (1999) BioChem Journal 338:305-10.).

[0078] There are also many affinity moieties that are not epitope based, and these can also be used in the invention. GST (Glutathione-S-transferase) is an affinity moiety envisioned for use in the instant invention (U.S. Pat. Nos. 5,654,176; 6,303,128 and 6,013,462). The poly-histidine affinity moiety is a non-natural consecutive sequence of histidine amino acid residues including any corresponding peptides disclosed in U.S. Pat. Nos. 5,284,933 and 5,310,663. Typically such sequences comprise four to ten histidine residues (SEQ ID NO: 20).

[0079] In one embodiment, the affinity moiety is glutathione -S-transferase (GST), C-myc tag, Chitin-binding domain, Streptavidin binding protein (SBP), Cellulose-binding domain, Calmodulin-binding peptide, S-tag, Strep-tag II, FLA, Protein A, Protein G, histidine affinity tag (HAT), Poly-His, or maltose binding protein (MBP). In one embodiment, the affinity moiety is not GST, C-myc tag, Chitin-binding domain, SBP, Cellulose-binding domain, Calmodulin-binding peptide, S-tag, Strep-tag II, FLA, Protein A, Protein G, HAT, Poly-His, or MBP. In one embodiment, the affinity moiety is AviTagTM, V5, Myc, T7, FLAG, HSV, VSV-G, poly His (typically His₆ (SEQ ID NO: 1)), biotin, or STREP (WSHPQFEK (SEQ ID NO: 21)). In one embodiment, the affinity moiety is not AviTagTM, V5, Myc, T7, FLAG, HSV, VSV-G, poly His, biotin, or STREP.

[0080] Binding pair members that interact with or are found naturally within the mammalian (human) body such as antibodies that naturally bind to NTNHA, or molecules recognized by transporters in the liver and/or kidney, are excluded from the compositions described herein.

Binding Targets for Affinity Moieties

[0081] Binding targets are used to immobilize the NTNHA polypeptide through binding of the affinity moiety. The binding target will typically be specific for a given affinity moiety. Binding targets are attached to the matrix such that their binding affinity for the affinity moiety is preserved. For example, the binding target for an epitope tag is an antibody which specifically binds the epitope tag. The binding target for GST is glutathione. The binding target for biotin is avidin or streptavidin. The binding target for STREP is Strep-tactin. The binding target for

polyHis is bivalent nickel or cobalt ions. The binding target for protein G is the Fc portion of IgG. The binding target for protein A is the Fc portion of immunoglobulin of various species.

Matrix

[0082] Various inert substance typically used to immobilize a molecule through physical attachment can be used as the matrix in the invention. The matrix, otherwise referred to as a substrate, can be made from a wide variety of materials and may take a variety of forms. Materials include, without limitation metal, metal alloy, polymer, plastic, paper, glass, fabric, packaging material, biological material such as cells, tissues, hydrogels, proteins, peptides, nucleic acids, and any combinations thereof. Forms the matrix can take include, without limitation, beads (including polymer microbeads, magnetic microbeads, and the like), filters, fibers, screens, mesh, tubes, hollow fibers, scaffolds, plates, channels, and any combination thereof. Other examples of substrate matrixes known in the art include, but are not limited to, nucleic acid scaffolds, protein scaffolds, lipid scaffolds, dendrimers, microparticles or microbeads, nanotubes, and microtiter plates. In one embodiment, the matrix components are in the form of a column.

[0083] In one embodiment, the NTNHA polypeptide is attached to the matrix by the coupling of an affinity moiety present on the NTNHA to a binding target present on the matrix surface. Various affinity moieties and binding targets are available for use, example of which are discussed herein. In one embodiment, the matrix is coated with glutathione as the binding target (e.g., glutathione-linked agarose beads). In one embodiment, the glutathione coated matrix is in the form of a column.

[0084] In one embodiment, the NTNHA polypeptide is conjugated directly to a matrix surface through a covalent or non-covalent interaction. This can occur through the N-terminus, the C-terminus, or internally to the molecule. It may further be useful to include a linker on the NTNHA polypeptide in order to facilitate attachment to the substrate.

[0085] Conjugation to the substrate can be accomplished using a variety of methods in the art. Examples of covalent attachment include, without limitation silane coupling (Weetall, 15

Adv. Mol. Cell Bio. 161 (2008); Weetall, 44 Meths. Enzymol. 134 (1976)), and use of NHS reaction or a conjugation agent. Non-covalent attachment can be based on ionic interactions, van der Waals interactions, dipole-dipole interactions, hydrogen bonds, electrostatic interactions, and/or shape recognition interactions. Without limitations, conjugation can include either a stable or a labile bond or conjugation agent. Exemplary conjugations include, but are not limited to, covalent bond, amide bond, additions to carbon-carbon multiple bonds, azide alkyne Huisgen cycloaddition, Diels-Alder reaction, disulfide linkage, ester bond, Michael additions, silane bond, urethane, nucleophilic ring opening reactions: epoxides, non-aldol carbonyl chemistry, cycloaddition reactions: 1,3-dipolar cycloaddition, temperature sensitive, radiation (IR, near-IR, UV) sensitive bond or conjugation agent, pH-sensitive bond or conjugation agent, non-covalent bonds (e.g., ionic charge complex formation, hydrogen bonding, pi-pi interactions, cyclodextrin/adamantly host guest interaction) and the like. As used herein, the term "conjugation agent" means an organic moiety that connects two parts of a compound. Linkers typically comprise a direct bond or an atom such as oxygen or sulfur, a unit such as NR₁, C(O), C(O)NH, SO, SO₂, SO₂NH or a chain of atoms, where one or more methylenes can be interrupted or terminated by O, S, S(O), SO₂, NH, C(O)N(R₁)₂, C(O), cleavable linking group, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocyclic; where R₁ is hydrogen, acyl, aliphatic or substituted aliphatic.

[0086] A variety of conjugation chemistry is available for conjugating two molecules together and can be used for linking the NTNHA polypeptide to a matrix. Exemplary coupling molecules and/or functional groups for conjugating at least one engineered microbe-targeting molecule to a substrate include, but are not limited to, a polyethylene glycol (PEG, NH₂-PEGX-COOH which can have a PEG spacer arm of various lengths X, where 1 < X < 100, e.g., PEG-2K, PEG-5K, PEG-10K, PEG-12K, PEG-15K, PEG-20K, PEG-40K, and the like), maleimide conjugation agent, PASylation, HESylation, Bis(sulfosuccinimidyl) suberate conjugation agent, DNA conjugation agent, peptide conjugation agent, silane conjugation agent, polysaccharide conjugation agent, hydrolyzable conjugation agent, and any combinations thereof.

[0087] The amount of NTNHA bound to the matrix can be determined and optimized by the skilled practitioner. In one embodiment, the matrix has about 20 mg/ml of NTNHA

polypeptide. In one embodiment, the matrix has about 5 mg/ml polypeptide, or about 2 mg/ml polypeptide.

Proteases

[0088] Any protease that will cleave the BoNT can be used in the herein described methods. Such proteases include, without limitation trypsin, pepsin, Lys-C endoproteinase, Lys-N endoproteinase, arginyl endopeptidase, plasmin, omptin and a clostridial protease as described in EP2524963. In one embodiment, the protease is trypsin or Lys-C endoproteinase. In one embodiment, the protease is a protease that cleaves a BoNT non-native (i.e. exogenous) cleavage site. In such clostridial toxins, the native protease cleavage site (also known as the activation site) is modified or replaced with a protease cleavage site that is not native to that clostridial toxin. Non-native proteases that may be employed include Enterokinase (DDDDK↓(SEQ ID NO: 2)), Factor Xa (IEGR↓(SEQ ID NO: 3)/ IDGR↓(SEQ ID NO: 4)), TEV(Tobacco Etch virus) (ENLYFQ↓G (SEQ ID NO: 5)), Thrombin (LVPR↓GS (SEQ ID NO: 6)) and PreScission (LEVLFQ↓GP (SEQ ID NO: 7)), (the ↓ denotes the cleavage site).

Nucleic Acid Vectors

[0089] Another aspect of the invention relates to a nucleic acid vector comprising the nucleic acid molecule encoding the NTNHA polypeptide described herein. The vector can be a vector solely for propagation of a nucleic acid sequence in an organism or cell or can also be for expression of the nucleic acid sequence as a polypeptide in that organism or cell.

[0090] In one embodiment the vector is an expression vector. Such an expression vector is referred to herein as an expression construct, and comprises a nucleic acid molecule disclosed herein operably-linked to the expression vector useful for expressing the nucleic acid molecule in a cell or cell-free extract. A wide variety of expression vectors can be employed for expressing a nucleic acid molecule encoding a NTNHA polypeptide described herein including, without limitation, a viral expression vector (e.g., retroviruses, vaccinia virus, adeno-associated virus,

herpes viruses, or bovine papilloma virus), a prokaryotic expression vector, a eukaryotic expression vectors, such as, e.g., a yeast expression vector, an insect expression vector, a mammalian expression vector, and a cell-free extract expression vector. In one embodiment, the expression vector is a baculovirus expression vector. Suitable expression vectors include, without limitation, Okayama-Berg cDNA expression vector pcDV1 (Pharmacia), pBluescript (Stratagene), pCDM8, pRc/CMV, pcDNA1, pcDNA3 (Invitrogen) or pSPORT1 (Invitrogen) or baculovirus-derived vectors. Expression vectors derived from viruses may be used for delivery of the nucleic acids of the invention into a targeted cell population. A number of expression vectors for producing fusions with affinity moieties such as those described herein are available in the art. The selection, making and use of an appropriate expression vector are routine procedures undertaken by the skilled practitioner.

Host Cells

[0091] Another aspect of the invention relates to a cell in which one or more of the molecules described herein (e.g., the NTNHA polypeptide and/or the BoNT polypeptide) is propagated and/or expressed. Such a cell is referred to as a host cell. Host cells may be genetically modified to express the molecules described herein, such as by transfection with a vector encoding the proteins, and/or may express one or more of the molecules (e.g., the BoNT) naturally. In one embodiment, the host cell comprises a nucleic acid that encodes the NTNHA polypeptide (e.g., in the context of a vector). In one embodiment, the host cell expresses the nucleic acid (e.g. from an expression vector). In some embodiments, cells used in accordance with the present invention include prokaryotic cells and eukaryotic cells. Non-limiting examples of prokaryotic cell are *Escherichia coli* cells, *Clostridium botulinum* cell, *Clostridium tetani* cells, *Clostridium beratti* cells, *Clostridium butyricum* cells, or *Clostridium perfringens* cells. Non-limiting examples of eukaryotic cells are insect cells, yeast cells, amphibian cells, mammalian cell, plant cells. Non-limiting examples of insect cells are *Spodoptera frugiperda* cells, *Aedes albopictus* cells, *Trichoplusia ni* cells, *Estigmene acrea* cells, *Bombyx mori* cells and *Drosophila melanogaster* cells. Non-limiting examples of yeast cells are *Saccharomyces*

cerevisiae cells, *Schizosaccharomyces pombe* cells, *Pichia pastoris* cells, *Hansenula polymorpha* cells, *Kluyveromyces lactis* cells and *Yarrowia lipolytica* cells.

[0092] Unless otherwise defined herein, scientific and technical terms used in connection with the present application shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

[0093] It should be understood that this invention is not limited to the particular methodology, protocols, and reagents, etc., described herein and as such may vary. The terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which is defined solely by the claims.

[0094] Other than in the operating examples, or where otherwise indicated, all numbers expressing quantities of ingredients or reaction conditions used herein should be understood as modified in all instances by the term “about.” The term “about” when used to described the present invention, in connection with percentages can mean $\pm 1\%$, or $\pm 5\%$, or $\pm 10\%$.

[0095] In one respect, the present invention relates to the herein described compositions, methods, and respective component(s) thereof, as essential to the invention, yet open to the inclusion of unspecified elements, essential or not (“comprising). In some embodiments, other elements to be included in the description of the composition, method or respective component thereof are limited to those that do not materially affect the basic and novel characteristic(s) of the invention (“consisting essentially of”). This applies equally to steps within a described method as well as compositions and components therein. In other embodiments, the inventions, compositions, methods, and respective components thereof, described herein are intended to be exclusive of any element not deemed an essential element to the component, composition or method (“consisting of”).

[0096] All patents, patent applications, and publications identified are expressly incorporated herein by reference for the purpose of describing and disclosing, for example, the methodologies described in such publications that might be used in connection with the present invention. These publications are provided solely for their disclosure prior to the filing date of the present application. Nothing in this regard should be construed as an admission that the

inventors are not entitled to antedate such disclosure by virtue of prior invention or for any other reason. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicants and does not constitute any admission as to the correctness of the dates or contents of these documents.

[0097] The invention is further illustrated by the following examples, which should not be construed as further limiting.

EXAMPLES

Example 1

A new method for purifying and activating BoNTs

[0098] Herein is proposed a new method to purify and activate un-tagged, natural forms of BoNTs through simple steps of affinity purification. This method is based on a unique feature of BoNTs: these toxins naturally form a dimer complex with its chaperon protein, known as NTNHA. The biological purpose of this dimer is to protect toxins from proteases and the harsh acidic environment in the gastrointestinal (GI) tract. The interactions between BoNTs and NTNHA are pH-dependent: they bind at pH <7, and dissociate from each other at pH >7.4. Therefore, introducing an affinity tag onto the NTNHA can be utilized to isolate the natural forms of BoNTs in solutions with pH < 7. Bound BoNTs can then be released by simply raising the pH of the solution to > pH 7.4. In another words, instead of putting an affinity tag onto BoNTs, its binding partner can be tagged. This allows the production of natural forms of BoNTs through convenient affinity purification method.

[0099] In addition to purification, BoNTs need to be activated by limited proteolysis. Recombinant BoNTs are usually activated post-purification with an endoproteinase (such as trypsin). This method has several drawbacks: 1) there are chances for non-specific cutting by the endoproteinase, which compromises toxin activity and yield; 2) the endoproteinase needs to be removed after the reaction is completed, requiring an additional separation step that compromises yield and activity of toxins.

[0100] The activation site on BoNTs is still exposed on the surface of BoNT-NTNHA complex, whereas other susceptible sites of BoNTs are often protected in the complex. This provides an opportunity to treat toxins with the endoproteinase while the toxin is still in the complex with NTNHA. This approach addresses both problems in previous methods: 1) NTNHA will protect toxins from non-specific cutting by the endoproteinase; 2) the endoproteinase can be easily removed in a single washing step along with all other non-toxin proteins that do not bind to NTNHA.

Results

[0101] Each naturally occurring BoNT has its own, naturally occurring NTNHA partner. BoNT/B and NTNHA/B were used as prototypes to establish the feasibility of our approach. Briefly, the NTNHA/B was expressed as a fusion protein with the commonly used GST tag (Glutathione-S-transferase). The GST-NTNHA/B was purified, immobilized on Glutathione beads, and was subsequently equilibrated with the toxin binding buffer (pH = 6). This resin was then added to *E.Coli* cell lysate containing recombinantly expressed BoNT/B and incubated for 1 hour at 4°C to allow for complex formation under pH 6 conditions. Subsequently, the bead-bound complex was washed with the binding buffer to remove non-specific contaminants and unbound proteins. Bound BoNT/B was either eluted from beads using a pH 8 elution buffer, or subjected to trypsin treatment to be activated.

[0102] The purification principle and steps are illustrated schematically in FIG. 1A and FIG. 1B. The results indicated that BoNT/B can be efficiently purified from crude bacterial lysates using this method (FIG. 2A), with high yield and purity of final protein (FIG. 2B). A fraction of the resin containing the NTNHA/B:BoNT/B complex was subjected to trypsin-mediated cleavage. The results, shown in FIG. 3A and FIG. 3B, indicate BoNT can be efficiently activated within a few hours on beads in the complex, and can be subsequently eluted from the beads to produce native, active toxin.

[0103] Whether NTNHA that is specific to one serotype can be used to purify chimeric toxins that contain a segment of that toxin, particularly the receptor binding domain was

explored. The receptor binding domain mediates the majority of interactions between NTNHA and BoNT. The results, shown in FIG. 4, indicate the successful use of NTNHA/B to purify a hybrid toxin (BoNT/A1B) containing the BoNT/B receptor binding domain.

[0104] These experimental results serve as proof of concept of a method that can be used to purify widely used therapeutic toxin: BoNT/A (with NTNHA/A) and BoNT/B (with NTNHA/B), purify other serotypes of BoNTs (with suitable NTNHAs), purify recombinant BoNTs containing mutations, purify chimeric BoNTs (with NTNHA that binds to the receptor binding domain or specifically designed chimeric NTNHA proteins). Advantages of this method are 1) the ability to purify BoNTs with natural N- and C-terminals that are expressed recombinantly, through convenient affinity purification, 2) mild buffer conditions (pH 6-8) minimize any potential damage to toxins, 3) specific pH dependent binding and elution yields highly pure toxins conveniently, reducing the need for further purification, 4) protection from NTNHA reduces non-specific cutting by the activating protease during the activation step, and 5) activation by protease prior to elution of toxins, abolishes the need to separately remove the protease.

Materials and Methods

[0105] Protein Expression and Purification. NTNHA/B was expressed in *E.Coli* as a Glutathione-S-Transferase fusion protein (GST-NTNHA/B) with the GST being fused to the N-terminus of the NTNHA/B protein; BoNT/B was expressed in *E.Coli* with a C-terminal His₆ tag (SEQ ID NO: 1). Bacterial cultures (1 L) were grown at 37 degree and protein expression was induced with the addition of Isopropyl β -D-1-thiogalactopyranoside (IPTG) (250 μ M) when the culture optical density at 600 nm (OD₆₀₀) reached ~0.6 AU. The cultures were then transferred to a 20 degree shaking incubator for overnight expression (~16 hrs). Bacteria were harvested by centrifugation at 5500 xg and the resulting pellets were frozen until purification.

[0106] BoNT/B pellets were thawed and solubilized in binding buffer (50 mM MES, 150 mM NaCl, pH 6) with 5 ml/gram of dry bacterial pellet; NTNHA/B pellet were thawed and solubilized in a different binding buffer (50 mM Tris, 150 mM NaCl, pH 8). 1 mM of

phenylmethylsulfonyl fluoride (PMSF) was added before lysis by sonication on ice (Branson Sonifier 250) for 15 min (3 X 5 min, 50% power). The crude lysate was then cleared by centrifugation (30,000 xg, 15 min) and the supernatant was filtered using 0.45 µm syringe filters (Nalgene).

[0107] GST-NTNHA/B purification. 600 µL Pierce Glutathione-Agarose beads (50 % slurry; Thermo) equilibrated with binding buffer was added to ~20 mL of GST-NTNHA/B supernatant and allowed to batch bind for 1 hr at 4 degree. The beads were recovered by centrifugation (700 xg) and washed twice with 3 resin bed volumes of binding buffer (50 mM Tris, 150 mM NaCl, pH 8). The estimated concentration of purified GST-NTNHA/B was ~0.6 mg/mL (BCA assay and SDS-PAGE analysis).

[0108] pH-dependent complexation; protease activation; and elution of purified BoNT. The agarose beads harboring GST-NTNHA/B were added to ~ 5 mL of BoNT/B cleared *E.Coli* lysate for batch binding for 2 hrs at 4 degree in a rocking conical tube. The beads were harvested by (700 xg) and washed twice with 3X resin bed volumes of binding buffer (50 mM MES, 150 mM NaCl, pH 6).

[0109] Trypsin or Lys-C endoproteinase (Sigma-Aldrich) was added at a molar ratio of 1:10 at pH 6 (on beads) to activate the NTNHA-bound toxin in a final volume of 500 µL. The reaction proceeded on a rotating platform at room temperature and monitored for 4 hrs by sampling small aliquots for subsequent analysis. The resin was washed twice with binding buffer to remove the proteases and unbound impurities. The purified and activated BoNT was eluted with two resin volumes of high pH buffer (50 mM Tris, 150 mM NaCl, pH 8).

[0110] SDS-PAGE and WB analysis. 10 µL of all samples (with or without the reducing agent DTT) were applied to 9% SDS-PAGE gels. After separation, the gel was stained with Coomassie stain or subjected to standard immunoblotting analysis. A human monoclonal antibody was used to detect BoNT/B and a polyclonal rabbit antibody was used to detect BoNT/A1B chimeric toxin.

Example 2

Facile, direct isolation of recombinant BoNTs from crude bacterial lysates

[0111] The association between BoNT and NTNHA is facilitated by numerous pH sensors on the two molecules that form specific surface recognition (Gu et al. 2012). This interlocked complex protects active toxin from the harsh acidic environment through which it must traverse to reach its cellular destinations.

[0112] The present Example confirms feasibility of isolating a recombinant full-length BoNT (inactive BoNT/B, hereafter termed BoNT/B_{RY}) that is expressed in *E.coli*, as described herein. The complex partner that facilitates toxin isolation is a GST-tagged compatible serotype of its recombinant complex partner, NTNHA/B. The GST-tagged NTNHA-B molecule and the BoNT/B_{RY} were expressed separately in *E.coli* hosts and protein production was achieved using standard auto-induction methods (Studier 2005). For GST-NTNHA-B isolation, a one-step batch purification with agarose-glutathione beads was performed as described in methods. The immobilized GST-NTNHA-B was stable for short-medium term storage at 4 degree for approximately one week, although longer term storage lead to possible spontaneous nicking as reported previously (Sagane et al. 2002; Gu et al. 2012). This reagent was subsequently used to isolate BoNT/B_{RY} and the chimeric BoNT/A1_{RY}B1 in a simple workflow (FIG. 6B), where the agarose beads were the bait in pulling the recombinant toxins out of crude lysates under favorable conditions (e.g. pH 6.0, 150 mM NaCl). SDS-Page analysis of relevant fractions from the purification scheme is shown in FIG. 6C. The regenerated GST-NTNHA/B after elution can be readily used in another cycle of purification to isolate more compatible toxins from fresh or alternative extracts. The eluted full-length toxin is selectively released from the complex upon buffer exchange on the beads and can be visualized on SDS-PAGE or by western blot (WB) analysis (FIG. 7A). Moreover, such mild conditions for isolating full-length (FL) toxins are more likely to preserve its protease activity and functional roles in binding its cellular targets. As the canonical neuronal receptor for BoNT/B, a Synaptotagmin derived labeled peptide is shown to interact with the isolated full-length toxin in an *in-vitro* fluorescence anisotropy binding assay (FIG. 7B).

The complexed toxin is efficiently activated by exogenous proteases

[0113] As dichain (AB) toxins, BoNTs are expressed as a single polypeptide chain that undergoes activation to generate a functional molecule linked by a disulfide bridge between the heavy and light chains. “Nicking” by exogenous or endogenous proteases that cleave the polypeptide chain between two conserved cysteines that maintain a covalent bridge between the LC and HC can improve potency, and may be required for maximum potency (FIG. 6A). The present Example documents that addition of such proteases (e.g., specifically of an exogenous protease) can be incorporated into purification protocol workflows as described herein and, in some embodiments, can help maximize recovery of active toxins. For example, complexed GST-NTNHA/B:BoNT/B_{RY} can be cleaved by catalytic amounts of trypsin or Lys-C endoproteinase under mild conditions at room temperature and the nicked toxin can be selectively released in higher pH buffer. FIGs. 8A-8C show time courses of activating complexed single chain BoNT//B_{RY} to release the ~50kD protease domain (LC) and the ~100 kDa HC in samples that contain dithiothreitol (DTT). The lower pH binding conditions and the lower activity of the proteases (Kasserra & Laidler 1969; Jekel et al. 1983) play a protective role from nonspecific/excessive degradation of the toxin and/or NTNHA (FIG. 8B). The purity and extent of toxin nicking can be visualized either on SDS-PAGE gel or detected by WB analysis (FIG. 8A and FIG. 8C).

Chimeric recombinant toxins can be isolated using a common NTNHA serotype

[0114] The present Example confirms that a chimeric recombinant botulinum neurotoxin can be purified using a complex-based purification protocol as described herein using various targets that may serve as therapeutic backbones for future biologics. The receptor binding domain of BoNTs mediates most polar contacts with NTNHA (Gu et al. 2012). The present Example confirms that recombinant botulinum neurotoxin can be purified via complex formation with NTNHA. A chimeric recombinant protein (BoNT/A1_{RY}B1) that is constructed from inactive BoNT/A LC, BoNT/A H_N, and BoNT/B H_C was used as a proof-of-concept. Using the same recombinant GST-NTNHA/B above, complexation and enrichment of the chimeric toxin

on the NTNHA beads could be detected despite low expression levels of the toxin (FIG. 9). The cleared lysate for the chimeric toxin contained degradation products and large impurities that often preclude efficient complexation with the immobilized NTNHA. Therefore, the BoNT/A1_{RY}B1 lysate was passed once over, and eluted from a Ni-NTA resin before exposure to the immobilized NTNHA on agarose resin. Subsequent activation profiles with this chimeric toxin may be similar to that of BoNT/B_{RY}, possibly with varying efficiencies.

Discussion

[0115] This Example demonstrates isolation of recombinantly expressed BoNTs using a NTNHA/B as a noncovalent progenitor complex partner. Both BoNT/B_{RY} and NTNHA/B were overexpressed separately in *E.coli* hosts. The NTNHA/B was expressed as a fusion protein with a GST tag appended to its N-terminus as an affinity moiety towards the solid agarose-glutathione resin. BoNT/B_{RY} (and the chimeric BoNT/A1_{RY}B1) were expressed as wild-type sequences except for the inactivation mutations and a C-terminal His_{6X} tag. Bacterial lysis in a low pH buffer released these toxins in a lysate that was incubated with the agarose beads harboring GST-NTNHA/B. After complex formation, the solid media is washed extensively to remove impurities; after which the toxins can either be eluted by high pH buffer exchange or activated through an additional step where an exogenous endoprotease is applied to the resin-bound complex.

[0116] As confirmed by the findings documented in the present Example, the present disclosure provides a solution for efficiently isolating active, therapeutic BoNTs from various sources under mild conditions. Enhanced methodology to isolate, activate, and elute purified BoNTs can be immensely useful, such as in large-scale production of therapeutic BoNTs. Potential benefits include the following: 1) efficient isolation of recombinant BoNTs from crude lysates under mild conditions unlike those in current practices (Malizio et al. 2000; Donovan 2007); 2) high-purity, activated toxins can be produced using a single purification scheme as it enables extensive washing of contaminants and avoids multiple chromatography steps; 3) the immobilized NTNHA can properly protect from non-specific cleavage of toxin in the activation step, which can be readily incorporated in the purification protocol (as opposed to the common

post purification activation). This can increase final yields and homogeneity of the final activated toxin; and 4) the immobilized GST-NTNHA can serve multiple sequential purifications as it is regenerated at the end of every cycle with little loss; and 5) such methodology can be expanded to isolation of chimeric therapeutic toxins with compatible receptor binding domains.

Materials and Methods

Protein Expression and Purification

[0117] NTNHA/B was expressed as a Glutathione-S-Transferase fusion protein (GST-NTNHA/B) in a pGEX vector; BoNT/B_{RY} and BoNT/A1_{RY}B1 were expressed with a C-terminal (His_{6x}) tag in a pET32-a vector in *E.coli* (BL21DE3). Cell cultures (typically 300 mL) were grown in autoinduction media (Formedium™, UK) in baffled 2L flasks at 37°C with vigorous shaking (>250 RPM). When cultures reached OD of ~0.6, the cell cultures were transferred to a 20°C shaking incubator for overnight expression (~16 hrs). Cells were harvested by centrifugation at 5500 xg and the resulting pellets were frozen at -20°C until purification.

[0118] BoNT/B_{RY} cell pellets were thawed and solubilized in binding buffer (50 mM MES, 150 mM NaCl, pH 6) with 5 ml/gram of dry cell pellet. GST-NTNHA/B cells were thawed and solubilized in TBS binding buffer (50 mM Tris, 150 mM NaCl, pH 8). Phenylmethylsulfonyl fluoride (PMSF) was added to the solubilized cells at a final concentration of 0.1 mM before lysis by sonication on ice (Branson Sonifier 250) for 15 min (3 X 5 min); 30% power. The crude lysates were then cleared by centrifugation (30,000 xg, 15 min) and the supernatant was filtered using 0.45 µm syringe filters (Nalgene).

GST-NTNHA/B purification

[0119] 600 µL Pierce Glutathione-Agarose beads (50 % slurry; Thermo) were equilibrated with binding buffer and added to ~20 mL of GST-NTNHA/B supernatant and allowed to batch bind for 1 hr at 4°C on a gently rocking platform. The beads were recovered by centrifugation (700 xg) and washed twice with 3X resin bed volumes of binding buffer (1X

TBS). The estimated concentration of purified GST-NTNHA/B was typically ~0.5 mg/mL (BCA assay and SDS-PAGE analysis).

Binding, Activation, and Elution of Purified BoNTs

[0120] The agarose beads harboring GST-NTNHA/B were added to 10-25 mL of BoNT/B_{RY} or BoNT/A1_{RY}B1 cleared lysates (in MES, pH 6) buffer and allowed batch binding to proceed for 2 hours at 4°C in a 50-mL conical tube on a rocking platform. The beads were harvested by (700 xg) and washed twice with 3X resin bed volumes of binding buffer (MES, pH 6). If no activation is desired, bound purified toxins may be eluted at this stage as described below.

[0121] Trypsin or Lys-C endoproteinase (Sigma-Aldrich) were added at a molar ratio of 1:10 endoproteinase:GST-NTNHA/B at pH 6 (on beads) to activate the bound toxin in a final volume of 500-1000 uL. The reaction proceeded on a rotating (tumbling) platform at room temperature and monitored (either 2-4 hours as in FIG. 8A and FIG. 8C; or overnight at 4°C as in FIG. 8B) by sampling small aliquots for subsequent analysis. The resin was washed twice with binding buffer to remove the proteases and impurities. The purified and activated BoNT/B_{RY} was eluted in fractions of two resin volumes of high pH buffer (TBS: 50 mM Tris, 150 mM NaCl, pH 8).

SDS-PAGE and WB analysis

[0122] 10 µL of all samples (with or without the reducing agents DTT or βME) were applied to 8-12% SDS-PAGE gels. After separation, gels were stained with Coomassie stain or subjected to standard Western blotting procedure. A monoclonal rabbit antibody (1:5000) was used to detect BoNT/B_{RY} and a polyclonal rabbit antibody (1:2000) raised against BoNT/A was used to detect BoNT/A1_{RY}B1.

Fluorescence Anisotropy

[0123] Human Synaptotagmin 1 (Syt 1) derived peptide (AA 33-53) was synthesized with an N-terminal FITC label (GenScript, Piscataway NJ) and used as a receptor in the binding experiment at 50-100 nM. The eluted full-length toxins were concentrated in Vivaspin 6

filtration units (10K MWCO, GE). Binding experiments (50 uL) were measured in black 96-well plate (Corning) using a filter-based plate reader (485/520 nm excitation/emission). BoNT/A and BoNT/B Hc were separately expressed and purified and served as negative and positive controls, respectively.

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What is claimed:

1. A molecule comprising a non-toxic non-hemagglutinin (NTNHA) polypeptide covalently linked to a heterologous affinity moiety.
2. The molecule of claim 1, wherein the NTNHA and affinity moiety are expressed as a fusion protein.
3. The molecule of any claim 1-2, wherein the affinity moiety is located at a position selected from the group consisting of the N-terminus of NTNHA amino acid sequence, the C-terminus of NTNHA amino acid sequence, and internal to the NTNHA amino acid sequence.
4. The molecule of any one of claims 1-3, wherein the affinity moiety effectively binds a binding target under conditions of about pH 6 to about pH 8.
5. The molecule of any one of claims 1-4, wherein the affinity moiety is selected from the group consisting of glutathione -S-transferase (GST), C-myc tag, Chitin-binding domain, Streptavidin binding protein (SBP), Cellulose-binding domain, Calmodulin-binding peptide, S-tag, Strep-tag II, FLA, Protein A, Protein G, histidine affinity tag (HAT), Poly-His, and maltose binding protein (MBP) .
6. The molecule of any one of claims 1-5, wherein the NTNHA is from serotype B, A, C1, D, E, F, or G.
7. The molecule of any one of claims 1-6, wherein the NTNHA is from serotype B.
8. The molecule of any one of claims 1-7, wherein the molecule is in a complex with a compatible Botulinum neurotoxin (BoNT) or a polypeptide comprising a receptor binding domain thereof.

9. The molecule of claim 8, wherein the BoNT or the polypeptide comprises a modified receptor binding domain of Clostridial botulinum serotype B (B-H_c).
10. The molecule of any one of claims 1-9, wherein the molecule is further bound to a binding target through the affinity moiety.
11. The molecule of claim 9, wherein the binding target is stably attached to a matrix.
12. An aqueous solution comprising the molecule of any one of claims 1-11.
13. A nucleic acid that encodes the functional NTNHA and affinity moiety fusion protein described in any one of claims 2-7.
14. An expression vector comprising the nucleic acid of claim 13.
15. A host cell that comprises and expresses the nucleic acid of one of claims 13 – 14.
16. The host cell of claim 14 that further expresses a compatible Botulinum neurotoxin (BoNT).
17. The host cell of claim 16, wherein the BoNT comprises a modified receptor binding domain of Clostridial botulinum serotype B (B-H_c).
18. The host cell of any one of claims 15-16, that is prokaryotic or eukaryotic.
19. The host cell of any one of claims 15-16, that is a bacterial cell, a yeast cell, a mammalian cell, an insect cell, a plant cell, or an amphibian cell.
20. A method of purifying Botulinum neurotoxin (BoNT) comprising the steps:

- a) contacting the BoNT to a compatible non-toxic non-hemagglutinin (NTNHA), under conditions appropriate for binding of the NTNHA to the BoNT to thereby form a NTNHA-BoNT complex.

21. The method of claim 20, wherein the BoNT is in solution, and the NTNHA is attached to a matrix, whereby the solution is contacted to the matrix to thereby contact the BoNT to the NTNHA.

22. The method of claim 21, further comprising:

- b) washing the matrix to thereby remove unbound materials; and
- c) eluting the BoNT from the matrix by contacting the matrix with an aqueous solution that dissociates the BoNT from the NTNHA-BoNT complex.

23. The method of claim 21, further comprising:

- b) washing the matrix to thereby remove unbound materials;
- c) contacting the matrix with a protease under conditions that preserve the NTNHA-BoNT complex and are appropriate for cleavage of the BoNT within the NTNHA-BoNT complex;
- d) washing the matrix to thereby remove the protease and unbound materials; and
- e) eluting the BoNT from the matrix by contacting the matrix with an aqueous solution that dissociates the BoNT from the NTNHA-BoNT complex.

24. The method of claim 21-23, wherein the NTNHA is covalently linked to an affinity moiety, the matrix is linked to a binding target of the affinity moiety, and the NTNHA is non-covalently bound to the matrix through interactions of the affinity moiety and the binding target.

25. The method of claims 20-24, wherein the BoNT comprises a modified receptor binding domain of Clostridial botulinum serotype B (B-H_c).

26. The method of claim 22 or 23, wherein the aqueous solution has a pH of ≥ 7.5 .

27. The method of any one of claims 21-26 wherein the solution comprising the BoNT is a cleared cell extract from BoNT expressing cells.

28. The method of claim 27 wherein the cleared cell extract further comprises 1 mM of phenylmethylsulfonyl fluoride (PMSF).

29. The method of any one of claims 20-27 wherein conditions appropriate for binding comprise contacting the BoNT in the context of a binding buffer which has a physiological ionic strength and a pH of < 7.5 .

30. The method of any one of claims 20-29 wherein washing is with a wash buffer that is of physiological ionic strength with a pH of < 7.5 .

31. The method of claim 29 or 30, wherein the binding buffer and/or wash buffer is between 100-200 mM KCl or NaCl.

32. The method of any one of claims 29-31, wherein the binding buffer and/or wash buffer has a pH of about 6.

33. The method of any one of claims 29-32 wherein the binding buffer and/or wash buffer comprises 50 mM MES, 150 mM NaCl, pH 6.

34. The method of any one of claims 29-33 wherein the aqueous solution is an elution buffer of about 50 mM Tris, 150 mM NaCl.

35. The method of any one of claims 29-34, wherein the aqueous solution is an elution buffer of about pH 8.

36. The method of any one of claims 24-35, wherein the affinity moiety is selected from the group consisting of glutathione -S-transferase (GST), C-myc tag, Chitin-binding domain,

Streptavidin binding protein (SBP), Cellulose-binding domain, Calmodulin-binding peptide, S-tag, Strep-tag II, FLA, Protein A, Protein G, histidine affinity tag (HAT), Poly-His, and maltose binding protein (MBP).

37. The method of any one of claims 24-36, wherein the affinity moiety is GST, and the binding target is glutathione.

38. The method of any one of claims 24-37, wherein the protease is trypsin or Lys-C endoproteinase.

39. The method of any one of claims 24-38, wherein the protease is added at a molar ratio of from about 1:2 to about 1:1000 to the NTNHA.

40. The method of any one of claims 24-39, wherein the protease is contacted to the matrix at room temperature.

41. The method of any one of claims 24-39, wherein the protease is contacted to the matrix for about 10 minutes to 18 hours.

42. A method of purifying Botulinum neurotoxin (BoNT) comprising:

- a) contacting a cleared cell extract comprising the BoNT to a glutathione coated matrix which has attached thereto a compatible non-toxic non-hemagglutinin (NTNHA) fused to glutathione-S-transferase, in a binding buffer with a pH of about 6 to thereby form a NTNHA-BoNT complex;
- b) washing the matrix with a wash buffer with a pH of about 6 to thereby remove unbound materials;
- c) eluting the BoNT from the matrix by contacting the matrix with an elution buffer that has a pH of ≥ 7.5 to thereby dissociate the BoNT from the NTNHA-BoNT complex.

43. A method of purifying Botulinum neurotoxin (BoNT) comprising:

- a) contacting a cleared cell extract comprising the BoNT to a glutathione coated matrix which has attached thereto a compatible non-toxic non-hemagglutinin (NTNHA) fused to glutathione-S-transferase, in a binding buffer with a pH of about 6 to thereby form a NTNHA-BoNT complex;
- b) washing the matrix with a wash buffer with a pH of about 6 to thereby remove unbound materials;
- c) contacting the matrix with a protease in a buffer with a pH of about 6 to thereby cleave the BoNT within the NTNHA-BoNT complex;
- d) washing the matrix with a wash buffer with a pH of about 6 to thereby remove the protease and unbound materials;
- e) eluting the BoNT from the matrix by contacting the matrix with an elution buffer that has a pH of ≥ 7.5 to thereby dissociate the BoNT from the NTNHA-BoNT complex.

44. The method of any one of claims 42 or 43, wherein the BoNT comprises a modified receptor binding domain of Clostridial botulinum serotype B (B-H_c).

45. The method of any one of claims 42 or 43, wherein the binding buffer and/or wash buffer comprises 50 mM MES, 150 mM NaCl.

46. The method of claim 45, wherein the binding buffer further comprises 1 mM phenylmethylsulfonyl fluoride (PMSF).

47. The method of any one of claims 42-46, wherein the elution buffer comprises 50 mM Tris, 150 mM NaCl, and has a pH of about 8.

48. The method of any one of claims 42-47, wherein the glutathione coated matrix is glutathione-linked agarose beads.

49. The method of any one of claims 42-47, wherein the glutathione coated matrix is a column.

50. The method of claim 42-49, wherein the glutathione coated matrix has about 5 mg/ml bound NTNHA.

51. The method of any one of claims 43-50 wherein the protease is trypsin or Lys-C endoproteinase.

52. A method of purifying a polypeptide comprising a receptor binding domain (Hc polypeptide) of Botulinum neurotoxin, comprising the steps:

- a) contacting a solution comprising the Hc polypeptide to a matrix which has attached thereto compatible non-toxic non-hemagglutinin (NTNHA), under conditions appropriate for binding of the NTNHA to the Hc-polypeptide to thereby form a NTNHA-Hc polypeptide complex;
- b) washing the matrix to thereby remove unbound materials;
- c) eluting the Hc polypeptide from the matrix by contacting the matrix with an aqueous solution that dissociates the Hc polypeptide from the NTNHA-Hc polypeptide complex.

53. The method of claim 52, wherein the receptor binding domain of the Hc polypeptide is a modified receptor binding domain of Clostridial botulinum serotype B (B-H_c).

54. The method of claim 52-53, wherein the Hc polypeptide is a Botulinum neurotoxin (BoNT) polypeptide.

55. The method of claim 52-53, wherein the Hc polypeptide is a chimeric Botulinum neurotoxin (BoNT) polypeptide.

56. Use of a molecule according to any one of claims 1 to 19, in a method of purifying a Botulinum neurotoxin (BoNT) polypeptide.

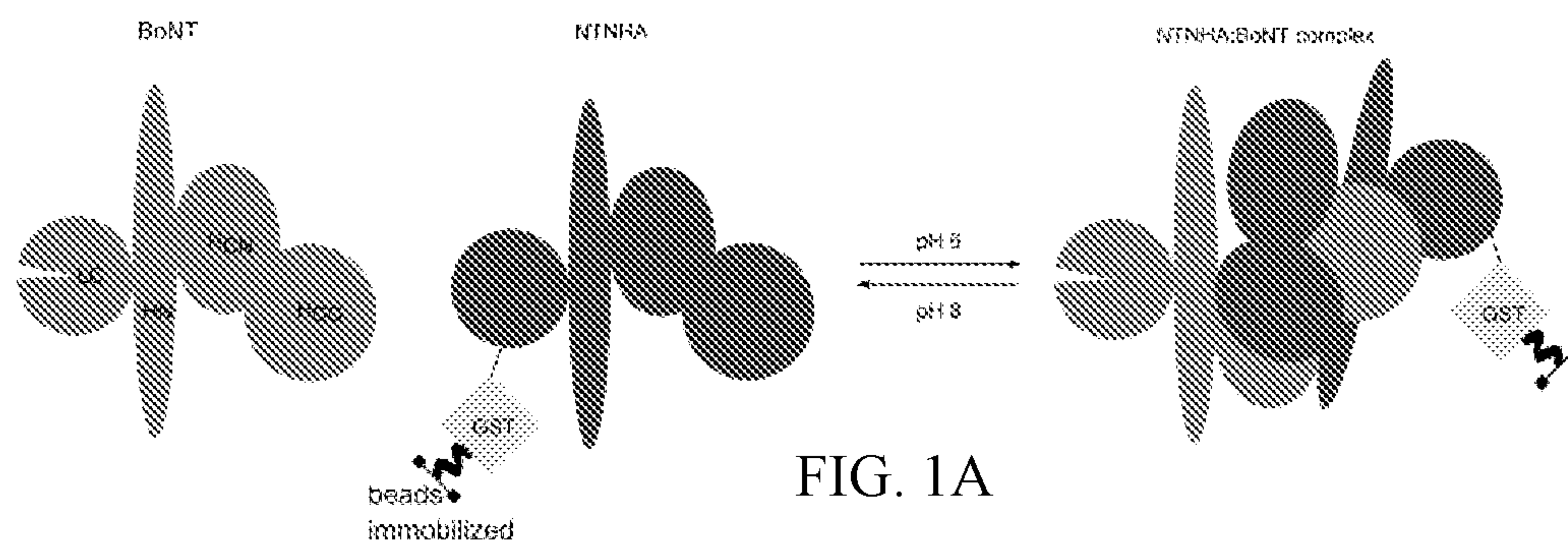


FIG. 1A

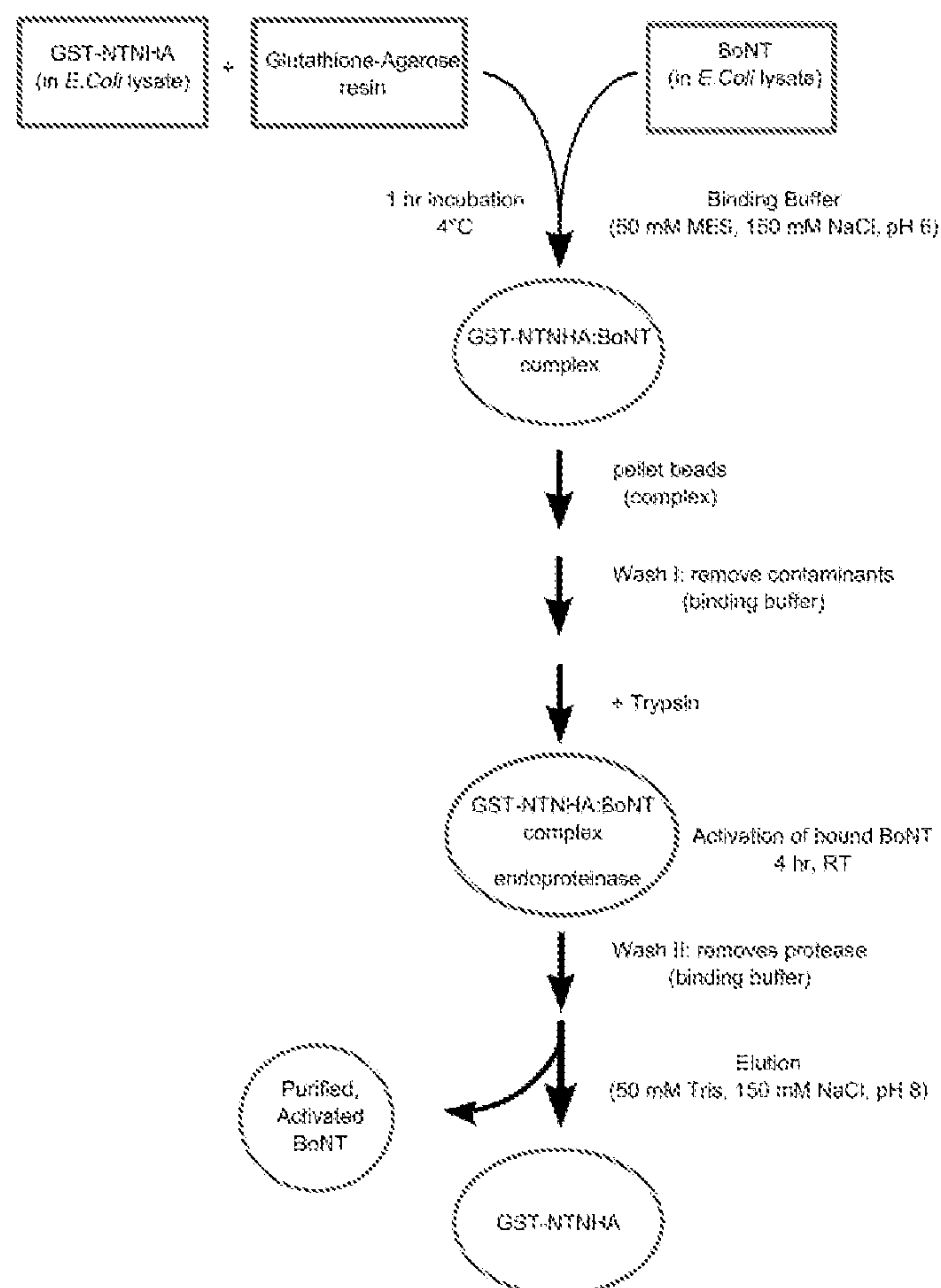


FIG. 1B

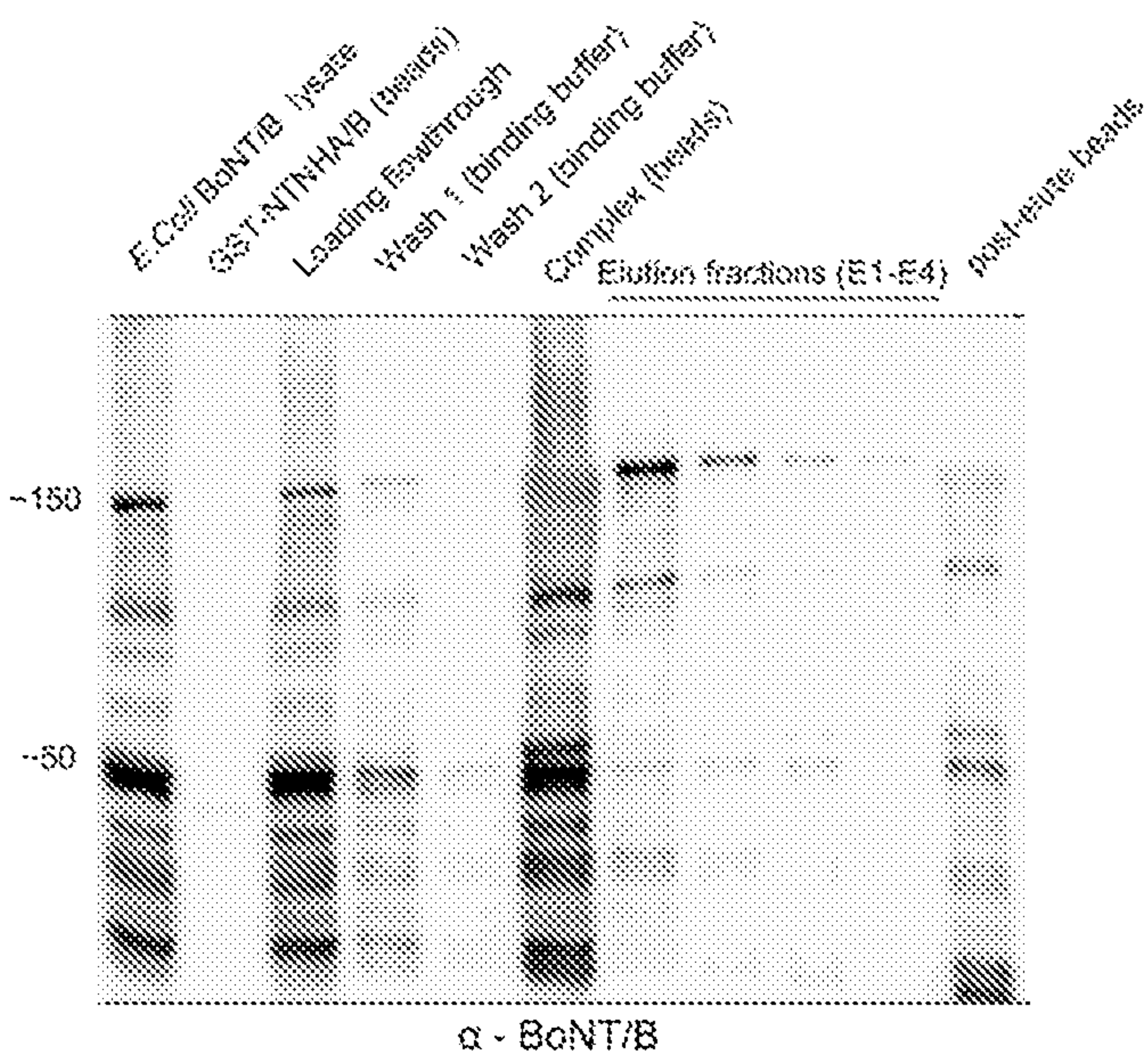


FIG. 2A

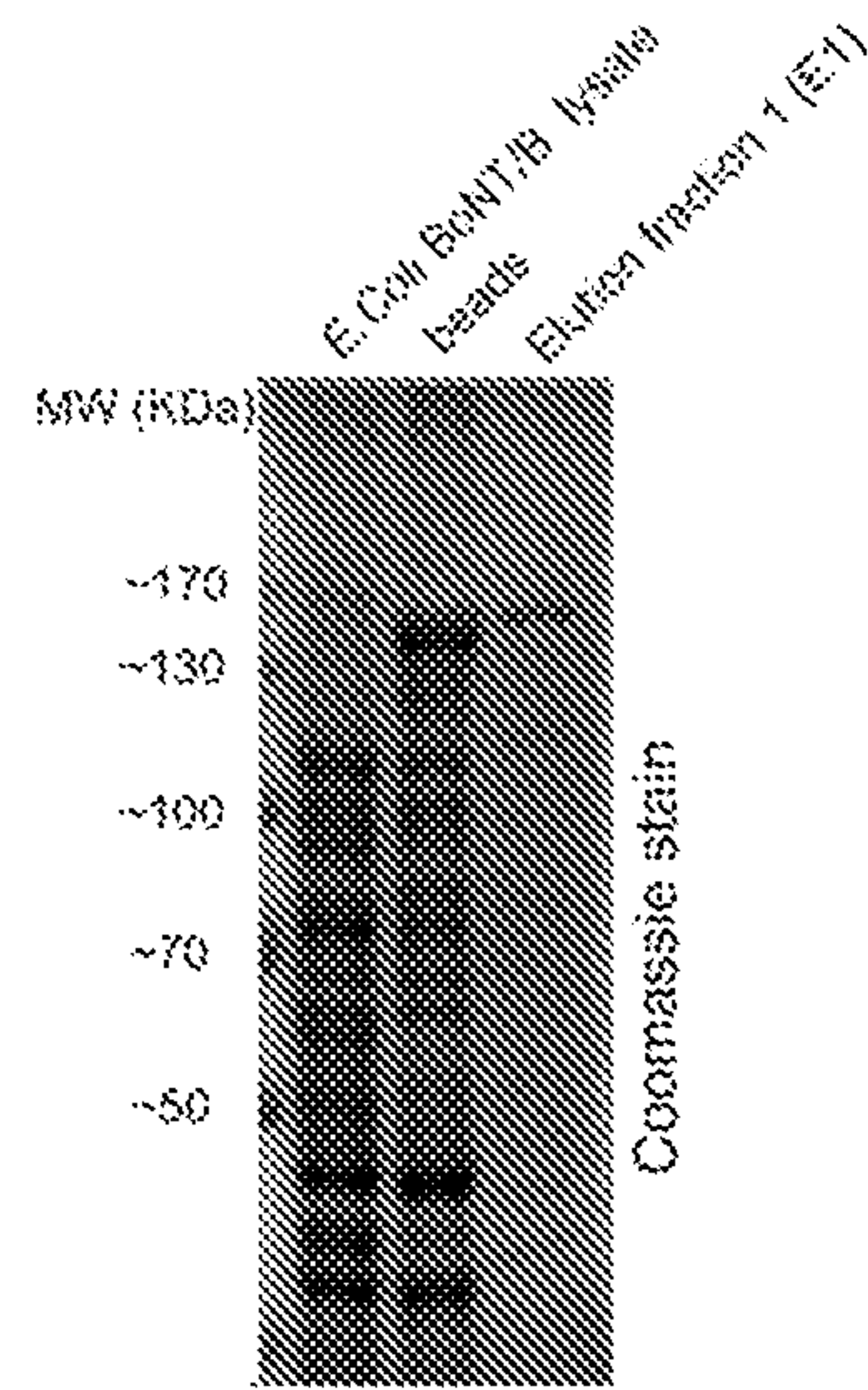


FIG. 2B

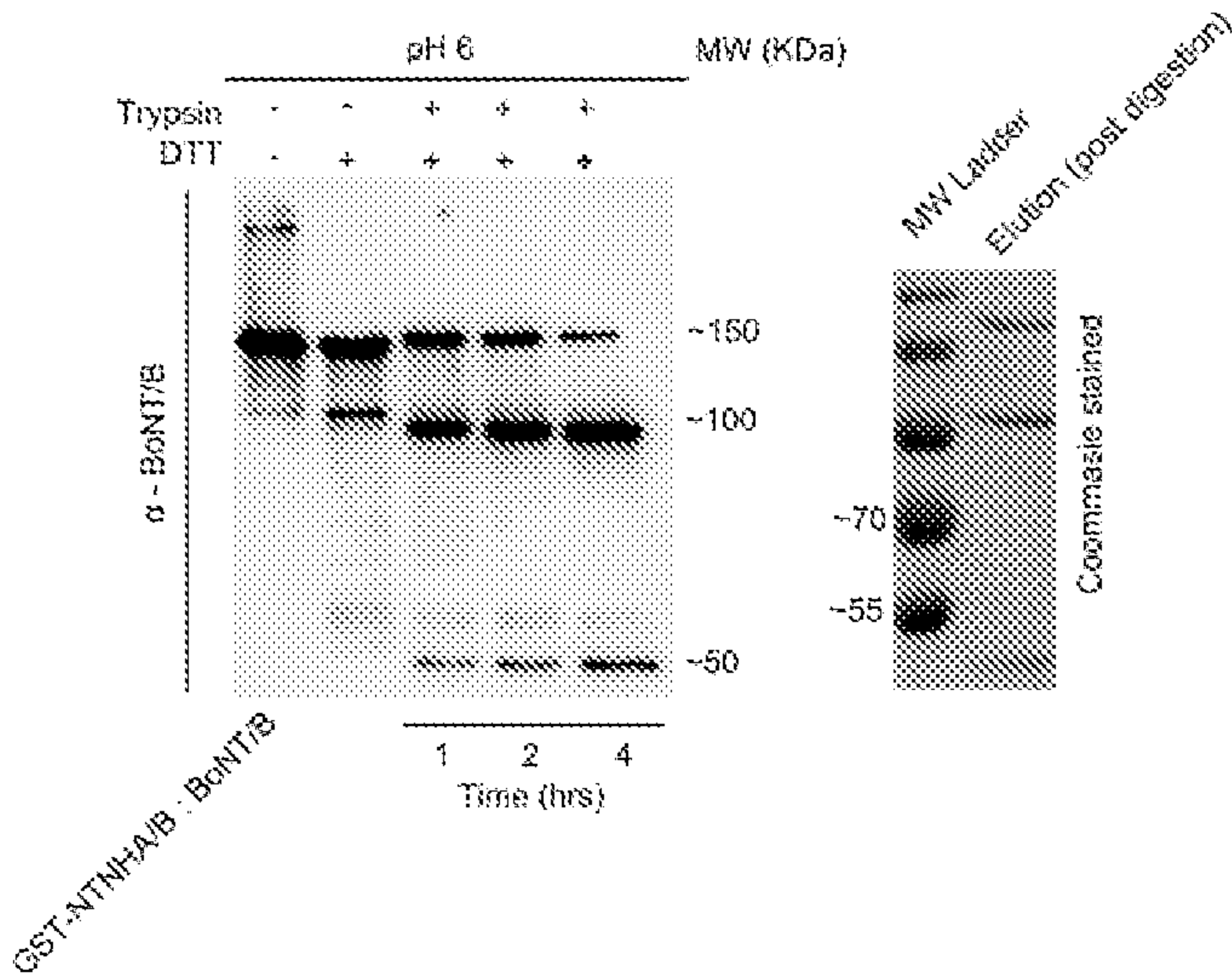


FIG. 3A

FIG. 3B

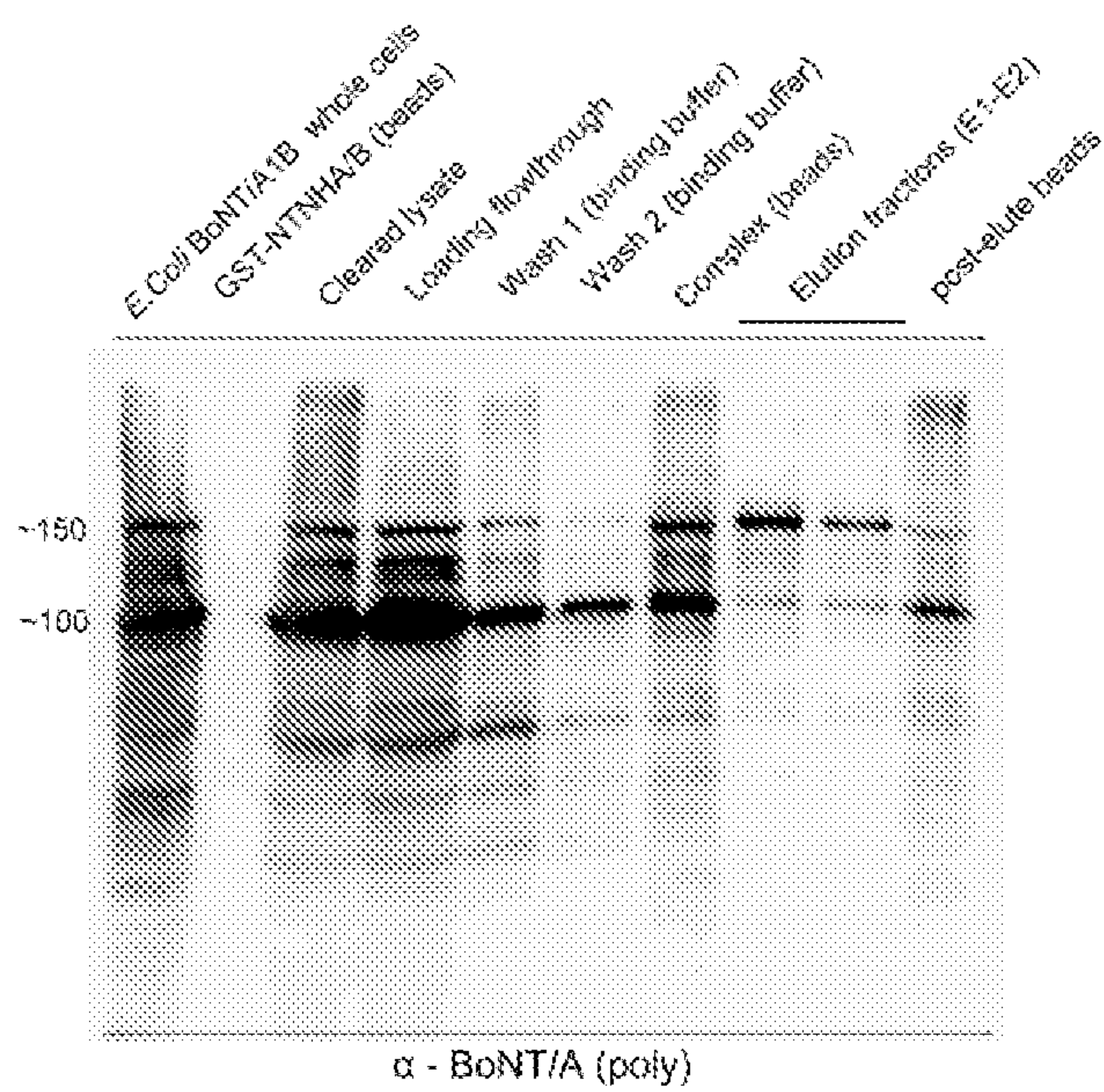


FIG. 4

NTNHA A1: *Clostridium botulinum* A str. ATCC 350. (YP_001253341.1) (SEQ ID NO: 22)

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1 mnindnlsin spvdknkvvv vrarktdtvf kafkvapniw vaperyyges lsideeykvd
61 ggiydsnfls qdsekdkflq aiitllkrin stnageklls listaipfpy gyigggyyap
121 nmitfgsapk snkklslis stipfpyagy retnylssed nksfyasniv ifgpganive
181 nntvfykked aengmgtmte iwfpqfityk ydefyidpai elikcliksl yflygikpsd
241 dlvipyrlrs elenieysql nivdllvsgg idpkfintdp ywftdnyfsn akkvfedhrn
301 iyeteiegnn aigndiklrl kqkfrinind iwelnlnyfs kefsimmpdr fnnalkhfyr
361 kqyykidype nysingfvng qinaqlslsd rnqdiinkpe eiinllngnn vslmrsniyg
421 dglkstvddf ysnykipynr ayeyhfnnsn dsslndvnig vidnipeiid vnpykencdk
481 fspvqkitst reintnipwp inylqaqntn nekfsllssdf vevvsskdks lvysflsnvm
541 fyldsikdns pidtdkkyyl wlreifrnyf fditatqein tncginkvvt wfgkalniln
601 tsdsfveefq nlgaisslink kenlsmpiie syeipndmlg lplndlnekl fnyiyskntay
661 fkkiyyfnld qwwtqyysqy fdlicmakrs vlaqetlikr iiqkklsyli gnsnissdnl
721 almnltttnt lrdissnesqi amnnvdsfln naaicvfesn iypkfisfme qcinninikt
781 kefiqkctni nedeklqlin qnvfnslde flniqnmxsl fssetallik eetwpyelvl
841 yafkepgnnv igdasgknts ieyskdiglv yginsdaly ngnsqsisfs ndffengltn
901 sfsiyfwlrn lgkdtiksl igskedncgw eiylqdtglv fnmidsngne kniylsdvsn
961 nswhyitisv drlkeqllif iddnlvanes ikeilniyss niisllsenn psyiegltil
1021 nkpttsqevl snyfevlnds yirdsneerl eynktyqln yvfssdkpice vkqnnniylt
1081 inntnnlnlq askfkllsin pnkqyvqkld eviisvldnm ekyidisedn rlqlidnknn
1141 akkmiisndi fisncltly ngkyiclsmk denhnwmicn ndmskylylw sfk

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FIG. 5A

NTNHA A2: *Clostridium botulinum* A2 str. Kyoto (WP_012704905) (SEQ ID NO: 23)

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1 mkinnnfnid slidnrsvai vrgrktdtff kvfqvapniw iaperyyges lninedqksd
61 ggiydsnfls tndekdeflq atvkilqrin nnvigaklls listaipfpy eykpgdyrqt
121 nylvskdnqh yytanlvifg pgtnivena iyykkedsen gmgtmseiwf qpfltykygg
181 fyvdpaleli kclikslyyl ygikpsddls ipyrlrseln sfeyseldmi dflisggtey
241 klltdnpywf tdnyfidapk nfekykndye tkiknnndia nsiklyleqk fktnaqdiwe
301 lnlsyfstef eimmpeifnn alnhyyrkey yvidyfkyn ingfingqik tilplskeyn
361 niinkpelvv nlinenntvl mksnvvgdgl kgtmdnfya ykipynigde yhinysynn
421 vnveeinnip pindadiypy rknsdpfipv ynitetkein tttplsvnyl qaqvtnsndi
481 slssdfskvi ssksrslvys fldntidyld sikydepidt dkkyyllwke ifrnysfdmt
541 etqevntpcg inkvvpwlgk alnilntgns fieefkslpg islinkkeni tmpkieidei
601 pnsmlnlsfk dlsenlnrf sknsyfeke yydfldqwwt qyysqyfdli cmakksilaq
661 etlikkiqk klsylignsn issdnlalmn lttntnlrdi snesqiamnn vdsflnsaai
721 cvfegniysk fisfmeqcin ninkntrefi qkctnitene klqlinqnif ssldfdflni
781 enklslfsse tallikeets pyelvlyafq epdnnaigda sakntsieys kdidlvysin
841 sdalyngsn qsisfsndff engltnsfsi yfwlrnlgkd tikskligsk edncgweiyf
901 qdtglvfnmi dsngnekniy lsdvsnnsw yitisvdrk eqllifiddn lvanesikei
961 lniiysnniis llsennpsyi egltilnkpt tsqevlnnyf kvlnnsyird sneerleynk
1021 tyqlynyvfs dkpicevkqn nniyltinnt nlnlqpskf kllsinsnkq yvqkfdevii
1081 silgnmekyi disednrlql idnkngakkm iisndmfisn cltlscggky iclsmkdenh
1141 nwmicnndms kylylwsfk

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FIG. 5B

NTNHA B: *Clostridium botulinum* B1 str. Okra (WP_003404192.1) (SEQ ID NO: 24)

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1 mnindnlsin spvdknkvvv vrarktdtvf kafkvapniw vaperyyges lsideeykvd

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FIG. 5C

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61  ggiydsnfls qdsekdkflq aiitllkrin stnageklls listaipfpy gyiggggyap
121 nmitfgsapk snkklnslis stipfpyagy retnylssed nksfyasniv ifgpganive
181 nntvfykked aengmgtmte iwfgpflytk ydefyidpai elikcliksl yflygikpsd
241 dlvipyrlrs elenieysql nivdllvsgg idpkfintdp ywftdnyfsn akkvfedhrn
301 iyetqiegnn aigndiklrl kqkfrinind iwelnlnyfs kefsimmpdr fnnalkhfyr
361 kqyykidype nysingfvng qinvqlslsd rnqdiinkpe eiinllngnn vslmrsniyg
421 dglkstvdff ysnykipynr ayeyhfnnsn dssldnvnig vidnipeiid vnpykencdk
481 fspvqkitst reintnipwp inylqaqntn nekfsllsdf vevvsskdkd lvysflsnvm
541 fyldsikdns pidtdkkyyl wlreifrnyf fditatqein tdcginkvvt wfgkalniln
601 tsdsfveefq nlgpislink kenlsmpiie iygipndmlg lplndlnekl fnyiylknily
661 fkkvyfnfld qwwteyysqy fdlicmakqs ilaqeklikq iiqnklqdlf kadismdkln
721 lmnlatektf idlsnesqia innindflnk saicvfdtni ypkfisfmeq cinsvnsnvt
781 afiqkctnit edeklqlikl ntfmnidfef fdiqsikdli tsetdlikee kesdynlflf
841 tlqednnkvi edisgkntlv kysdsislvy gvngdalylk epdesvsfsn kafengltns
901 fsicfwlrnl gediitskli enkadncgwe iyfennglvf sivdcngnee niylsdvisk
961 nwyysisid rlrnqllifi ndkliangsi eqilniyssn tislvnennp iyieglstiln
1021 rsitseevvn nyfsylnnsy irdisgerle ynktyelyny vfpenslyev tennniylsi
1081 kdtnnlniqg akfklinida nkqyvqkwde gvvcllgdee kyvdisenn riqlvnskd
1141 akriifnndi fmpncltfay nnkylslslr drnynwmicn nndnipkaah lwalkgi

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FIG. 5C Continued

NTNHA C1: Clostridium botulinum C1 (YP 398515.1) (SEQ ID NO: 25)

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MDINDDLNIN SPVDNKNVVI VRARKTNTFF KAFKVAPNIW VAPERYYGEP LDIAEEYKLD
GGIYDSNFLS QDSERENFLQ AIIILLKRIN NTISGKQLLS LISTAIPFPY GYIGGGYSSP
NIFTFGKTPK SNKKLNSLVT STIPFPFGGY RETNYIESQN NKNFYASNII IFGPGSNIVE
NNVIYYKKND AENGMGTMMAE IVFQPLLYTK YNKFYIDPAM ELTKCLIKSL YFLYGIKPSD
NLVVPYRLRT ELDNKQFSQL NIIDLLISGG VDLEFINTNP YWFTNSYFPN SIKMFEKYKN
IYKTEIEGNN AIGNDIKLRL KQKFQINVQD IWNLNLNYFC QSFNSIIPDR FSNALKHFYR
KQYYTMDYTD NYNINGFVNG QINTKLPLSN KNTNIIISKPE KVVNLVNENN ISLMKSNIYG
DGLKGTTEDF YSTYKIPYNE EYEYRFNDS DNFPLNNISIE EVDSIPEIID INPYKDNSDN
LVFTQITSMT EEVTTHTALS INYLQAQITN NENFTLSSDF SKVVSSKDKS LVYSFLDNLM
SYLETIKNDG PIDTDKYYL WLKEVFKNYS FDINLTQEID SMCGINEEVL WFGKALNILN
TSNSFVEEYQ DSGAISLISK KDNLREPNI EIDDISDLLG LSFKDLNNKL YEIYSKNIVY
FKKIYFSFLD QWWTEYYSQY FELICMAKQS ILAQESLVKQ IVQNKFTDLS KASIPDPTLK
LIRETTEKTF IDLSNESQIS MNRVDNFLNK ASICVFVEDI YPKFISYMEK YINNINIKTR
EFIQRCTNIN DNEKSILINS YTFKTIDFKF LDIQSIKNFF NSQVEQVMKE ILSPYQLLLF
ASKGPNSNII EDISGKNTLI QYTESIELVY GVNGESLYLK SPNETIKFSN KFFTNGLTNN
FTICFWLRFT GKNDDKTRLI GNKVNNCGWE IYFEDNGLVF EIIDSNGNQE SVYLSNIIND
NWYIYSISVD RLKDQLLIFI NDKNVANVSI DQILSIYSTN IISLVNKNNS IYVEELSVLD
NPITSEEVIR NYFSYLDNSY IRDSSKSLE YNKNYQLYNY VFPETSLYEV NDNNKSYLSL
KNTDGINISS VKFKLINIDE SKVYVQKWDE CIICVLDGTE KYLDISPENN RIQLVSSKDN
AKKITVNTDL FRPDCITFSY NDKYFSLSLR DGDYNWMICN DNNKVPKGAH LWILES

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FIG. 5D

NTNHA D: *Clostridium botulinum* D (BAA75083.1) (SEQ ID NO: 26)

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1  mdinddl nin spv d n k n v v i v r a r k t n t f f k a f k v a p n i w v a p e r y y g e p l d i a e e y k l d
61 g g i y d s n f l s q d s e r e n f l q a i i l l k r i n n t i s g k q l l s l i s t a i p f p y g y i g g g y s s p
121 n i f t f g k t p k s n k k l n s l v t s t i p f p f g g y r e t n y i e s q n n k n f y a s n i v i f g p g s n i v e
181 n n v i y y k k n d a e n g m g t m a e i v f q p l l t y k y n k f y i d p a m e l t k c l i k s l y f l y g i k p s d
241 n l v v p y r l r t e l d n k q f s q l n i i d l l i s g g v d l e f i n t n p y w f t n s y f p n s i k m f e k y k n
301 i y k t e i e g n n a i g n d i k l r l k q k f q i n v q d i w n l n l n y f c q s f n s i i p d r f s n a l k h f y r
361 k q y y t m d y t d n y n i n g f v n g q i n t k l p l s n k n t n i i s k p e k v v n l v n e n n i s l m k s n i y g
421 d g l k g s t e d f y s t y k i p y n e e y e y r f n d s d n f p l n n i s i e e v d s i p e i i d i n p y k d n s d n
481 l v f t q i t s m t e e v t t h t a l s i n y l q a q i t n n e n f t l s s d f s k v v s s k d k s l v y s f l d n l m
541 s y l e t i k n d g p i d t d k k y y l w l k e v f k n y s f d i n l t q e i d s m c g i n e v v l w f g k a l n i l n
601 t s n s f v e e y q d s g a i s l i s k k d n l r e p n i e i d d i s d s l l g l s f k d l n n k l y e i y s k n i v y
661 f k k i y f s f l d q w w t e y y s q y f e l i c m a k q s i l a q e s l v k q i v q n k f t d l s k a s i p p d t l k
721 l i r e t t e k t f i d l s n e s q i s m n r v d n f l n k a s i c v f v e d i y p k f i s y m e k y i n n i n i k t r
781 e f i q r c t n i n d n e k s i l i n s y t f k t i d f k f l d i q s i k n f f n s q v e q v m k e i l s p y q l l l f
841 a s k g p n s n i i e d i s g k n t l i q y t e s i e l v y g v n g e s l y l k s p n e t i k f s n k f f t n g l t n n
901 f t i c f w l r f t g k n d d k t r l i g n k v n n c g w e i y f e d n g l v f e i i d s n g n q e s v y l s n i i n d
961 n w y y i s i s v d r l k d q l l i f i n d k n v a n v s i d q i l s i y s t n i i s l v n k n n s i y v e e l s v l d
1021 n p i t s e e v i r n y f s y l d n s y i r d s s k s l l e y n k n y q l y n y v f p e t s l y e v n d n n k s y l s l
1081 k n t d g i n i s s v k f k l i n i d e s k g y v q k w d e c i i c v l d g t e k y l d i s p e n n r i q l v s s k d n
1141 a k k i t v n t d l f r p d c i t f s y n d k y f s l s l r d g d y n w m i c n d n n k v p k g a h l w i l e s

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FIG. 5E

NTNHA E: *Clostridium botulinum* type E (WP_003409842) variant (SEQ ID NO: 27)

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1  m k i n g n l n i d s p v d n k n v a i v r s r k s d v f f k a f q v a p n i w i a p e r y y g e s l k i n e d q k s d
61 g g i y d s n f l s t n n e k d e f l q a t i k l l q r i n n n v v g a k l l s l i s t a i p f p y e n n t e d y r q t
121 n y l s s k n n e h y y t a n l v i f g p g s n i i k n n v i y y k k e y a e n g m g t m l e i w f q p f l t h k y d e
181 f y v d p a l e l i k c l i k s l y y l y g i k p n d n l n i p y r l r n e f n s l e y s e l d m i d f l i s g g i d y
241 k l l n t n p y w f i d k y f i d t s k n f e k y k n d y e i k i k n n n y i a n s i k l y l e q k f k i n v k d i w e
301 l n l s y f s k e f q i m m p e r y n n a l n h y y r k e y y v i d y f k n y n i n g f k n g q i k t k l p l s k y n k
361 e i i n k p e l i v n l i n q n n t v l m k s n i y g d g l k g t v d n f y s n y i i p y n l n y e h s i n y s y l d n
421 v n i e e i e k i p p i n d e d i y p y r k n a d t f i p v y n i t k a k e i n t t t p l p v n y l q a q m i d s n d i
481 n l s s d f l k v i s s k g s l v y s f l n n t m d y l e f i k y d k p i d t d k k y y k w l k a i f r n y s l d i t e
541 t q e i s n q f g d t k i i p w i g r a l n i l n t n n s f v e e f k n l g p i s l i n k k e n i t i p k i k i d e i p
601 s s m l n f s f k d l s e n l f n i y c k n n f y l k k i y y n f l d q w w t q y y s q y f d l i c m a s k s v l a q e
661 k l i k k l i q k q l r y l m e n s n i s s t n l i l i n l t t t n t l r d i s n q s q i a i n n i d k f f n n a a m c
721 v f e n n i y p k f t s f m e q c i k n i n k s t k e f i l k c t n i n e t e k s h l i m q n s f s n l d f d f l d i q
781 n m k k l f n s y t e l l i k e q t s p y e l s l y a f q e q d n n v i g d t s g k n t l v e y p k d i g l v y g i n n
841 n a i h l t g a n q n i k f t n d y f e n g l t n n f s i y f w l r n l n q n t i k s k l i g s k e d n c g w e i y f e
901 n n g l v f n i i d s n g n e k n i y l s n i s n k s w h y i v i s i n r l k d q l l i f i d n i l v a n e d i k e i l
961 n i y s s d i i s l l s d n n n v y i e g l s v l n k t i n s n e i l t d y f s d l n n s y i r n f d e e i l q y n r t
1021 y e l f n y v f p e i a i n k i e q n n n i y l s n n n e n s l n f k p l k f k l l n t n p n k q y v q k w d e v i f s
1081 v l d g t e k y l d i s i d n n r i q l v d n k n n a k t f i i n n d i f i s n c l t l t y n n v n v y l s i k n q d y
1141 n w v i c d l n h d i p k k s y l w i l k n i

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FIG. 5F

NTNHA F: *Clostridium botulinum* type F (YP_001390122.1) (SEQ ID NO: 28)

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1 M K I N D D L N I N S P V D N K N V V I V R A R K T N I F F
31 K A F Q V A P N I W V A P E R Y Y G E P L N I S D Q E K S D
61 G G I Y D E N F L K E N S E K E E F L Q A I I L L L K R I N
91 N N I I G Q K L L S L M C T S I P F L H E Y K Q G D Y R Q S
121 N Y L G S K N S E Y L Y S A N I V I F G P G S N I V K N N T
151 I Y Y K K N F A E N G M G T M A E I L F Q P F L T Y K Y N Q
181 F Y A D P A L E L I K C L I K A I Y F L Y G I K P N D N L N
211 I P Y R L R N E F S N V E Y S E L N I I D F L I S G G I D Y
241 K F I N T N P Y W F I D N Y F I D V P K V F E K H K N D Y E
271 I N I K N N S E I G T S I K L Y L E Q K F K T N V Q D I W E
301 L N L S Y F S K E F Q I M M P E K H N N A L K H Y Y R K E Y
331 Y K I N Y S K Q Y D I N G F V N G Q I A T K L L L S E K N Q
361 Y I I N K P Q L I I N L I N K S N N S L L M K S N I Y G D G
391 L N G T T D N F Y R N Y K I P D N I A Y Q Y H P N N T Y L D
421 N V N I E E I N N I P Q I T D A D I Y P Y T N N C D T F I P
451 I Y N I T Q S R E I N T T V P Y S I N Y L Q S Q I M N S D D
481 I T L S S D F W E V V C S N D K S L V Y S Y L D N V I N Y L
511 D S I K N N T P I N T D K K Y Y L W L K E I F R N Y S F D I
541 T A T E E I T T E C G I N K I V S W F G K A F N I L N T D N
571 S F K I E F Q N S G A I A L I N K K D N I I I P K I E I D E
601 M P N S M L N L S F E D L N E Q L Y S I Y S K N I T Y F K K
631 I Y Y N F L D Q W W T E Y Y S Q Y F D L I C M A K K S I L A
661 Q E N L I K K I I Q K K I S Y L I G A S N I P D D I L A V M
691 R L T T T N T L R D I S V E S Q I A M N N L N N F L N K A A
721 M C V F Q S N I Y P K F I S F M E Q C I K H I N K S T K E F
751 I Q K C T N I N E T E K L Q L I M Q N S F S N L D F D F L D
781 I Q N M K N L F N S Y T E L L I K E Q T S P Y E L S L Y A F
811 E E Q D N N V I G D A S G K N T L V E Y P K G I E L V Y G I
841 N N S A L Y L N G S N Q S I I F T N D Y F E N G L T N S F S
871 I Y F W L R N L G Q D T I K S K L I G S K E Y N C G W E I Y
901 F Q E I G H V F N M I D S N G N E K N I Y L S D V S N N S W
931 H Y I T I S V D R L K E Q L L I F I D D N L V V N E S I K D
961 I L N I Y S S N I I S L L S D N K A S Y I E G L T I L N K P
991 T T G E E V L R N Y F K N L N N S Y V R D S N D E R L E Y N
1021 K T Y Q L Y D Y V F P D N P I C E V K Q D N N I Y L T I N N
1051 I N N L N M K P C K F K L L S I N S N K Q Y V Q K W D E V I
1081 I S V L Y D T E K Y V C I S N E N N R V K I I D N K I M Q V
1111 K F I I S N D I F I S N C L T H A H N N K Y I C L S M K D E
1141 N Y N W M I C N N E S N I P K K A Y L W I L K E V

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FIG. 5G

NTNHA G: *clostridium botulinum* (*Clostridium argentinense*) type G ATCC
27322 (CAA61228.1) (SEQ ID NO: 29)

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1 mkinsnltin spidnknvvi vraretskff kafkvapniw vaperyyyges lsieeskkvn
61 ggvydsnfls qnnekdkflq aiitllkrin sniageklls lvstaipfpy gyiggggyycp
121 niwtfgstik ynkkinslis ttipfpyggy retnylsskd tenfyaaniv ifgpganive
181 nntvfykked aengmgtmae icfqpfltyk ydqfyvdpal elmecliksl yflygikpnn
241 nltvpyrlrn elsniefsqsl sivdllisgg idskfintdp ywfidsyfsn akttfeehks
301 iyeteikgnn aigndiklrl kqkfqtthvd iwqlnldyfs kefqimmpyr fnnalkyyyr
361 keyykidype kysiagfvdg qlntqlslsd knqyiinkpe livnlisenn islmrsniyg
421 dglkyttndf ystykipynr ayeyhfnss tsslenvnve eisnipeiid inpyrensdi
481 fspveniiet kevntktpw inylqaqipn neeftlssdf sqvvsyktqs lvysflsnvi
541 sylsvkdtn pidtdekyyl wlreifrnyf fditaieein tscginkvvs wfgkalniln
601 tsnsfvkefk nlgpislink kenlsmpiie vneipndmlg lslkdlnekl fnyiylknily
661 fkkvyfsfld qwwteyysqy fglicmakqs ilagenlikk ivqkklsdls kqsnisnekl
721 nlmnltekt fidlsnqsqi amnninnfln kaaicvfesn iypkfisfme qyinninikt
781 tafirkctni tekeklqlin qntfnnldfe ffdiqtienl ltsetnliik ektspydlla
841 fslqeadrkv ikdisgkdtl vqysdtidls ygvngdaly kepnqsvnfs nnifengltn
901 sfsicfwlrn lgqdnhlssn ignivnncgw qiyfennglv fsmvdcngne kniylsdvls
961 kywyyisvsv drrlnkllif indklivnes ieqlniyys niislvnenn picieelsil
1021 nkaltseevl nsyftnltns yirdsygarl eynknyelyn yvfpenslye viennnmyls
1081 iknikntnil gakfklintd eskqyvqkw eviicvlgt ekyadiqagn nriqlvnskd
1141 narkiivnnn ifrpncvlf ynnkylslsl rnrnynwmic ndnsfipkha hlwilkki

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FIG. 5H

NTNHA E: *Clostridium botulinum* type E (WP_012450714.1) (SEQ ID NO: 30)

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1 mkingnlid spvdnknvai vrarkedvff kafqvapniw ivparyyyges lkinedqkfd
61 ggiydsnfls tnnkddflq atikllqrin nwwvgaklls listaipfpy enntedyrgt
121 nylsaknneh yytanlvifg pganiiknnv iyykkeyaes gmgtmleiwf qpilthkyde
181 fyvdpaleli kclikslyyl ygikpndaln ipyrlrnefn sleyseldmi dflisggidy
241 kllntnpywf idkyfidtsk nfekykndye ikiknnnyia nsiklylegk fkinvkdawe
301 lnlsyfskef qimmperynn alnhyyrkey yvidyfkyn ingfkagqik tkilplakynk
361 eiinkpeliv nlinqntvl mksniygqgl kgtvdnfyen yiipynlnye hsinyeyldn
421 vniecekip pindediypy rkndatfipv ynithakein ttgplpvnvl qagmidendi
481 nlssdfkvi sskgslyvaf lnntmdylef ikydkipdtd kkykwlkai frnyaldite
541 tqeianqfgd tkilpwigra lnilttnnaf veefknigpi alinkkenit ipkikideip
601 samlafsfd lsenlfniyc knnfyllkiy ynfldgwwtg yysqyfdlic maskavlage
661 klikkligkq lrylmeneni setnlilini tttntlrdis ngsqiainni dkifnnaamc
721 vfenniypkf tsfmagcikn inkstkefil kctninetek shlmqnsfs nldfdfldig
781 nmknlfnsyt ellikeqtsp yelalyafge qdnvvgdts gkntiveypk diglvyginn
841 naikltgang nikftndyfa ngltnnfaiy fwlrnlkqnt ikskligake dncgwaiyfe
901 ndglvfniid sngnekniyl snisnnawhy ivisinrlkd qllifidnil vanedikeil
961 niyssdiel lsdnnnyie glsvlnktin aneiltayfs alnnsyirnf deeilqynrt
1021 yelfnyvfge isinkiegnn niylsinne nlnfkplkfk lntnnpnkqy vqkwdevife
1081 vldgtekyld isttnnriql vdnknagif iindifian cltltynnv vylslnqdy
1141 nwwicdlnd ipkksylwil kni

```

FIG. 5I

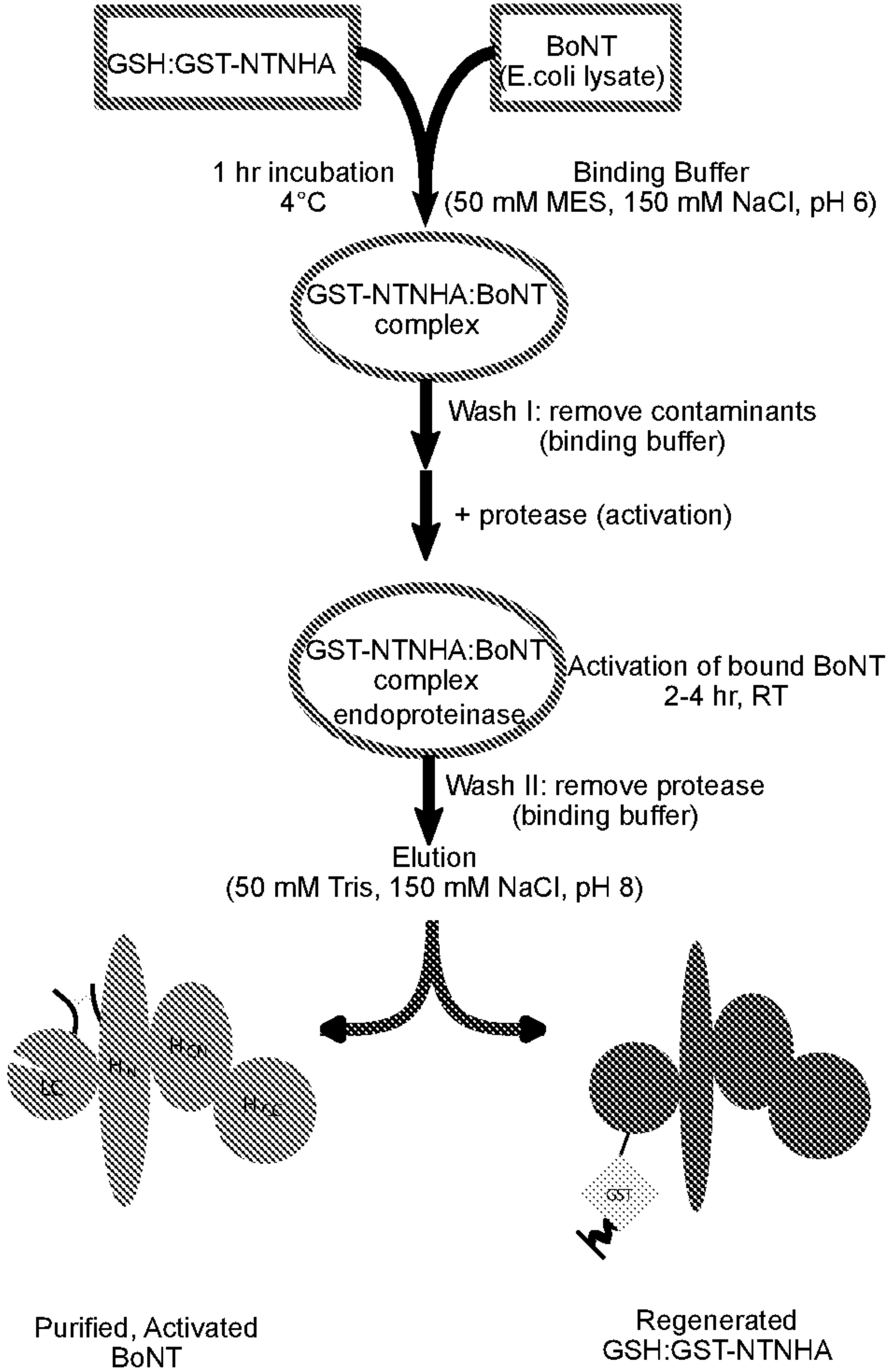
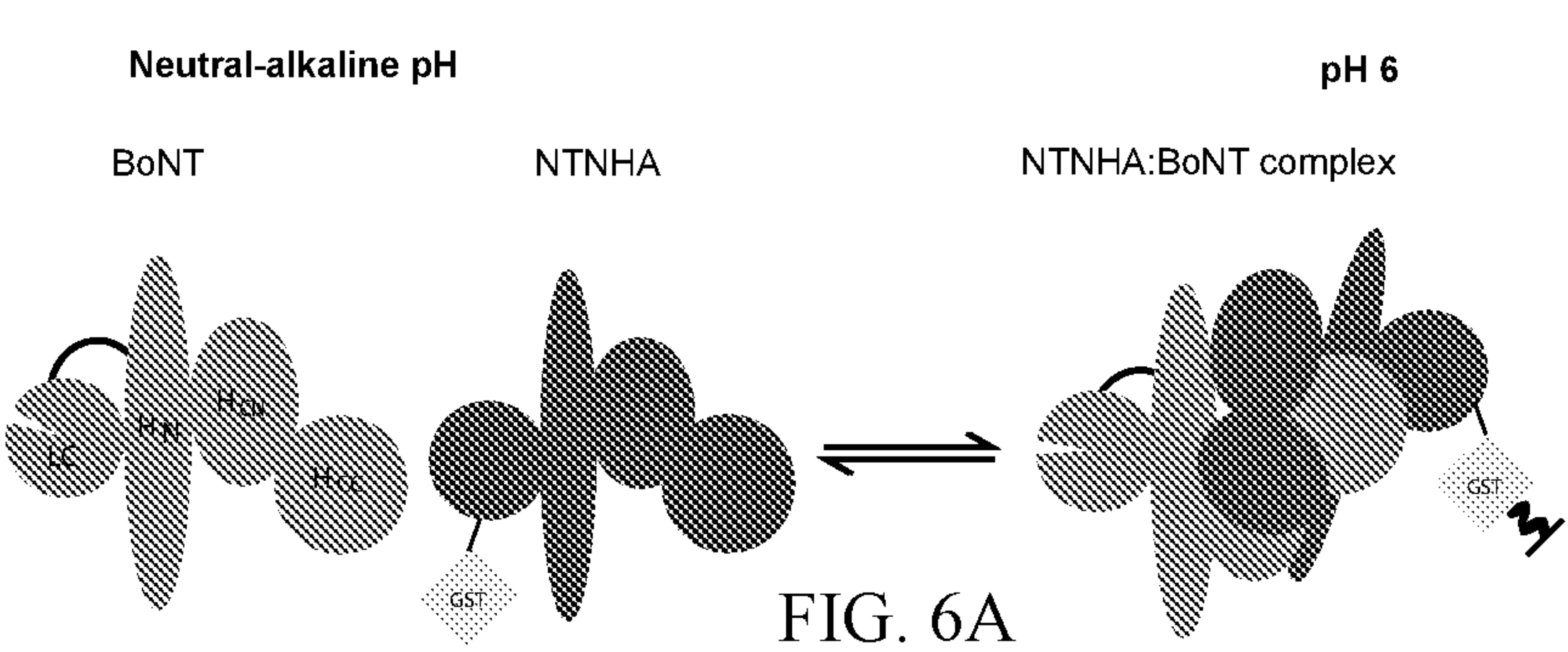


FIG. 6B

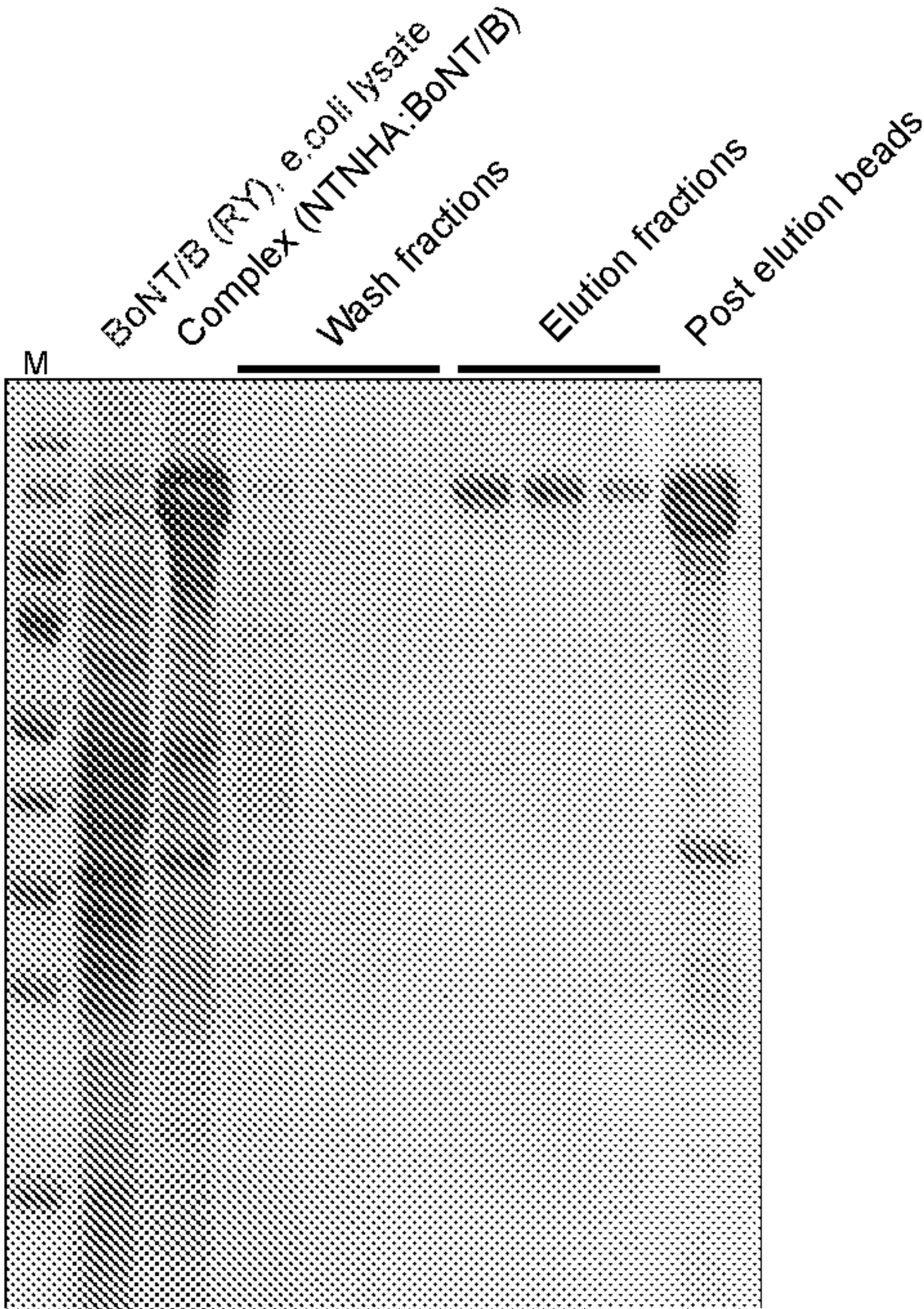


FIG. 6C

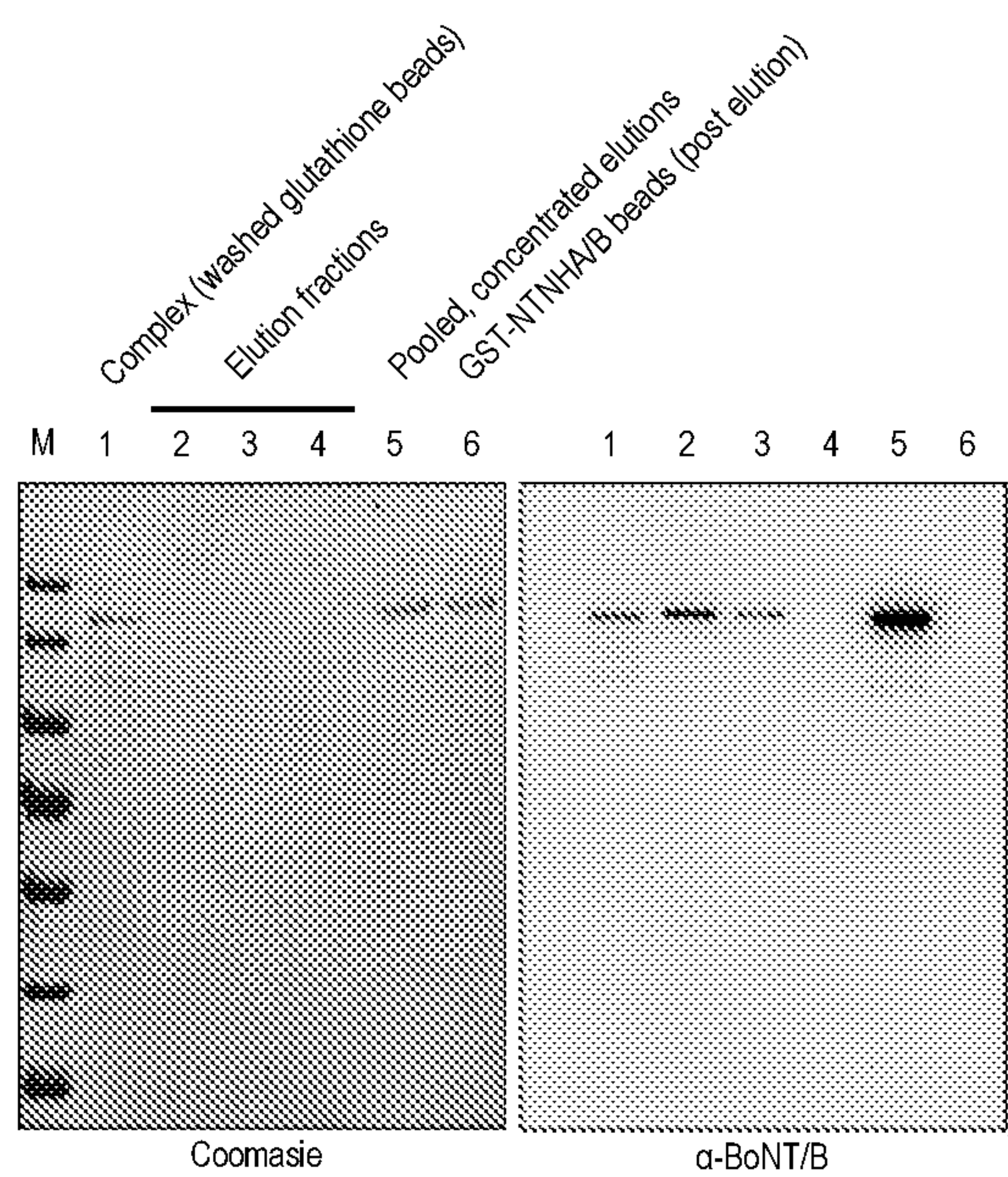


FIG. 7A

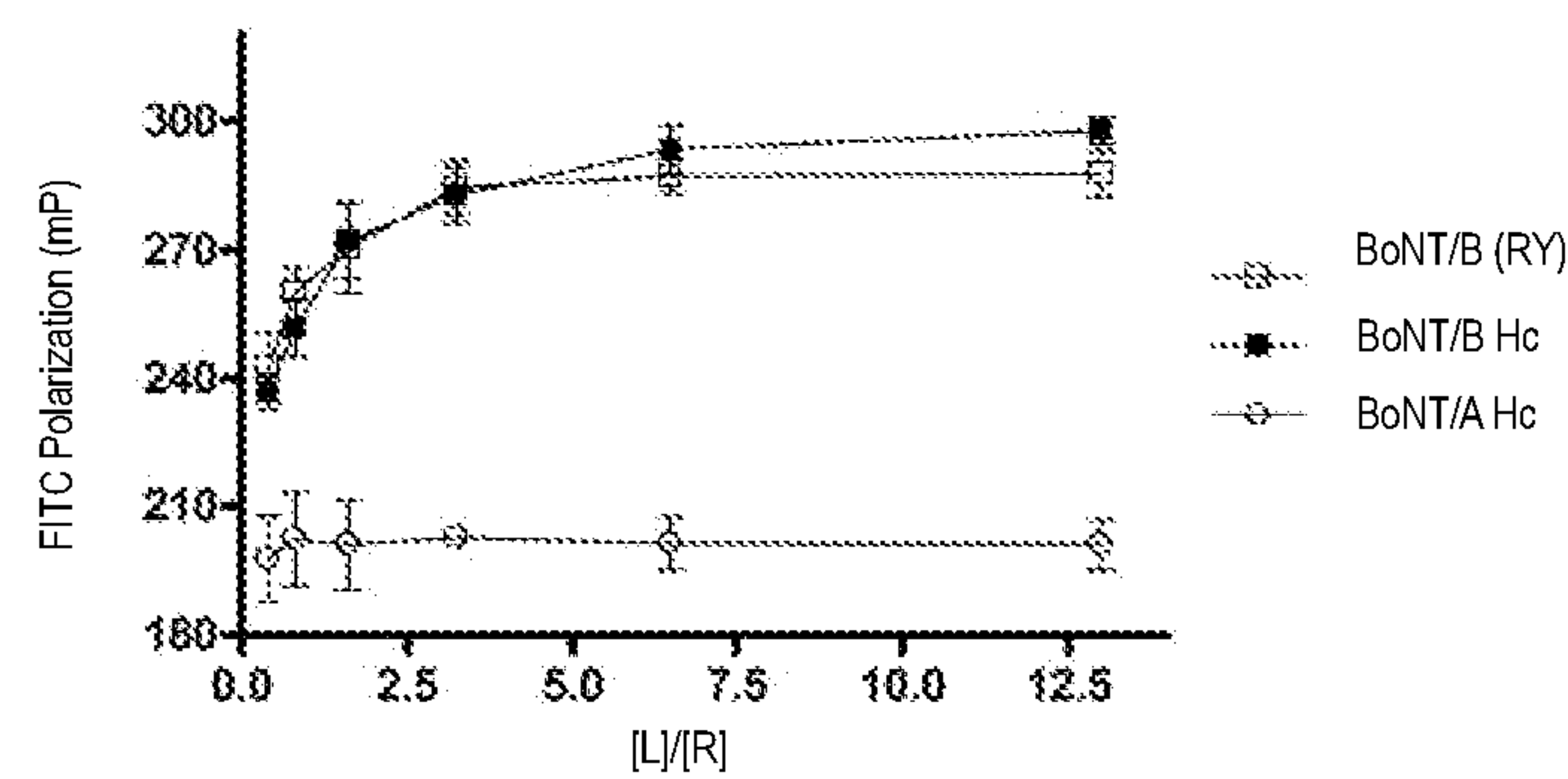


FIG. 7B

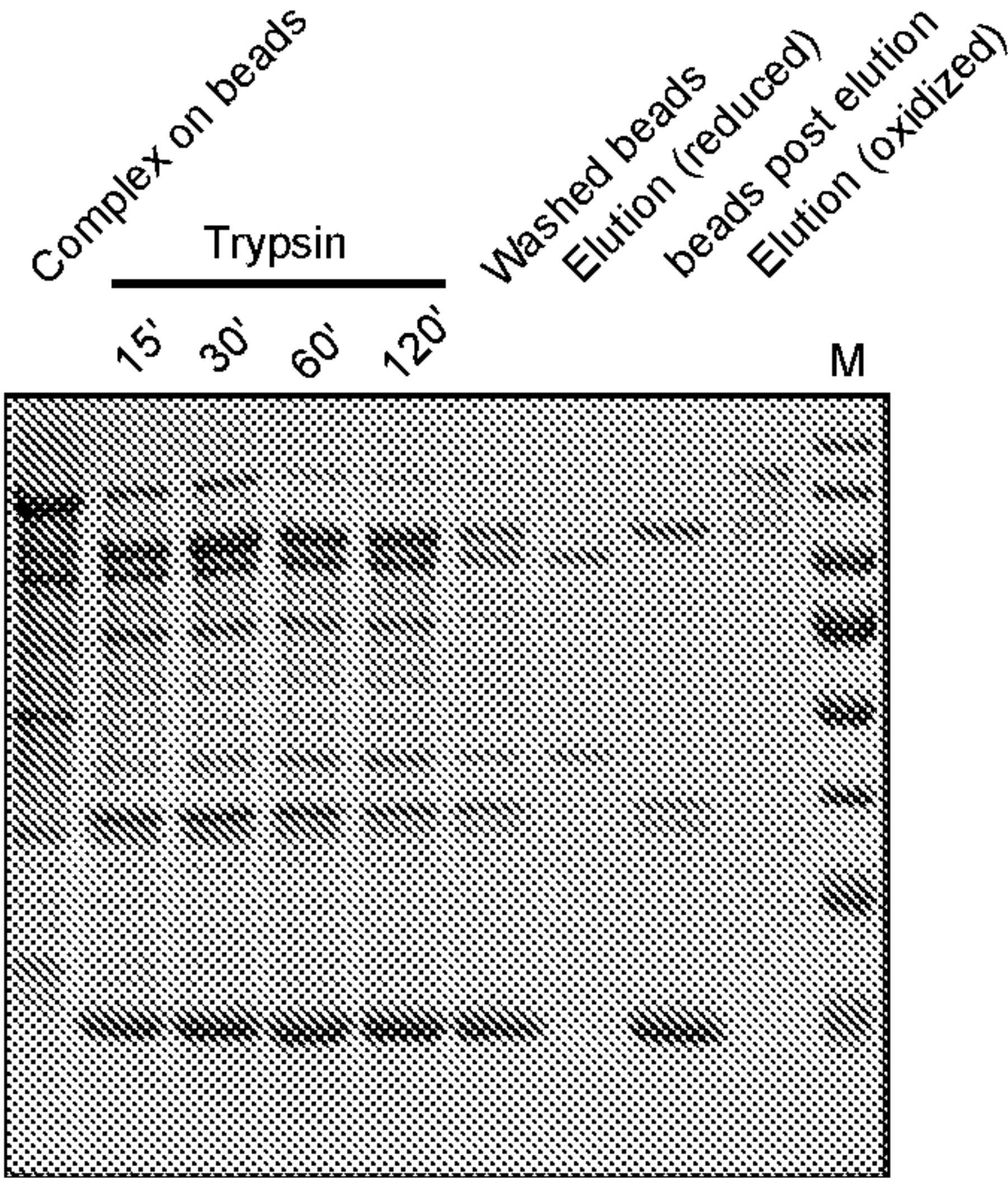


FIG. 8A

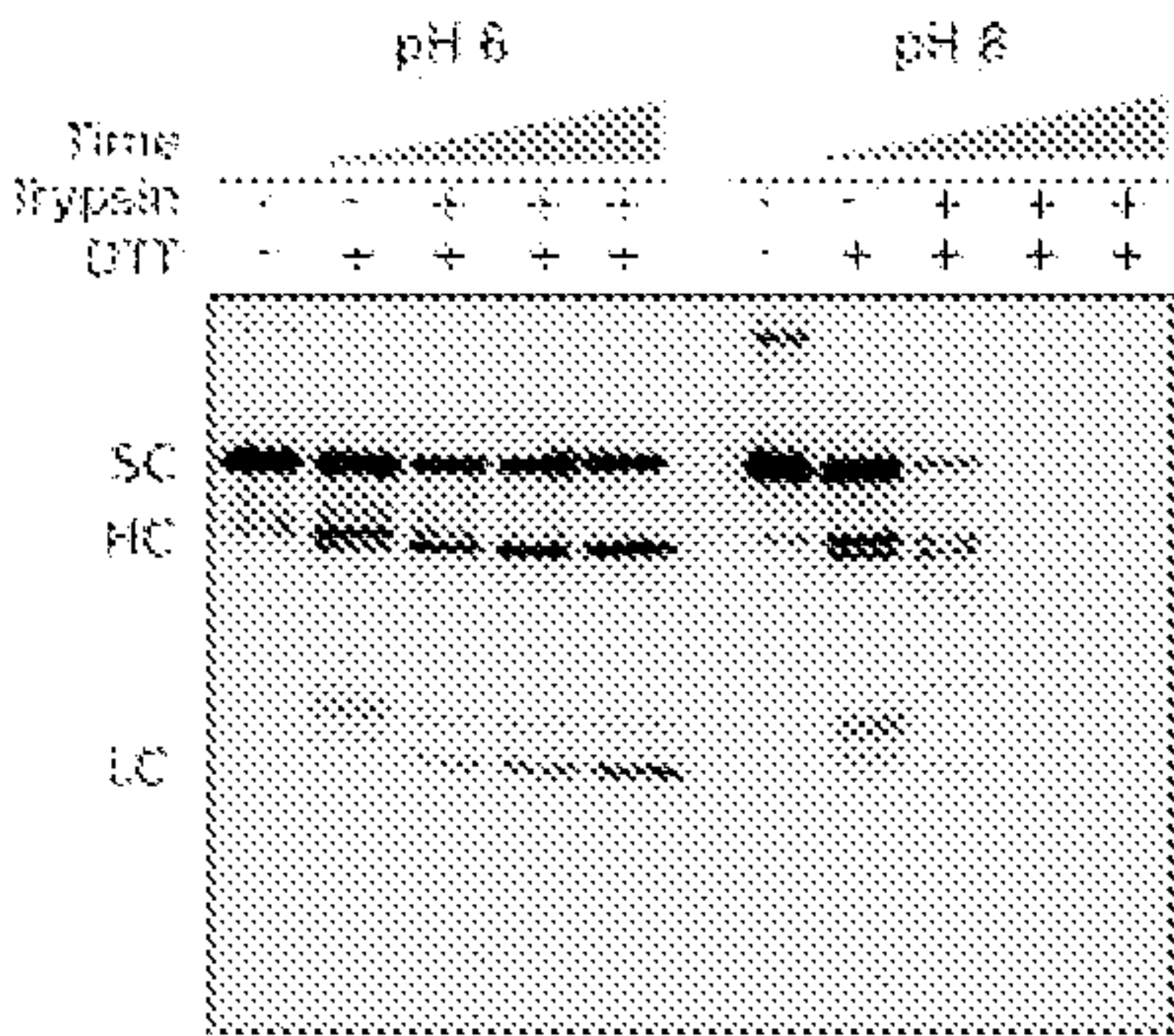


FIG. 8B

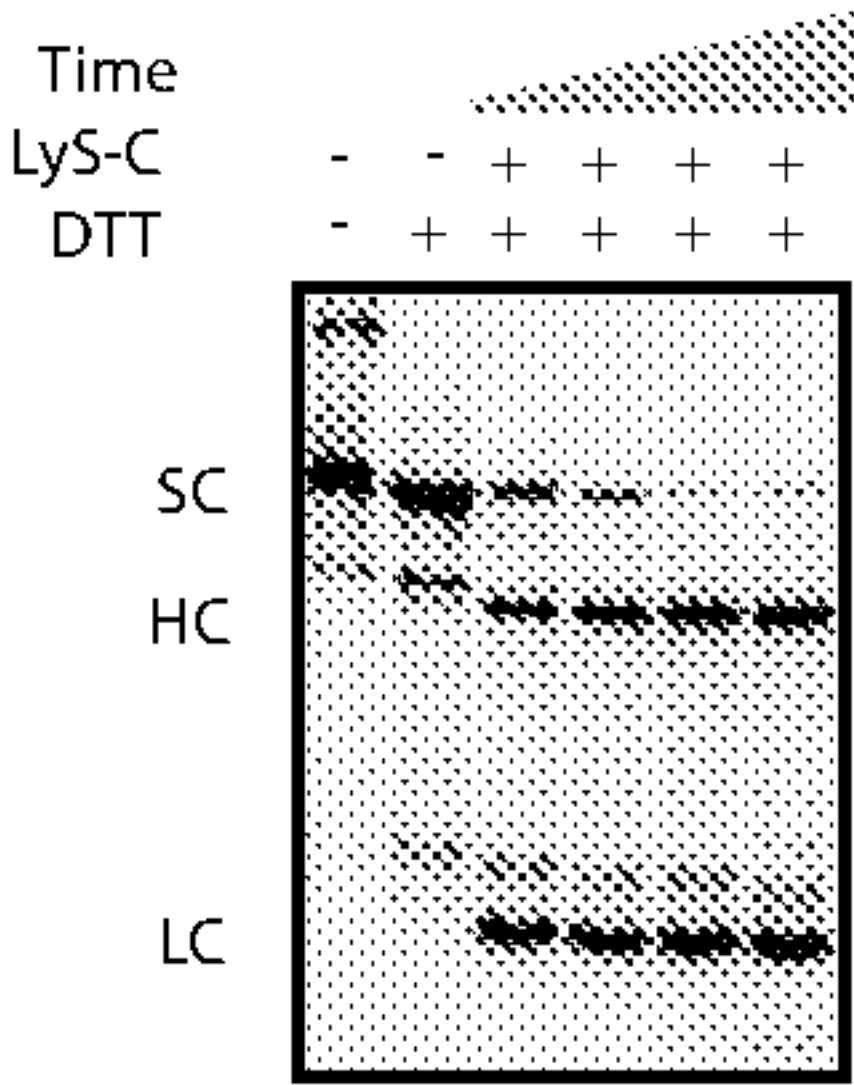


FIG. 8C

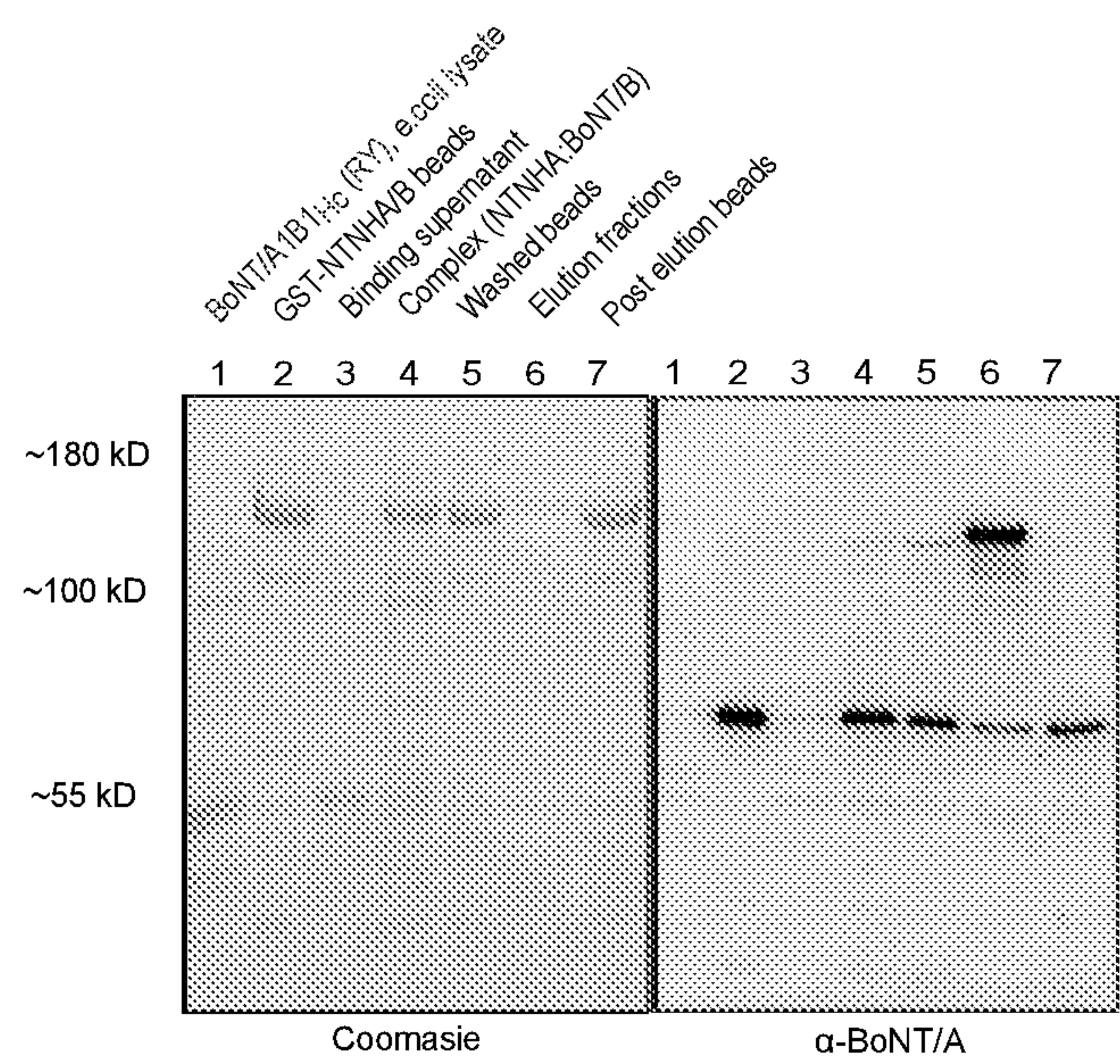


FIG. 9

