

PREPARATION METHOD FOR BOTULINUM TOXIN TYPE A AND USE OF SAME

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the priority of Chinese Patent Application NO.202310681461.9, filed with the China National Intellectual Property Administration on June 08, 2023, and titled with “PREPARATION METHOD FOR BOTULINUM TOXIN TYPE A AND USE OF SAME”, which is hereby incorporated by reference in its entirety.

FIELD

[0002] The present disclosure relates to the technical field of polypeptide preparation, and in particular to efficient production of a botulinum neurotoxin type A and use thereof in the manufacture of a medicament.

BACKGROUND

[0003] *Clostridium botulinum* and *Clostridium tetani* produce highly potent neurotoxins, which are botulinum neurotoxin (BoNT) and tetanus neurotoxin (TeNT), respectively. These clostridial neurotoxins (CNTs) specifically bind to neurons and interfere with neurotransmitter release. *Clostridium botulinum* secretes seven antigenically distinct serotypes of botulinum neurotoxin (BoNT), designated as A to G. All the serotypes are Zn^{2+} -endoproteases that block synaptic exocytosis by cleaving proteins involved in the formation of a SNARE complex that controls cell membrane fusion.

[0004] In 1989, botulinum neurotoxin serotype A (BoNT/A) was approved in the United States for human use to treat strabismus, blepharospasm, and other diseases. It is commercially available as botulinum toxin A protein preparations, for example, sold under the trade name BOTOX (Allergan) and the trade name DYSPORT (Ipsen). Efficient production and product application of BoNT/A have shown great commercial value in the medical field.

[0005] Naturally occurring clostridial toxins are each translated as a single-chain polypeptide of about 150 kDa. A disulfide loop of the single-chain polypeptide is subsequently cleaved by

proteolytic cleavage by naturally occurring proteases, such as endogenous clostridial proteases or naturally occurring proteases produced in the environment. Post-translational processing generates a mature double-chain molecule, which comprises a light chain (LC) of about 50 kDa and a heavy chain (HC) of about 100 kDa, linked together by a single interchain disulfide bond and non-covalent interactions. A linker region or a loop region is present between the cysteine residues involved, a length of which varies greatly among various serotypes of clostridial toxins. The loop is cleaved at the latest when the toxin is released from the clostridia during cell lysis, wherein a ratio of cleaved and uncleaved species varies among serotypes. Cleavage by the protease (in the loop region and at an end of the heavy chain) is random, thereby generating many undesired intermediate products, which increases purification difficulty and reduces product yield. There is a method for separately expressing and purifying heavy and light chains of TeTx and BoNTs in *Escherichia coli* (see Li, et al., Biochemistry 33: 7014-7020 (1994); Zhou, et al., Biochemistry, 34: 15175-15181 (1995)). In the absence of the light chain, the isolated heavy chain has poor water solubility and is very susceptible to proteolytic degradation, and is therefore not practical. Patent US7709228 discloses modifiable enzymatic cleavage sites and proteases that can specifically recognize these sites, but the toxicity of its recombinant product is two-fifths of that of natural TeTx, and the decrease is not very significant. Meanwhile, those skilled in the art generally use a restriction endonuclease construction method, which introduces 2 to 3 amino acid residues after constructing a protein of interest into a region of interest, thereby creating a risk of immunogenicity. Therefore, there is an urgent need in the art for an efficient and safe method for producing a botulinum neurotoxin to obtain a polypeptide product with high safety and high yield, so that it can be applied to medical treatments more safely and effectively.

SUMMARY

[0006] To address the deficiencies of the prior art, a purpose of the present disclosure is to provide a method for producing a botulinum neurotoxin and use thereof.

[0007] A technical solution of the present disclosure is as follows:

[0008] In an aspect of the present disclosure, provided is a recombinant botulinum neurotoxin type A (BoNT/A), having an amino acid sequence as set forth in SEQ ID NO: 7.

[0009] Preferably, the recombinant botulinum neurotoxin type A (BoNT/A) comprises amino acid sequences as set forth in SEQ ID NO: 1, SEQ ID NO: 3, and SEQ ID NO: 5:

[0010] (a) a LC region of BoNT/A having an amino acid sequence as set forth in SEQ ID NO: 1;

5 **[0011]** (b) a thrombin enzymatic cleavage site having an amino acid sequence as set forth in SEQ ID NO: 3; and

[0012] (c) a HC region of BoNT/A having an amino acid sequence as set forth in SEQ ID NO: 5.

10 **[0013]** Preferably, a combination tag is inserted at N-terminal of the amino acid sequence of the BoNT/A via a homologous recombination method, to obtain a recombinant botulinum neurotoxin type A (BoNT/A) having an amino acid sequence as set forth in SEQ ID NO: 13.

[0014] Preferably, the combination tag is a His-tag and a SUMO tag.

[0015] The His-tag has an amino acid sequence as set forth in SEQ ID NO: 9, and a coding nucleotide sequence as set forth in SEQ ID NO: 10.

15 **[0016]** The SUMO tag has an amino acid sequence as set forth in SEQ ID NO: 11, and a coding nucleotide sequence as set forth in SEQ ID NO: 12.

[0017] In a second aspect of the present disclosure, provided is a method for producing a recombinant botulinum neurotoxin type A (BoNT/A), comprising:

20 **[0018]** (1) designing and constructing a recombinant botulinum neurotoxin type A (BoNT/A) having an amino acid sequence as set forth in SEQ ID NO: 13;

[0019] (2) designing, ligating and producing a BoNT/A expression vector;

[0020] (3) performing induced expression of a BoNT/A protein and collecting cells;

[0021] (4) performing affinity purification on a supernatant of cell lysate; and

[0022] (5) removing the SUMO tag by enzymatic cleavage and activating the BoNT/A protein.

25 **[0023]** Preferably, the step of removing the SUMO tag by enzymatic cleavage and activating the BoNT/A protein comprises: performing enzymatic cleavage with a SUMO protease, and subsequently adding 1 U of thrombin per 1 mg of the BoNT/A protein.

[0024] Preferably, the step of induced expression of the BoNT/A protein comprises: after OD600 of cell culture reaching 0.6, adding IPTG to a final concentration of 1 mM, and performing induced culture at 30 °C for 12 h.

[0025] Preferably, the step of collecting cells comprises: collecting cell pellet after centrifugation at 4000 rpm, resuspending the cell pellet with 40 mL of 20 mM PB + 150 mM NaCl at pH 7.2, performing ultrasonic disruption in ice bath at 420 W with a sonication pulse for 3 s and an interval of 5 s for a total duration of 15 min, and collecting a supernatant after centrifugation at 12000 rpm and 4 °C for 20 min; and

[0026] the affinity purification of the supernatant of cell lysate comprises: filtering the collected supernatant through a 0.22 µm filter membrane, and performing affinity enrichment and purification on a Ni column, wherein 20 mM PB + 150 mM NaCl is used as equilibration buffer, 20 mM PB + 150 mM NaCl + 5 mM imidazole, 20 mM PB + 150 mM NaCl + 20 mM imidazole, 20 mM PB + 150 mM NaCl + 50 mM imidazole, and 20 mM PB + 150 mM NaCl + 80 mM imidazole are used as washing buffer, 20 mM PB + 150 mM NaCl + 300 mM imidazole is used as elution buffer, and all the reagents of the affinity purification have a pH of 8.0.

[0027] In a third aspect of the present disclosure, provided is a recombinant BoNT/A polypeptide produced by the aforementioned method.

[0028] In a fourth aspect of the present disclosure, provided is use of a recombinant BoNT/A polypeptide produced by the aforementioned method in the manufacture of a medicament.

[0029] Beneficial Effects

[0030] The present disclosure provides a method for producing a recombinant botulinum neurotoxin type A (BoNT/A). A BoNT/A amino acid sequence is designed based on the protein with an accession number of P0DPI0 in protein database Uniprot. By inserting a His-tag (having an amino acid sequence as set forth in SEQ ID NO: 9 and a coding nucleotide sequence as set forth in SEQ ID NO: 10) and a SUMO tag (having an amino acid sequence as set forth in SEQ ID NO: 11 and a coding nucleotide sequence as set forth in SEQ ID NO: 12) at N-terminal of the BoNT/A amino acid sequence via a homologous recombination method, and then cleaving GG-M by a SUMO protease, there will be no residual amino acids from any tag or the SUMO sequence.

[0031] When thrombin was used at an amount of 0 U, the protein of interest did not present as

two fragments, indicating that the BoNT/A protein was not activated, i.e., an inactive BoNT/A protein was obtained. When thrombin was used at amounts of 0.5 U, 1 U, 2 U, and 3 U, respectively, only when thrombin was added at 1 U, 2 U, and 3 U per 1 mg of protein, the protein of interest presented as two fragments after cleavage by thrombin, and more BoNT/A proteins purified from the supernatant were activated. Furthermore, considering that the larger the amount of thrombin used, the greater the safety risk, it is therefore preferred to add 1 U of thrombin per 1 mg of protein in the present disclosure.

[0032] In addition, through comparative experiments, the results show that, when SUMO tag was used, it was more efficient for both tag removal and polypeptide activation to use the supernatant of cell lysate to perform the enzymatic cleavage for tag removal and the activation of the botulinum neurotoxin type A protein polypeptide. Animal experiments show that the recombinant botulinum neurotoxin type A polypeptide prepared, purified, and activated from the above supernatant had stronger toxicity, and thus had significantly reduced side effects and irritation in practical applications, exhibiting higher safety.

BRIEF DESCRIPTION OF DRAWINGS

[0033] FIG. 1 shows a schematic diagram of a SUMO-BoNT/A expression vector.

[0034] FIG. 2 shows an identification result of an extracted pKMD-SUMO blank plasmid.

[0035] FIG. 3 shows an identification result of PCR products of BoNT/A fragments.

[0036] FIG. 4 shows an identification result of a PCR product of a BoNT/A transformant.

[0037] FIG. 5 shows an enzymatic cleavage identification result of a pKMD-SUMO-BoNT/A expression vector.

[0038] FIG. 6 shows transformation and identification of an expression strain.

[0039] FIG. 7 shows a purification result of BoNT/A protein of interest from the supernatant.

[0040] FIG. 8 shows a purification result of BoNT/A protein of interest from the inclusion bodies.

[0041] FIG. 9 shows a result of enzymatic cleavage of the SUMO tag from the protein of

interest.

[0042] FIG. 10 shows a result of activation of the protein of interest from the supernatant by thrombin enzymatic cleavage.

[0043] FIG. 11 shows a result of activation of the protein of interest by thrombin.

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DETAILED DESCRIPTION

[0044] The technical solutions in the embodiments of the present disclosure are described clearly and completely below. It should be understood that the described embodiments are merely some, but not all, of the embodiments of the present disclosure. All other embodiments obtained by those of ordinary skill in the art based on the embodiments of the present disclosure without making inventive efforts shall fall within the protection scope of the present disclosure.

Example 1: Design and Construction of an Amino Acid Sequence of BoNT/A

[0045] (1) A BoNT/A was designed and constructed, which had an amino acid sequence as set forth in SEQ ID NO: 7 and a nucleotide sequence as set forth in SEQ ID NO: 8. The BoNT/A molecule comprised the following components sequentially from the 5' end:

[0046] (a) a LC region of BoNT/A having an amino acid sequence as set forth in SEQ ID NO: 1 and a corresponding nucleotide sequence as set forth in SEQ ID NO: 2;

[0047] (b) a thrombin enzymatic cleavage site having an amino acid sequence as set forth in SEQ ID NO: 3 and a corresponding nucleotide sequence as set forth in SEQ ID NO: 4; and

[0048] (c) a HC region of BoNT/A having an amino acid sequence as set forth in SEQ ID NO: 5 and a corresponding nucleotide sequence as set forth in SEQ ID NO: 6.

[0049] Information of the BoNT/A protein was obtained from the UniProt website, where its unique protein database accession number is P0DPI0, as detailed at <https://www.uniprot.org/uniprotkb/P0DPI0/entry>.

[0050] (2) A combination tag was inserted at N-terminal of the amino acid sequence of BoNT/A via a homologous recombination method. The resulting designed recombinant

botulinum neurotoxin type A (BoNT/A) had an amino acid sequence as set forth in SEQ ID NO: 13 and a corresponding nucleotide sequence as set forth in SEQ ID NO: 14.

[0051] The combination tag was a His-tag and a SUMO tag.

[0052] The His-tag had an amino acid sequence as set forth in SEQ ID NO: 9, and a coding nucleotide sequence as set forth in SEQ ID NO: 10.

[0053] The SUMO tag had an amino acid sequence as set forth in SEQ ID NO: 11, and a coding nucleotide sequence as set forth in SEQ ID NO: 12.

Example 2: Design, Ligation, and Preparation of a BoNT/A Expression Vector

[0054] (1) Selection of an expression vector: A blank plasmid was selected as a backbone vector for BoNT/A expression. After the obtained amino acid sequence was subjected to codon optimization, a gene codon sequence that can be favorably expressed in a prokaryotic host was obtained. Then, the plasmid construction design software Snapgene was used to perform a theoretical sequence assembly, that is, to design and construct a schematic diagram of the vector, and the complete schematic diagram of the constructed expression vector obtained is as shown in FIG. 1.

[0055] (2) Preparation of the expression vector: First, the blank plasmid was extracted and identified. The identification result is shown in FIG. 2, where the left lane showed that the plasmid was extracted successfully.

[0056] (3) Synthesis and pre-amplification of the target fragment: Nucleotide sequences SEQ ID NO: 2, SEQ ID NO: 4, and SEQ ID NO: 6 corresponding to the amino acid sequences of SEQ ID NO: 1, SEQ ID NO: 3, and SEQ ID NO: 5 of the chemically synthesized BoNT/A amino acid sequence, were subjected to pre-amplification in fragments by PCR. Primer sequences were internal primers in company. Specifically:

[0057] forward primer G0268322-1F: SEQ ID NO: 15,

[0058] TATCGAAGCCCATCGCGAACAGATTGGTGGTATGCCGTTTGTTAATAAGCAGT
TTAACTATAAG;

[0059] reverse primer G0268322-1R1: SEQ ID NO: 16,

[0060] CACCGGAATGGCAATTTCCGGAATGAATTCCAG.

[0061] The expected amplified fragment has a length of 2100 bp, and gel electrophoresis identification showed two amplified fragments as shown in FIG. 3.

5 **[0062]** (4) Self-ligation of the target fragment and its ligation with the vector:

[0063] After the synthesized BoNT/A nucleotide fragments were ligated, a reaction system for ligation with the vector was prepared as shown in Table 1. Using the principle of ligation with a homologous recombinase, ligation was carried out at a constant temperature of 52 °C for 30 min, and then transformation was performed.

10 Table 1. Ligation Reaction System with Homologous Recombinase

Reactant	Volume
Self-ligated and purified PCR product	5 μ L
Vector after enzymatic cleavage	5 μ L
Seamless assembly MIX	10 μ L
Total volume 20 μ L	
The above ligation mixture was allowed to react at a constant temperature of 52 °C for 30 min	

[0064] (5) Transformation of the recombinant plasmid:

15 **[0065]** (a) 1 to 3 μ L of the plasmid with a concentration of about 100 ng/ μ L was added to about 100 μ L of BL21 strain competent cells, gently shaken and swirled to mix evenly, and placed on ice for 3 min. For a plasmid with a high concentration, the addition amount was reduced appropriately, and for a plasmid with a low concentration, the addition amount was increased appropriately.

[0066] (b) The mixture was subjected to a water bath at 42 °C for 90 s without shaking, and then placed in an ice bath for about 3 min.

20 **[0067]** (c) 500 to 800 μ L of LB medium pre-warmed to 37 °C was added to each tube

containing the above mixture, and the tubes were gently agitated at 200 rpm for 40 min in a 37 °C shaker.

[0068] (6) Verification of the recombinant plasmid:

[0069] (a) Agar plates containing a corresponding antibiotic were prepared.

5 **[0070]** (b) 100 µL of the BL21 bacterial culture was taken and then spread on an agar plate containing the corresponding antibiotic. The bacteria were gently spread on the surface of the plate with a sterile glass spreader, and the plate was placed at 37 °C for 15 min.

[0071] (c) The plates were inverted and cultured at 37 °C for 12 to 16 h until colonies appeared.

[0072] (d) Colonies were picked from the plates and cultured with shaking at 250 rpm and
10 37 °C for 14 h. The bacterial culture was subjected to PCR identification, and the bacterial culture of a positive clone was sequenced.

[0073] Forward primer: SEQ ID NO: 15,

[0074] TATCGAAGCCCATCGCGAACAGATTGGTGGTATGCCGTTTGTTAATAAGCAGT
TTAACTATAAG;

15 **[0075]** Reverse primer: SEQ ID NO: 16,

[0076] CACCGGAATGGCAATTTCCGGAATGAATTCCAG

[0077] (7) PCR identification reaction system:

[0078] Identification of PCR product of BoNT/A transformant: Primers were designed with an expected amplified fragment length of 2100 bp (a partial sequence of the target gene). The PCR
20 reaction was performed using a 20 µL system: 0.5 µL of primers, 2 µL of template bacterial culture, 0.5 µL of polymerase buffer, 3 µL of buffer, and 14 µL of ddH₂O. Cycling parameters: pre-denaturation at 96 °C for 3 min; 23 cycles of 95 °C for 15 s, 58 °C for 15 s and 72 °C for 20 s; and a final extension at 72 °C for 1 min. As shown in FIG. 4, 8 BoNT/A transformants were selected for PCR product identification. The primers used were the aforementioned G0268322-1F
25 and G0268322-1R1. A positive transformant was selected and obtained.

[0079] Enzymatic cleavage identification: After the positive transformant was obtained, the successfully constructed pKMD-SUMO-BoNT/A expression plasmid (gene ID: G0268322-1)

was identified for its plasmid structure through an enzymatic cleavage experiment. Enzyme EcoRI was used, and two fragments of 1851 bp and 7599 bp were expected to be obtained. The reaction system is shown in Table 2, and the experimental result is shown in FIG. 5.

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Table 2. Enzymatic Cleavage Reaction System

pKMD-SUMO-BoNT/A plasmid	5 μ L
EcoRI endonuclease	0.25 μ L
10x Buffer	1.0 μ L
ddH ₂ O	to a final volume of 10 μ L
Reaction temperature	37 $^{\circ}$ C, 1 h

Example 3: Induced Expression of BoNT/A Protein and Collection of Cells

[0080] (1) Transformation of expression strain

10 **[0081]** One cryotube (100 μ L) of Rosetta BL21 competent cells was thawed on ice. In a clean bench, 1.5 mL sterile centrifuge tubes were taken, and 50 μ L of the competent cells was aliquoted into each tube.

[0082] 20 μ L of the vector constructed in Example 2 was taken, and in the clean bench, the plasmid was added to the competent cells, and the mixture was gently mixed, and then incubated
15 on ice for 30 min.

[0083] After a heat shock at 42 $^{\circ}$ C for 90 s, the mixture was immediately placed on an ice bath for 5 min. 30 μ L of the mixture was taken and spread on a TB plate (containing 50 μ g/mL Kana), and after culture at 37 $^{\circ}$ C for 14 h (overnight culture), a single colony was picked for activation culture.

20 **[0084]** (2) Identification of expression

[0085] A single colony was picked from the TB plate of the transformed expression strain and added to 2 mL of TB liquid medium (containing 50 μ g/mL Kana). After activation until OD600

of the cell culture reached 0.6 to 0.8, the culture was transferred to 5 mL of TB liquid medium (containing 50 µg/mL Kana).

[0086] After the cells were cultured to OD₆₀₀ of 0.6 to 0.8, IPTG was added to a final concentration of 1 mM, and induced expression was performed at 37 °C for 4 h, after which the cells were collected.

[0087] Samples of cells from each condition before and after induced expression were taken for SDS-PAGE analysis. The experimental results are shown in FIG. 6, where lanes 2 and 3 both show obvious expression.

[0088] (3) Large-scale expression and collection of cells

[0089] The BL21 strain capable of expressing the protein of interest was selected after the expression identification and inoculated into 1 L of TB medium (containing 50 µg/mL Kana). The culture was scaled up at 37 °C with shaking at 220 rpm until OD₆₀₀ reached 0.6 to 0.8. Then, IPTG was added to a final concentration of 1 mM, and induced culture was performed at 30 °C for 12 h. The cells were then collected after centrifugation at 4000 rpm for future use.

[0090] The collected cell pellet was resuspended with 40 mL of 20 mM PB + 150 mM NaCl at pH 7.2 and disrupted by ultrasonic disruption on an ice bath at 420 W with a single sonication for 3 s and an interval of 5 s for a total duration of 15 min,. The supernatant and pellet were collected respectively after centrifugation at 12000 rpm at 4 °C for 20 min for future use.

Example 4: Affinity Purification from Cell Lysate Supernatant and Pellet Inclusion Bodies

[0091] (1) Purification of protein from supernatant: The supernatant collected by centrifugation was filtered through a 0.22 µm filter membrane and then subjected to affinity enrichment and purification using a Ni column.

[0092] Equilibration buffer: 20 mM PB + 150 mM NaCl, pH 8.0.

[0093] Washing buffer: 20 mM PB + 150 mM NaCl + 5 mM imidazole, pH 8.0;

[0094] 20 mM PB + 150 mM NaCl + 20 mM imidazole, pH 8.0;

[0095] 20 mM PB + 150 mM NaCl + 50 mM imidazole, pH 8.0; and

[0096] 20 mM PB + 150 mM NaCl + 80 mM imidazole, pH 8.0.

[0097] Elution buffer: 20 mM PB + 150 mM NaCl + 300 mM imidazole, pH 8.0.

[0098] The above buffers flew through Ni column sequentially to purify samples. After a loading buffer was added, the samples were boiled at 100 °C and then subjected to SDS-PAGE analysis. The results are shown in FIG. 7, where the BoNT/A protein of interest was obtained in lanes 2 to 9.

[0099] (2) Purification of protein from pellet inclusion bodies: The pellet collected by centrifugation was resuspended with 20 mM PB + 150 mM NaCl, pH 7.2, and then subjected to affinity enrichment and purification using a Ni column.

[00100] Equilibration buffer: 20 mM PB + 150 mM NaCl, pH 8.0.

[00101] Washing buffer: 20 mM PB + 150 mM NaCl + 5 mM imidazole, pH 8.0;

[00102] 20 mM PB + 150 mM NaCl + 20 mM imidazole, pH 8.0;

[00103] 20 mM PB + 150 mM NaCl + 50 mM imidazole, pH 8.0; and

[00104] 20 mM PB + 150 mM NaCl + 80 mM imidazole, pH 8.0.

[00105] Elution buffer: 20 mM PB + 150 mM NaCl + 300 mM imidazole, pH 8.0

[00106] The above buffer flew through to purify samples. After a loading buffer was added, the samples were boiled at 100 °C and then subjected to SDS-PAGE analysis. The experimental results are shown in FIG. 8, where the BoNT/A protein of interest was obtained in lanes 2 to 10.

[00107] (3) Enzymatic cleavage to remove SUMO tag and activate BoNT/A protein: After cleavage by a SUMO protease, the SUMO tag was completely removed. The experimental results are shown in FIG. 9. Compared to the BoNT/A protein purified from inclusion bodies, the BoNT/A protein purified from the supernatant was cleaved more completely after the cleavage by SUMO protease.

[00108] An optimization of thrombin enzymatic cleavage for the BoNT/A protein was then performed as follows:

[00109] Thrombin: Shanghai Yuan Ye Bio-Technology Co., Ltd., Cat. No.: S10117-1ku, Thrombin, Lot: M25HS179066

[00110] Experimental Group 1: 0 U of thrombin was added per 1 mg of protein, and cleavage was performed for 16 h.

[00111] Experimental Group 2: 0.5 U of thrombin was added per 1 mg of protein, and cleavage was performed for 16 h.

5 **[00112]** Experimental Group 3: 1 U of thrombin was added per 1 mg of protein, and cleavage was performed for 16 h.

[00113] Experimental Group 4: 2 U of thrombin was added per 1 mg of protein, and cleavage was performed for 16 h.

[00114] Experimental Group 5: 3 U of thrombin was added per 1 mg of protein, and cleavage
10 was performed for 16 h.

[00115] Thrombin is obtained by artificial synthesis and not naturally occurred. Typically, thrombin is used for enzymatic cleavage to cleave protein chains; and when using thrombin, dosage is a very important factor. An excessively high dosage may lead to unsafe issues such as the formation of thrombosis or bleeding, and environmental pollution.

15 **[00116]** The results of thrombin enzymatic cleavage and activation of the protein of interest from the supernatant are shown in FIG. 10: when the amount of thrombin was 0 U, the protein of interest did not show two fragments, representing that the BoNT/A protein was not activated, i.e., an inactive BoNT/A protein was obtained; when the amounts of thrombin were 0.5 U, 1 U, 2 U, and 3 U, respectively, only when thrombin was added at 1 U, 2 U, and 3 U per 1 mg of protein
20 did the protein of interest show two fragments after thrombin cleavage, and a greater portion of the BoNT/A protein purified from the supernatant was activated. Considering the risk that the larger the amount of thrombin used, the less safe it is, the present solution preferably adopted the amount of 1 U of thrombin per 1 mg of protein.

[00117] For the thrombin activation results of the protein of interest from the supernatant and
25 inclusion bodies, 1 U of thrombin was added per 1 mg of protein, and cleavage was performed for 16 h. As shown in FIG. 11, after both the BoNT/A protein purified from the supernatant and the BoNT/A protein purified from inclusion bodies were activated by thrombin, only the BoNT/A protein purified from the supernatant was completely activated.

Example 5: Comparative Verification of Activity and Safety of the Polypeptide

[00118] (1) Test substance: The test sample was a lyophilized protein powder of the recombinant botulinum toxin type A active polypeptide purified from supernatant or pellet inclusion body, activated by thrombin. One vial (50 µg) of the test sample was taken and dissolved with 1 mL of sterile water for injection.

[00119] Animals: Kunming mice, SPF grade, 26 to 30 days old, sourced from the Guangdong Medical Experimental Animal Center were used. The experimental animal production license was No. SCXK (Yue) 2022-0002. The mice were under a quality certificate of No. 44007200115254, and were purchased on 2023-03-23. The experimental animal use license was No. SYXK (Yue) 2021-0253. The quarantine period was from 2023-03-23 to 2023-03-26.

[00120] Instruments: MS8001TS electronic balance (20213248); 902 ultra-low temperature freezer (20141115); HYC-390F medical refrigerator (20212898); and DW-25L262 medical low-temperature storage box (20212917).

[00121] (2) Experimental method:

[00122] (a) Sample preparation: One vial (50 µg) of the test sample was taken and dissolved by adding 1 mL of sterile water for injection. The sample was serially diluted by 10^3 , 10^4 , 10^5 , and 10^6 using a 10-fold serial dilution method.

[00123] (b) Animal injection: A total of 4 dilutions, 10^3 , 10^4 , 10^5 , and 10^6 , were taken. For each dilution, 4 SPF-grade Kunming mice of 26- to 30-day-old were intraperitoneally injected with 0.1 mL each. The survival and death of the mice were recorded for 4 days, and the virulence of this product was calculated in units of LD₅₀/mL using a statistical method (Reed-Muench).

[00124] (3) Experimental results: The protein safety data of the recombinant botulinum toxin type A active polypeptide purified from the supernatant and the pellet inclusion bodies are shown in Table 3.

Table 3. Protein Safety Data of the Recombinant botulinum toxin type A Active Polypeptides

Protein safety data of the recombinant botulinum toxin type A active polypeptide purified

from the supernatant							
Dilution	Number of mice	Survival	Death	Cumulative survival	Cumulative death	Death ratio	Death rate
		Number	Number	Number	Number		(%)
10^3	4	0	4	0	4	4/4	100
10^4	4	0	4	0	4	4/4	100
10^5	4	0	4	0	4	4/4	100
10^6	4	4	0	4	0	0/4	0
protein safety data ofr the recombinant botulinum toxin type A active polypeptide purified from the pellet inclusion bodies							
Dilution	Number of mice	Survival	Death	Cumulative survival	Cumulative death	Death ratio	Death rate
		Number	Number	Number	Number		(%)
10^1	4	2	2	2	2	2/4	50
10^2	4	4	0	4	0	0/4	0
10^3	4	4	0	4	0	0/4	0
10^4	4	4	0	4	0	0/4	0

[00125] Calculation of LD₅₀: LD₅₀ was calculated according to the Reed-Muench method. Proportionate distance = (Mortality rate (%) above 50% - 50%) / (Mortality rate (%) above 50% - Mortality rate (%) below 50%) i.e., Proportionate distance = (100% - 50%) / (100% - 0%) = 50/100 = 0.5. Logarithm of LD₅₀ = logarithm of the dilution above 50% + proportionate distance × logarithm of the dilution factor, i.e., Log(LD₅₀) = -5 + 0.5 × (-1) = -5.5. Therefore, the LD₅₀ value of $1 \times 10^{5.5}$ refers to the virulence unit of the sample under the condition of an injection volume of 0.1 mL, i.e., the virulence was $1 \times 10^{5.5}$ LD₅₀/0.1 mL. In the Chinese Pharmacopoeia, an injection volume of 0.5 mL was injected to mice, i.e., $5 \times 10^{5.5}$ LD₅₀/mL. Based on the principle of Chinese Pharmacopoeia 3.1, the final LD₅₀ of the recombinant toxin would be $1 \times 10^{7.5}$ LD₅₀/mg (Dilution factor: 1000/50=20, the calculated LD₅₀ for diluted sample is $5 \times 10^{5.5}$, the final LD₅₀

of sample would be $5 \times 10^{5.5} \times 20 = 1 \times 10^{7.5}$).

[00126] Therefore, the virulence of recombinant botulinum toxin type A active polypeptide purified from supernatant under an injection volume of 0.1 mL was $1 \times 10^{5.5}$ LD₅₀, while the protein virulence of the recombinant botulinum toxin type A active polypeptide purified from the pellet inclusion bodies was significantly weaker. That is, in use, the recombinant botulinum toxin type A active polypeptide purified from the supernatant can exert its effect at a low dose, and has significantly higher safety compared to the recombinant botulinum toxin type A active polypeptide purified from the pellet inclusion bodies which requires a large dose.

[00127] In summary, the present disclosure has verified through comparative experiments that the recombinant botulinum toxin type A active polypeptide purified from the supernatant, when using a SUMO tag, allows for efficient removal of the tag, shows a high proportion of activation after enzymatic cleavage, and possesses significant safety in use under its outstandingly potent virulence.

[00128] The present disclosure may be implemented in other specific forms without departing from the spirit or essential characteristics of the present disclosure. Therefore, the foregoing examples are considered, in all respects, to be illustrative and not limiting. The scope of the present disclosure is indicated by the appended claims rather than by the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be encompassed within the claims of the present disclosure.

CLAIMS

1. A recombinant botulinum neurotoxin type A (BoNT/A) having an amino acid sequence as set forth in SEQ ID NO: 7.

5 2. The recombinant botulinum neurotoxin type A (BoNT/A) according to claim 1, comprising amino acid sequences as set forth in SEQ ID NO: 1, SEQ ID NO: 3, and SEQ ID NO: 5;

(a) a light chain (LC) region of BoNT/A having an amino acid sequence as set forth in SEQ ID NO: 1;

10 (b) a thrombin enzymatic cleavage site having an amino acid sequence as set forth in SEQ ID NO: 3; and

(c) a heavy chain (HC) region of BoNT/A having an amino acid sequence as set forth in SEQ ID NO: 5.

15 3. The recombinant botulinum neurotoxin type A (BoNT/A) according to claim 2, wherein a combination tag is inserted at N-terminal of the amino acid sequence of the BoNT/A via a homologous recombination method, to obtain a recombinant botulinum neurotoxin type A (BoNT/A) having an amino acid sequence as set forth in SEQ ID NO: 13.

4. The recombinant botulinum neurotoxin type A (BoNT/A) according to claim 3, wherein the combination tag is a His-tag and a SUMO tag.

20 5. A method for producing the recombinant botulinum neurotoxin type A (BoNT/A) according to claim 4, comprising:

(1) designing and constructing a recombinant botulinum neurotoxin type A (BoNT/A) having an amino acid sequence as set forth in SEQ ID NO: 13;

(2) designing, ligating and producing a BoNT/A expression vector;

25 (3) performing induced expression of a BoNT/A protein and collecting cells;

(4) performing affinity purification on a supernatant of cell lysate; and

(5) removing the SUMO tag by enzymatic cleavage and activating the BoNT/A protein.

6. The method according to claim 5, wherein the step of removing the SUMO tag by enzymatic cleavage and activating the BoNT/A protein comprises: performing enzymatic cleavage with a SUMO protease, and subsequently adding 1 U of thrombin per 1 mg of the BoNT/A protein.

5 7. The method according to claim 6, wherein the step of performing induced expression of the BoNT/A protein comprises: after OD600 of cell culture reaching 0.6, adding IPTG to a final concentration of 1 mM, and performing induced culture at 30 °C for 12 h.

8. The method according to claim 7, wherein:

10 the step of collecting cells comprises: collecting cell pellet after centrifugation at 4000 rpm, resuspending the cell pellet with 40 mL of 20 mM PB + 150 mM NaCl at pH 7.2, performing ultrasonic disruption in ice bath at 420 W with a sonication pulse for 3 s and an interval of 5 s for a total duration of 15 min, and collecting a supernatant after centrifugation at 12000 rpm and 4 °C for 20 min; and

15 the affinity purification of the supernatant of cell lysate comprises: filtering the collected supernatant through a 0.22 µm filter membrane, and performing affinity enrichment and purification on a Ni column, wherein 20 mM PB + 150 mM NaCl is used as equilibration buffer, 20 mM PB + 150 mM NaCl + 5 mM imidazole, 20 mM PB + 150 mM NaCl + 20 mM imidazole, 20 mM PB + 150 mM NaCl + 50 mM imidazole, and 20 mM PB + 150 mM NaCl + 80 mM imidazole are used as washing buffer, 20 mM PB + 150 mM NaCl + 300 mM imidazole is used
20 as elution buffer, and all the reagents of the affinity purification have a pH of 8.0.

9. A recombinant BoNT/A polypeptide produced by the method according to any one of claims 1 to 8.

10. Use of the recombinant BoNT/A polypeptide produced by the method according to any one of claims 1 to 9 in the manufacture of a medicament.