US 20190183988A1

(19) United States

(12) Patent Application Publication (10) Pub. No.: US 2019/0183988 A1

JARSTAD et al. (43) Pub. Date: Jun. 20, 2019

(54) **NEUROTOXIN FORMULATION**

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(21) Appl. No.: **16/304,986**

(22) PCT Filed: **May 26, 2017**

(86) PCT No.: **PCT/EP2017/062785**

§ 371 (c)(1),
(2) Date: **Nov. 27, 2018**

(30) **Foreign Application Priority Data**

May 27, 2016 (EP) PCT/EP2016/062085

Publication Classification

(51) **Int. Cl.**

A61K 38/48	(2006.01)
A61K 47/18	(2006.01)
A61K 47/02	(2006.01)
A61K 47/26	(2006.01)
A61K 8/19	(2006.01)
A61K 8/39	(2006.01)
A61K 8/49	(2006.01)
A61K 8/66	(2006.01)
A61K 9/00	(2006.01)
A61Q 19/08	(2006.01)

(52) **U.S. Cl.**

CPC ***A61K 38/4893*** (2013.01); ***A61K 47/183*** (2013.01); ***A61K 47/02*** (2013.01); ***A61K 47/26*** (2013.01); ***A61Q 19/08*** (2013.01); ***A61K 8/39*** (2013.01); ***A61K 8/492*** (2013.01); ***A61K 8/66*** (2013.01); ***A61K 9/0019*** (2013.01); ***A61K 8/19*** (2013.01)

(57) **ABSTRACT**

The invention relates to stable liquid neurotoxin formulations which are free of animal proteins, comprising a surfactant, an amino acid selected from tryptophan and tyrosine, a buffer comprising sodium, chloride and phosphate ions, which have a pH between 5.5 and 8, and which are stable for 2 months. These compositions are suitable for use in therapy and in particular for administration to a patient to achieve a desired therapeutic or aesthetic effect. The invention also relates to the use of an amino acid selected from tryptophan and tyrosine to protect a proteinaceous neurotoxin from degradation in a liquid composition which is free of animal derived proteins.

Specification includes a Sequence Listing.

NEUROTOXIN FORMULATION

[0001] The present invention relates to animal protein free liquid neurotoxin formulations. In particular, the present invention relates to animal protein free liquid botulinum neurotoxin formulations stabilized with non proteinaceous excipients.

[0002] The neurotoxin formulations described herein are suitable for use in therapy and in particular for administration to a patient to achieve a desired therapeutic or aesthetic effect.

BACKGROUND OF THE INVENTION

[0003] Clostridial neurotoxins naturally produced by clostridial strains are the most toxic biological agents known to date and at the same time are powerful tools for the treatment of a number of neuromuscular and endocrine disorders, including cervical dystonia, spasticity, blepharospasm, hyperhidrosis or sialorrhea. They also find uses in the aesthetic field for the smoothing of wrinkles.

[0004] In order to be suitable for use as a pharmaceutical product, a neurotoxin composition must be such that it can be stored without significant loss of neurotoxin activity.

[0005] In all currently approved formulations of botulinum neurotoxins, an animal (including human) protein, usually human serum albumin (HSA), is used as a stabiliser.

[0006] The presence of animal proteins such as HSA in pharmaceutical compositions is however undesirable because of the risk, even if low, of unwillingly transmitting animal borne infectious agents such as prions to a patient.

[0007] Animal protein free botulinum toxin formulations have been disclosed in the art. For example, WO0158472 describes lyophilized compositions in which a polysaccharide, such as 2-hydroxyethyl starch is used to stabilize a botulinum toxin. WO2005007185 describes compositions in which a surface active substance, and a mixture of at least two amino acids selected from Glu and Gln or Asp and Asn are used to stabilize a botulinum toxin.

[0008] Most prior art formulations are however not stable in liquid form and are therefore stored in lyophilized or freeze-dried form. Such formulations need to be reconstituted by the physician in a sterile saline solution before administration to a patient. This reconstitution step is associated with a loss of physician time, a risk of a dilution error and also a risk of contamination during the reconstitution process. The botulinum toxin provider must also train the physicians in order to ensure that the reconstitution step is performed adequately.

[0009] Liquid formulations are therefore advantageous as they obviate the loss of time for the physician, the risk of a dilution error, the contamination risk and the need for providing training for the provider.

[0010] Liquid HSA-free formulations are described for example in WO2006005910 which discloses liquid botulinum toxin formulations comprising a surfactant, sodium chloride and a disaccharide. WO2009008595 discloses liquid botulinum toxin formulations comprising polysorbate 20 and methionine.

[0011] It is an objective of the present invention to provide advantageous liquid animal protein free botulinum neurotoxin formulations, which are suitable for storage and for use in therapy. In particular, the stabilizing formulation should maintain product stability, be free of animal proteins and also be suitable for stabilising a neurotoxin which is free of complexing proteins.

SUMMARY OF THE INVENTION

[0012] A first aspect of the present invention is a liquid composition comprising or consisting essentially of a proteinaceous neurotoxin, a surfactant, an amino acid selected from tryptophan and tyrosine, a buffer comprising sodium, chloride and phosphate ions, which has a pH between 5.5 and 8, which is stable over time and which is free of animal derived proteins.

[0013] Another aspect is the use of the liquid compositions according to the invention in therapy and/or in cosmetics.

[0014] A further aspect of the present invention is the use of an amino acid selected from tryptophan and tyrosine to protect a proteinaceous neurotoxin from degradation in a liquid composition which is free of animal derived proteins.

DETAILED DESCRIPTION OF THE INVENTION

[0015] A first aspect of the present invention is a liquid composition comprising or consisting essentially of a proteinaceous neurotoxin, a surfactant, an amino acid selected from tryptophan and tyrosine, a buffer comprising sodium, chloride and phosphate ions, which has a pH between 5.5 and 8, which is stable over time and which is free of animal derived proteins.

[0016] "Animal protein free" is to be understood as comprising no protein of animal, including human, origin.

[0017] A neurotoxin is a substance that targets a nerve cell and affects a neurological function. Proteinaceous neurotoxins include botulinum toxins (BoNT) and tetanus toxin (TeNT). Preferably, the proteinaceous neurotoxin is a botulinum neurotoxin.

[0018] Botulinum neurotoxins are 150 kDa metalloproteases that consist in their active form of a 50 kDa light chain (L) and a 100 kDa heavy chain (H) linked by a disulfide bridge. The L chain is a zinc-protease which intracellularly cleaves one of the SNARE (Soluble NSF Attachment Protein REceptor) proteins involved in vesicle mediated neurotransmitter release, thereby disrupting neurotransmitter mediated mechanisms. The heavy chain encompasses two domains: an N-terminal 50 kDa translocation domain (H_N), and a C-terminal 50 kDa receptor-binding domain (H_C). The H_C domain of a botulinum neurotoxin comprises two distinct structural features that are referred to as the H_{CC} and H_{CN} domains. Amino acid residues involved in receptor binding are believed to be primarily located in the H_{CC} domain.

[0019] Botulinum neurotoxins have been classified in 7 antigenically distinct serotypes (A to G). Exemplary amino acid sequences for each serotype are provided herein as SEQ ID NO 1 to 7.

[0020] For each of the sequences, the different domains can for example be as follow.

Serotype	L chain	H_N domain	H_{CN} domain	H_{CC} domain
BoNT/A (SEQ ID NO 1)	1-448	449-871	872-1110	1111-1296
BoNT/B (SEQ ID NO 2)	1-440	441-858	859-1097	1098-1291
BoNT/C (SEQ ID NO 3)	1-441	442-866	867-1111	1112-1291
BoNT/D (SEQ ID NO 4)	1-445	446-862	863-1098	1099-1276
BoNT/E (SEQ ID NO 5)	1-422	423-845	846-1085	1086-1252
BoNT/F (SEQ ID NO 6)	1-439	440-864	865-1105	1106-1274
BoNT/G (SEQ ID NO 7)	1-441	442-863	864-1105	1106-1297

[0021] The skilled person will appreciate that there can be some variation in each of the botulinum neurotoxin domains.

[0022] BoNTs act for example on neuromuscular nerve junctions by preventing release of acetylcholine and thereby preventing muscular contraction. Nerve terminal intoxication is reversible and its duration varies for different BoNT serotypes.

[0023] Natural BoNTs are produced by *Clostridium botulinum*, and other Clostridial species such as *C. butyricum, C. baratii* and *C. argentinense* as part of multi-protein complexes that protect the neurotoxin from proteolytic degradation. By "botulinum neurotoxin in complex form" is meant a botulinum neurotoxin and one or more of the proteins which are part in nature of such multi-protein complexes (neurotoxin-associated proteins or "NAPs"). NAPs include non-toxic non-hemagglutinin (NTNH) protein and hemagglutinin proteins (HA-17, HA-33, and HA-70). By "high purity botulinum neurotoxin" is meant a botulinum neurotoxin essentially free of NAPs.

[0024] According to an embodiment of the invention, the botulinum neurotoxin is a botulinum neurotoxin in complex form. According to another embodiment, the botulinum neurotoxin is a high purity botulinum neurotoxin.

[0025] Method for producing BoNTs through culture of natural clostridial strains and purifying them either in complex form or high purity form are well known in the art and are described for example in Pickett, Andy. "Botulinum toxin as a clinical product: manufacture and pharmacology." Clinical Applications of Botulinum Neurotoxin. Springer New York, 2014. 7-49.

[0026] High purity or essentially pure botulinum neurotoxin can be obtained from a protein complex comprising botulinum toxin for example according to the method described in Current topics in Microbiology and Immunology (1995), 195, p. 151-154.

[0027] Alternatively, high purity botulinum neurotoxin can be produced by recombinant expression of a BoNT gene in a heterologous host such as *E. coli* and purified therefrom.

[0028] Preferably, the proteinaceous neurotoxin is a botulinum neurotoxin. According to an embodiment of the invention, the botulinum neurotoxin is a botulinum neurotoxin in complex form. According to another embodiment, the botulinum neurotoxin is a high purity botulinum neurotoxin.

[0029] According to an embodiment of the invention, the botulinum neurotoxin is a botulinum neurotoxin purified from its natural clostridial strain. According to another embodiment, botulinum neurotoxin is a botulinum neurotoxin produced recombinantly in a heterologous host such as *E. coli.*

[0030] According to the present invention, the Botulinum neurotoxin can be a BoNT of serotype A, B, C, D, E, F or G.

[0031] According to the present invention, a botulinum neurotoxin can be a modified botulinum neurotoxin. According to the present invention, a "modified BoNT" is a BoNT which has an amino acid sequence which has at least 50% sequence identity with SEQ ID NO 1, 2, 3, 4, 5, 6 or 7. Preferably, a modified BoNT has an amino acid sequence which has at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity with SEQ ID NO 1, 2, 3, 4, 5, 6 or 7. Preferably, a modified BoNT is a BoNT whose amino acid sequence differs from SEQ ID NO 1, 2, 3, 4, 5, 6 or 7 by less than 600, 400, 200, 150, 100, 50 or 20 amino acid substitutions, deletions or additions, for example by 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 amino acid substitutions, deletions or additions.

[0032] According to the present invention, a recombinant botulinum neurotoxin can be a chimeric botulinum neurotoxin. According to the present invention, a "chimeric BoNT" is constituted by an L, H_N, H_{CN}, and H_{CC} domain which do not all belong to the same serotype. For example a chimeric BoNT can consist of an L chain from one serotype and a full H chain (H_N, H_{CN}, and H_{CC} domains) from a different serotype. A chimeric BoNT can also consist of an L chain and an H_N domain ("LH_N") from one serotype and an H_C domain (H_{CN} and H_{CC}) from a different serotype. A chimeric BoNT can also consist of an L chain and H_N and H_{CN} domains ("extended LH_N") from one serotype and an H_{CC} domain from a different serotype.

[0033] According to the invention a Light chain domain (L) can have an amino acid sequence which has at least 50%, preferably at least 60%, 70%, 80%, 90% or 95% sequence identity to one of the following amino acid sequences and which retains the ability to cleave one of the SNARE proteins involved in vesicle mediated neurotransmitter release:

[0034] Amino acid 1-448 of SEQ ID NO:1

[0035] Amino acid 1-440 of SEQ ID NO:2

[0036] Amino acid 1-441 of SEQ ID NO:3

[0037] Amino acid 1-445 of SEQ ID NO:4

[0038] Amino acid 1-422 of SEQ ID NO:5

[0039] Amino acid 1-439 of SEQ ID NO:6

[0040] Amino acid 1-441 of SEQ ID NO:7

[0041] According to the invention an H_N domain can have an amino acid sequence which has at least 50%, preferably at least 60%, 70%, 80%, 90% or 95% sequence identity to one of the following amino acid sequences and which retains a translocation ability:

[0042] Amino acid 449-871 of SEQ ID NO:1

[0043] Amino acid 441-858 of SEQ ID NO:2

[0044] Amino acid 442-866 of SEQ ID NO:3

[0045] Amino acid 446-862 of SEQ ID NO:4

[0046] Amino acid 423-845 of SEQ ID NO:5

[0047] Amino acid 440-864 of SEQ ID NO:6

[0048] Amino acid 442-863 of SEQ ID NO:7

[0049] According to the invention an H_C domain can have an amino acid sequence which has at least 50%, preferably at least 60%, 70%, 80%, 90% or 95% sequence identity to one of the following amino acid sequences and which retains the ability to bind to a neuromuscular cell:

[0050] Amino acid 872-1296 of SEQ ID NO:1

[0051] Amino acid 859-1291 of SEQ ID NO:2

[0052] Amino acid 867-1291 of SEQ ID NO:3

[0053] Amino acid 863-1276 of SEQ ID NO:4

[0054] Amino acid 846-1252 of SEQ ID NO:5

[0055] Amino acid 865-1274 of SEQ ID NO:6

[0056] Amino acid 864-1297 of SEQ ID NO:7

[0057] According to the invention an H_{CC} domain can have an amino acid sequence which has at least 50%, preferably at least 60%, 70%, 80%, 90% or 95% sequence identity to one of the following amino acid sequences and which retains the ability to bind to a neuromuscular cell:

[0058] Amino acid 1111-1296 of SEQ ID NO:1

[0059] Amino acid 1098-1291 of SEQ ID NO:2

[0060] Amino acid 1112-1291 of SEQ ID NO:3

[0061] Amino acid 1099-1276 of SEQ ID NO:4

[0062] Amino acid 1086-1252 of SEQ ID NO:5

[0063] Amino acid 1106-1274 of SEQ ID NO:6

[0064] Amino acid 1106-1297 of SEQ ID NO:7

[0065] The above-identified reference sequences should be considered as a guide, as slight variations may occur according to sub-serotypes.

[0066] The "percent sequence identity" between two or more nucleic acid or amino acid sequences is a function of the number of identical nucleotides/amino acids at identical positions shared by the aligned sequences. Thus, % identity may be calculated as the number of identical nucleotides/amino acids at each position in an alignment divided by the total number of nucleotides/amino acids in the aligned sequence, multiplied by 100. Calculations of % sequence identity may also take into account the number of gaps, and the length of each gap that needs to be introduced to optimize alignment of two or more sequences. Sequence comparisons and the determination of percent identity between two or more sequences can be carried out using specific mathematical algorithms, such as BLAST, which will be familiar to a skilled person.

[0067] Surfactants (or surface active agents) are compounds that are able to lower the surface tension between a liquid and a solid or between two liquids. Surfactants can be non-ionic, anionic, cationic or amphoteric. In the compositions according to the invention, the surfactant is preferably a non-ionic surfactant. Non-ionic surfactants include Polyoxyethylene glycol alkyl ethers, such as Octaethylene glycol monododecyl ether or Pentaethylene glycol monododecyl ether; Polyoxypropylene glycol alkyl ethers; Glucoside alkyl ethers, such as Decyl glucoside, Lauryl glucoside or Octyl glucoside; Polyoxyethylene glycol octylphenol ethers, such as Triton X-100; Polyoxyethylene glycol alkylphenol ethers, such as Nonoxynol-9; Glycerol alkyl esters, such as Glyceryl laurate; Polyoxyethylene glycol sorbitan alkyl esters, such as Polysorbates; Sorbitan alkyl esters, such as Spans; Cocamide MEA, cocamide DEA; Dodecyldimethylamine oxide; Block copolymers of polyethylene glycol and polypropylene glycol, such as Poloxamers; Polyethoxylated tallow amine (POEA).

[0068] According to a preferred embodiment, the liquid composition according to the invention comprises a non-ionic surfactant which is a polysorbate, preferably polysorbate 20 (PS20), polysorbate 60 (PS60) or polysorbate 80 (PS80). Most preferably, the non-ionic surfactant is PS80. When the surfactant is a polysorbate, its concentration is preferably from 0.001% to 15% v/v, more preferably from 0.005 to 2% v/v, more preferably still from 0.01 to 1% for example 0.01, 0.05, 0.1, 0.2, 0.5 or 1% v/v. According to one embodiment, the surfactant is PS80 at a concentration from 0.05 to 0.2% v/v, for example about 0.05, 0.06, 0.07, 0.08, 0.09, 0.10, 0.11, 0.12, 0.13, 0.14, 0.15, 0.16, 0.17, 0.18, 0.19 or 0.20% v/v.

[0069] PS20 has a density of approximately 1.1 g/mL. PS60 has a density of approximately 1.044 g/mL. PS80 has a density of approximately 1.06 to 1.09 g/mL.

[0070] Polysorbates are believed to form micelles and prevent adsorption of proteins to surfaces and protein aggregation. Without wishing to be bound by theory, it is believed that upon degradation/oxidation, polysorbates may form peroxides and acids that may have an effect on protein stability. Therefore, it is considered preferable that the concentration of polysorbate be as low as possible in the formulation of the product. It is therefore considered preferable that the concentration of polysorbate should not exceed 200 times its critical micellar concentration (CMC), more preferably it should not exceed 100, 50, 20, 10 or 5 times its CMC.

[0071] For PS20 (Mw 1227.5 g/mol), the CMC is approximately 8×10^{-5} M at 21° C., i.e. approximately 0.01% w/v.

[0072] For PS60 (Mw 1309 g/mol), the CMC is approximately 21×10^{-6} M at 21° C., i.e. approximately 0.003% w/v.

[0073] For PS80 (Mw 1310 g/mol), the CMC is approximately 12×10^{-6} M at 21° C., i.e. approximately 0.002% w/v.

[0074] According to a preferred embodiment, the polysorbate concentration is between 1 and 200 times its CMC at a given temperature, for example about 21° C., preferably between 2 and 100 times its CMC, for example about 20 or 50 times its CMC.

[0075] The liquid composition according to the invention comprises an amino acid which is tryptophan or tyrosine. Without willing to be bound by theory, it is hypothesized that tryptophan or tyrosine can prevent oxidation of the active protein which would render it non-functional. Indeed, it is thought that the amino acid added in molar excess over the neurotoxin will be oxidized in the first place, saving the neurotoxin. It is also hypothesized that tryptophan or tyrosine can neutralize reactive degradation products of surfactants such as polysorbates.

[0076] Preferably the amino acid is tryptophan. More preferably, the amino acid is L-tryptophan.

[0077] The amino acid concentration is preferably from about 0.1 to 5 mg/mL, more preferably between 0.1 and 5 mg/mL, from 0.25 and 3 mg/mL for example about 0.25, 0.5, 1, 1.5, 2 or 3 mg/m L.

[0078] The composition according to the invention comprises a buffer which comprises sodium, chloride and phosphate ions. The inventors indeed surprisingly found that buffers without sodium, chloride and phosphate ions lowered the stability of the toxin. Preferably the buffer also comprises potassium ions.

[0079] The buffer can for example be obtained by combining sodium chloride, potassium chloride and sodium phosphate salts. The sodium chloride concentration is preferably from 10 to 500 mM, preferably from about 25 to 300 mM, for example about 25, 50, 75, 100, 140, 150, 200, 250 or 300 mM.

[0080] The sodium phosphate concentration is preferably from 1 to 100 mM, preferably from 2 to 50 mM, for example about 2, 5, 10, 20, 30, 40 or 50 mM.

[0081] The potassium chloride concentration is preferably from 1 to 50 mM, preferably from 1 to 10 mM for example about 1, 2, 3, 4, 5 or 10 mM.

[0082] The composition according to the invention has a pH between 5.5 and 8. According to a preferred embodiment, the pH is between 6.0 and 7.5, for example about 6.3, 6.35, 6.4, 6.45, 6.5, 6.55, 6.6, 6.65, 6.7, 6.75, 6.8, 6.85, 6.9, 6.95, 7.0, 7.05, 7.1, 7.15, 7.2, 7.25, 7.3, 7.35, 7.4, 7.45 or 7.5. Preferably the pH is within one unit from physiological pH (which is around 7.4).

[0083] The composition according to the invention is liquid. The composition preferably comprises an aqueous diluent, more preferably water, for example sterile water, water for injection, purified water, sterile water for injection.

[0084] Preferably the formulation is isotonic and is suitable for injection to a patient, in particular a human patient.

[0085] The quantity of botulinum neurotoxin is commonly expressed in mouse LD50 (lethal dose 50) units, defined as the median lethal intraperitoneal dose in mice.

[0086] The mouse LD50 (MLD50) unit for botulinum toxins is not a standardised unit. Indeed, assays used by different manufacturers of marketed toxins differ in particular in the choice of dilution buffer. For example the test used for Dysport® uses gelatine phosphate buffer, whereas the assay used for BOTOX® uses saline as a diluent. It is believed that gelatine buffers protect the toxin at the high dilutions used in LD50 assays. In contrast the use of saline as a diluent is thought to lead to some loss of potency. This could explain why when tested with the Dysport® assay, one BOTOX® unit is equivalent to approximately three units of Dysport (Straughan, D. W., 2006, ATLA 34(3), 305-313; Hambleton and Pickett, Hambleton, P., and A. M. Pickett., 1994, Journal of the Royal Society of Medicine 87.11: 719).

[0087] Preferably, the dilution buffer used to determine the mouse LD50 is a gelatine phosphate buffer. For example, the mouse LD50 can be determined as described in Hambleton, P. et al. Production, purification and toxoiding of *Clostridium botulinum* type A toxin. Eds. G. E. Jr Lewis, and P. S. Angel. Academic Press, Inc., New York, USA, 1981, p. 248. Briefly, botulinum toxin samples are serially diluted in 0.2% (w/v) gelatine 0.07M Na2HPO4 buffer at pH 6.5. Groups of mice (eg 4 to 8 mice per group) weighing about 20 g are injected intraperitoneally with a sample of diluted toxin (for example 0.5 ml per animal). Dilution groups, for example 5 dilution groups, are selected to span the 50% lethality dose. The mice are observed for up to 96 hours and the mouse lethal dose 50 (MLD50) is estimated.

[0088] The composition according to the invention preferably comprises from 4 to 10000 LD50 units of botulinum neurotoxin per mL, more preferably from 10 to 2000 LD50 units of botulinum neurotoxin per mL, for example 20, 30, 40, 50, 75, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 or 1500 LD50 units of botulinum neurotoxin per mL.

[0089] The quantity of botulinum neurotoxin can also be expressed in ng. The composition according to the invention preferably comprises from about 0.01 to 75 ng of botulinum neurotoxin per mL, more preferably from about 0.03 to 20 ng botulinum neurotoxin per mL, more preferably still from about 0.1 to 15 ng botulinum neurotoxin per mL, for example about 0.15, 0.3, 0.5, 0.75, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 ng botulinum neurotoxin per mL.

[0090] The formulation according to the invention is animal protein free. In particular, the compositions according to the invention comprise no albumin, and in particular no human serum albumin. Preferably, the composition according to the invention is animal product free, meaning that they comprise no constituent of animal (including human) origin. Preferably, the composition according to the invention comprises no protein other than the proteinaceous neurotoxin. According to another embodiment, the composition according to the invention comprises no protein other than the proteinaceous neurotoxin and one or more NAPs (neurotoxin-associated proteins). For the sake of doubt, it is noted amino acids are not proteins.

[0091] According to an embodiment of the invention, the composition comprises no saccharides, including no monosaccharides, no disaccharides and no polysaccharides.

[0092] The liquid composition according to the invention is stable over time. For example, it is stable for 2 months at 2 to 8° C. According to one embodiment, it is stable for 3 months at 2 to 8° C., for example at 5° C. According to a preferred embodiment, it is stable for 6 months at 2 to 8° C., for example at 5° C. According to one embodiment, it is stable for 12 months at 2 to 8° C., for example at 5° C. According to one embodiment, it is stable for 18 months at 2 to 8° C., for example at 5° C. According to one embodiment, it is stable for 24 months at 2 to 8° C., for example at 5° C. According to one embodiment, it is stable for 36 months at 2 to 8° C., for example at 5° C. According to one embodiment, it is stable for 3 months at room temperature, for example at 25° C. According to one embodiment, it is stable for 6 months at room temperature, for example at 25° C. According to one embodiment, it is stable for 2 months at 37° C.

[0093] The liquid composition according to the invention is preferably stored at a temperature between 0° and 30° C. In a preferred embodiment it is stored at 2-8° C., for example at 5° C. In another embodiment, it is stored at room temperature. Preferably it is not frozen.

[0094] Stability can be assessed through comparison of the activity of the botulinum neurotoxin over time. Activity of the botulinum neurotoxin may refer to the ability of the activity of the botulinum neurotoxin to bind to its target receptor on a cell, to translocate the light chain into a cell, and/or to cleave its target SNARE protein.

[0095] Methods for measuring Botulinum neurotoxin activity are well known in the art. Botulinum neurotoxin activity can be assessed for example by using a mouse lethality assay (LD50) as described above, a muscle tissue based assay such as the mouse phrenic nerve hemidiaphragm assay (for example as described in Bigalke, H. and Rummel A., Toxins 7.12 (2015):4895-4905), a cell based assay (for example as described in WO201349508 or in WO2012166943) or an extracellular proteolytic activity assay such as BoTest® (Botulinum Neurotoxin Detection Kit available from BioSentinel Inc.).

[0096] Preferably, a composition according to the invention is considered stable if there is no more than a given percentage of loss of activity over a given period of time and at a given temperature.

[0097] According to one embodiment, a composition according to the invention is considered stable if there is no more than 30% loss in extracellular proteolytic activity over 3, 6, 12, 18, 24 or 36 months at 2 to 8° C., for example no more than 30% loss in extracellular proteolytic activity over 6 months at 5° C. Preferably, a composition according to the invention is considered stable if there is no more than 20% loss in extracellular proteolytic activity over 3 months at 5° C., more preferably over 6, 12, 18, 24 or 36 months at 5° C. According to another embodiment, a composition according to the invention is considered stable if there is no more than 40% loss in extracellular proteolytic activity over 3 months at room temperature, for example at 25° C. Preferably, a composition according to the invention is considered stable if there is no more than 30% loss in extracellular proteolytic activity over 3 months at 25° C., more preferably over 6 months at 25° C. According to another embodiment, a composition according to the invention is considered stable if there is no more than 50% loss in extracellular proteolytic activity over 2 months at 37° C. Extracellular proteolytic activity can be measured with the BoTest® assay.

[0098] According to one embodiment, a composition according to the invention is considered stable if there is no more than 30% loss in MLD50 units over 2, 3, 6, 12, 18, 24

or 36 months at 2 to 8° C., for example no more than 30% loss in MLD50 units over 6 months at 5° C. Preferably, a composition according to the invention is considered stable if there is no more than 20% loss in MLD50 units over 2 months at 5° C., more preferably over 3, 6, 12, 18, 24 or 36 months at 5° C. According to another embodiment, a composition according to the invention is considered stable if there is no more than 40% loss in MLD50 units over 2 or 3 months at room temperature, for example at 25° C. Preferably, a composition according to the invention is considered stable if there is no more than 30% loss in MLD50 units over 3 months at 25° C., more preferably over 6 months at 25° C. According to another embodiment, a composition according to the invention is considered stable if there is no more than 50% loss in MLD50 units over 2 months at 37° C. MLD50 units can be measured as indicated above.

[0099] The liquid compositions according to the invention can be stored in sealed vials or syringes, for example glass vials or syringes, preferably type 1 (or "body neutral") glass vials or syringes. Preferably there is no or very little oxygen in the vial or syringe. The vials or syringes can for example be filled in an atmosphere with an oxygen below 100 ppm, preferably below 50 ppm, and nitrogen gas can be used as a protective atmosphere in the vials. When glass vials are used, they can for example be capped with chlorobutyl or bromobutyl rubber stoppers, which can be FluroTec® coated stoppers. Preferably, the liquid compositions according to the invention are stored in glass vials capped with FluroTec® coated stoppers.

[0100] According to one embodiment, a liquid composition according to the invention comprises or consists essentially of:

[0101] 4 to 10000 LD50 units of botulinum neurotoxin per mL,
[0102] 0.001 to 15% v/v polysorbate,
[0103] 0.1 to 5 mg/mL tryptophan,
[0104] 10 to 500 mM NaCl,
[0105] 1 to 50 mM KCl,
[0106] 1 to 100 mM Sodium phosphate,
[0107] has a pH between 5.5 and 8, and is stable for 6 months at 5° C.

[0108] According to one embodiment, a liquid composition according to the invention comprises or consists essentially of:

[0109] 10 to 2000 LD50 units of botulinum neurotoxin per mL,
[0110] 0.005 to 2% v/v polysorbate,
[0111] 0.1 to 5 mg/mL tryptophan,
[0112] 25 to 300 mM NaCl,
[0113] 1 to 10 mM KCl,
[0114] 2 to 50 mM Sodium phosphate,
[0115] has a pH between 6.0 and 7.5, and is stable for 12 months at 5° C.

[0116] According to one embodiment, a liquid composition according to the invention comprises or consists essentially of:

[0117] 10 to 2000 LD50 units of botulinum neurotoxin per mL,
[0118] 0.05 to 0.2% v/v polysorbate 80,
[0119] 0.1 to 5 mg/mL tryptophan,
[0120] 25 to 300 mM NaCl,
[0121] 1 to 10 mM KCl,
[0122] 2 to 50 mM Sodium phosphate,
[0123] has a pH between 6.0 and 7.5, and is stable for 12 months at 5° C.

[0124] According to one embodiment, a liquid composition according to the invention comprises or consists essentially of:

[0125] Botulinum neurotoxin A,
[0126] 0.2% v/v polysorbate 80,
[0127] 1 mg/mL tryptophan
[0128] 140 mM NaCl,
[0129] 3 mM KCl,
[0130] 10 mM Sodium phosphate,
[0131] wherein the pH of said composition is approximately 6.6.

[0132] According to another embodiment, a liquid composition according to the invention comprises or consists essentially of:

[0133] Botulinum neurotoxin A,
[0134] 0.04% v/v polysorbate 80,
[0135] 1 mg/mL tryptophan
[0136] 140 mM NaCl,
[0137] 3 mM KCl,
[0138] 10 mM Sodium phosphate,
[0139] wherein the pH of said composition is approximately 6.9.

[0140] According to another embodiment, a liquid composition according to the invention comprises or consists essentially of:

[0141] Botulinum neurotoxin B,
[0142] 0.25% v/v polysorbate 20,
[0143] 4 mg/mL tryptophan
[0144] 140 mM NaCl,
[0145] 3 mM KCl,
[0146] 10 mM Sodium phosphate,
[0147] wherein the pH of said composition is approximately 7.4.

[0148] According to another embodiment, a liquid composition according to the invention comprises or consists essentially of:

[0149] Botulinum neurotoxin A,
[0150] 0.01% v/v polysorbate 80,
[0151] 0.25 mg/mL tryptophan
[0152] 255 mM NaCl,
[0153] 2 mM Sodium phosphate,
[0154] wherein the pH of said composition is approximately 7.2.

[0155] According to another embodiment, a liquid composition according to the invention comprises or consists essentially of:

[0156] Botulinum neurotoxin A,
[0157] 0.01% v/v polysorbate 80,
[0158] 0.25 mg/mL tryptophan
[0159] 255 mM NaCl,
[0160] 10 mM KCl,
[0161] 50 mM Sodium phosphate,
[0162] wherein the pH of said composition is approximately 6.3.

[0163] According to another embodiment, a liquid composition according to the invention comprises or consists essentially of:

[0164] Botulinum neurotoxin A,
[0165] 1% v/v polysorbate 80,
[0166] 0.25 mg/mL tryptophan
[0167] 255 mM NaCl,
[0168] 50 mM Sodium phosphate,
[0169] wherein the pH of said composition is approximately 6.3.

[0170] According to another embodiment, a liquid composition according to the invention comprises or consists essentially of:

[0171] Botulinum neurotoxin A,
[0172] 1% v/v polysorbate 80,
[0173] 3 mg/mL tryptophan
[0174] 255 mM NaCl,
[0175] 10 mM KCl,
[0176] 50 mM Sodium phosphate,
[0177] wherein the pH of said composition is approximately 7.2.

[0178] According to another embodiment, a liquid composition according to the invention comprises or consists essentially of:
[0179] Botulinum neurotoxin A,
[0180] 0.1% v/v polysorbate 80,
[0181] 1.625 mg/mL tryptophan
[0182] 140 mM NaCl,
[0183] 3 mM KCl,
[0184] 10 mM Sodium phosphate,
[0185] wherein the pH of said composition is approximately 6.75.

[0186] According to another embodiment, a liquid composition according to the invention comprises or consists essentially of:
[0187] Botulinum neurotoxin A,
[0188] 0.01% v/v polysorbate 80,
[0189] 1 mg/mL tryptophan
[0190] 140 mM NaCl,
[0191] 3 mM KCl,
[0192] 10 mM Sodium phosphate,
[0193] wherein the pH of said composition is approximately 6.75.

[0194] According to another embodiment, a liquid composition according to the invention comprises or consists essentially of:
[0195] Botulinum neurotoxin A,
[0196] 0.1% v/v polysorbate 80,
[0197] 1 mg/mL tryptophan
[0198] 140 mM NaCl,
[0199] 3 mM KCl,
[0200] 10 mM Sodium phosphate,
[0201] wherein the pH of said composition is approximately 6.75.

[0202] According to another embodiment, a liquid composition according to the invention comprises or consists essentially of:
[0203] Botulinum neurotoxin A,
[0204] 1% v/v polysorbate 80,
[0205] 1 mg/mL tryptophan
[0206] 140 mM NaCl,
[0207] 3 mM KCl,
[0208] 10 mM Sodium phosphate,
[0209] wherein the pH of said composition is approximately 6.75.

[0210] According to another embodiment, a liquid composition according to the invention comprises or consists essentially of:
[0211] Botulinum neurotoxin B,
[0212] 15% v/v polysorbate 20,
[0213] 1 mg/mL tryptophan
[0214] 140 mM NaCl,
[0215] 3 mM KCl,
[0216] 10 mM Sodium phosphate,
[0217] wherein the pH of said composition is approximately 7.4.

[0218] According to another embodiment, a liquid composition according to the invention comprises or consists essentially of:
[0219] Botulinum neurotoxin B,
[0220] 15% v/v polysorbate 20,
[0221] 4 mg/mL tryptophan
[0222] 140 mM NaCl,
[0223] 3 mM KCl,
[0224] 10 mM Sodium phosphate,
[0225] wherein the pH of said composition is approximately 7.4.

[0226] According to another embodiment, a liquid composition according to the invention comprises or consists essentially of:
[0227] Botulinum neurotoxin B,
[0228] 0.25% v/v polysorbate 20,
[0229] 4 mg/mL tryptophan
[0230] 140 mM NaCl,
[0231] 3 mM KCl,
[0232] 10 mM Sodium phosphate,
[0233] wherein the pH of said composition is approximately 7.4.

[0234] Another aspect is the use of the liquid compositions according to the invention in therapy.

[0235] The liquid compositions according to the invention can be used in therapy to treat or prevent muscular disorders, neuromuscular disorders, neurological disorders, ophtalmological disorders, pain disorders, psychological disorders, articular disorders, inflammatory disorders, endocrine disorders or urological disorders.

[0236] For example, the liquid compositions according to the invention can be used for treating or preventing a disease, condition or syndrome selected from the following:

[0237] ophtalmological disorders selected from the group consisting of blepharospasm, strabismus (including restrictive or myogenic strabismus), amblyopia, oscillopsia, protective ptosis, therapeutic ptosis for corneal protection, nystagmus, estropia, diplopia, entropion, eyelid retraction, orbital myopathy, heterophoria, concomitant misalignment, nonconcomitant misalignment, primary or secondary esotropia or exotropia, internuclear ophthalmoplegia, skew deviation, Duane's syndrome and upper eyelid retraction;

[0238] movement disorders including hemifacial spasm, torticollis, spasticity of the child or of the adult (e.g. in cerebral palsy, post-stroke, multiple sclerosis, traumatic brain injury or spinal cord injury patients), idiopathic focal dystonias, muscle stiffness, Writer's cramp, hand dystonia, VI nerve palsy, oromandibular dystonia, head tremor, tardive dyskinesia, tardive dystonia, occupational cramps (including musicians' cramp), facial nerve palsy, jaw closing spasm, facial spasm, synkinesia, tremor, primary writing tremor, myoclonus, stiff-person-syndrome, foot dystonia, facial paralysis, painful-arm-and-moving-fingers-syndrome, tic disorders, dystonic tics, Tourette's syndrome, neuromyotonia, trembling chin, lateral rectus palsy, dystonic foot inversion, jaw dystonia, Rabbit syndrome, cerebellar tremor, III nerve palsy, palatal myoclonus, akasthesia, muscle cramps, IV nerve palsy, freezing-of-gait, extensor truncal dystonia, post-facial nerve palsy synkinesis, secondary dystonia, Parkinson's disease, Huntington's chorea, epilepsy, off period dystonia, cephalic tetanus, myokymia and benign cramp-fasciculation syndrome;

[0239] otorhinolaryngological disorders including spasmodic dysphonia, otic disorders, hearing impairment, ear click, tinnitus, vertigo, Meniere's disease, cochlear nerve dysfunction, stuttering, cricopharyngeal dysphagia, bruxism, closure of larynx in chronic aspiration,

vocal fold granuloma, ventricular dystonia, ventricular dysphonia, mutational dysphonia, trismus, snoring, voice tremor, aspiration, tongue protrusion dystonia, palatal tremor, deep bite of lip and laryngeal dystonia; First Bite Syndrome;

[0240] gastrointestinal disorders including achalasia, anal fissure, constipation, temperomandibular joint dysfunction, sphincter of Oddi dysfunction, sustained sphincter of Oddi hypertension, intestinal muscle disorders, puborectalis syndrome, anismus, pyloric spasm, gall bladder dysfunction, gastrointestinal or oesophageal motility dysfunction, diffuse oesophageal spasm and gastroparesis;

[0241] urogenital disorders including detrusor sphincter dyssynergia, detrusor hyperreflexia, neurogenic bladder dysfunction (e.g. in Parkinson's disease, spinal cord injury, stroke or multiple sclerosis patients), overactive bladder, neurogenic detrusor overactivity, bladder spasms, urinary incontinence, urinary retention, hypertrophied bladder neck, voiding dysfunction, interstitial cystitis, vaginismus, endometriosis, pelvic pain, prostate gland enlargement (Benign Prostatic Hyperplasia), prostatodynia, prostate cancer and priapism;

[0242] dermatological disorders including cutaneous cell proliferative disorders, skin wounds, psoriasis, rosacea, acne; rare hereditary skin disorders such as Fox-Fordyce syndrome or Hailey-Hailey disease; keloid and hypertrophic scar reduction; pore size reduction; inflammatory conditions of the skin; painful inflammatory conditions of the skin;

[0243] pain disorders including back pain (upper back pain, lower back pain), myofascial pain, tension headache, fibromyalgia, painful syndromes, myalgia, migraine, whiplash, joint pain, post-operative pain, pain not associated with a muscle spasm and pain associated with smooth muscle disorders;

[0244] inflammatory disorders including pancreatitis, neurogenic inflammatory disorders (including gout, tendonitis, bursitis, dermatomyositis and ankylosing spondylitis);

[0245] secretory disorders such as excessive gland secretions, hyperhidrosis (including axillary hyperhidrosis, palmar hyperhidrosis and Frey's syndrome), hypersalivation, sialorrhoea, bromhidrosis, mucus hypersecretion, hyperlacrimation, holocrine gland dysfunction; excess sebum secretion;

[0246] respiratory disorders including rhinitis (including allergic rhinitis), COPD, asthma and tuberculosis;

[0247] hypertrophic disorders including muscle enlargement, masseteric hypertrophy, acromegaly and neurogenic tibialis anterior hypertrophy with myalgia;

[0248] articular disorders including tennis elbow (or epicondilytis of the elbow), inflammation of joints, coxarthrosis, osteoarthritis, rotator muscle cap pathology of the shoulder, rheumatoid arthritis and carpal tunnel syndrome;

[0249] endocrine disorders like type 2 diabetes, hyperglucagonism, hyperinsulinism, hypoinsulinism, hypercalcemia, hypocalcemia, thyroid disorders (including Grave's disease, thyroiditis, Hashimoto's thyroiditis, hyperthyroidism and hypothyroidism), parathyroid disorders (including hyperparathyroidism and hypoparathyroidism), Gushing's syndrome and obesity;

[0250] autoimmune diseases like systemic lupus erythemotosus;

[0251] proliferative diseases including paraganglioma tumors, prostate cancer and bone tumors;

[0252] traumatic injuries including sports injuries, muscle injuries, tendon wounds and bone fractures; and

[0253] veterinary uses (e.g. immobilisation of mammals, equine colic, animal achalasia or animal muscle spasms).

[0254] The liquid compositions according to the invention can also be used in aesthetic medicine (that is for improving cosmetic appearance), in particular for treating or preventing skin wrinkles, in particular facial wrinkles such as facial frown lines, wrinkles of the contour of the eye, glabellar frown lines, downturned mouth, wrinkles of the neck (platysmal bands), wrinkles of the chin (mentalis, peau d'orange, dimpled chin), forehead lines, "scratched skin" wrinkles, nasal lift treatment or sleep lines. According to this aspect of the invention, the subject to be treated or prevented for improving cosmetic appearance is preferably not suffering from any of the disorders, conditions or syndromes that are described above. More preferably, said subject is a healthy subject (i.e. not suffering from any disease, condition or syndrome).

[0255] The liquid compositions according to the invention can be used in combination with another therapeutic compound. In one embodiment the liquid compositions according to the invention is administered in combination with an analgesic compound for treating pain, in particular in combination with an opioid derivative such as morphine as described in WO 2007/144493 the content of which is herein incorporated by reference. In another embodiment, the liquid compositions according to the invention is administered in combination with hyaluronic acid, for example for treating prostate cancer as described in WO 2015/0444416 the content of which is herein incorporated by reference.

[0256] A further aspect of the present invention is the use of an amino acid selected from tryptophan and tyrosine to protect a proteinaceous neurotoxin from degradation in a liquid composition which is free of animal derived proteins.

[0257] According to a preferred embodiment, the amino acid is tryptophan, more preferably L-tryptophan.

[0258] Preferably, the proteinaceous neurotoxin is a botulinum neurotoxin. According to an embodiment of the invention, the botulinum neurotoxin is a botulinum neurotoxin in complex form. According to another embodiment, the botulinum neurotoxin is a high purity botulinum neurotoxin. According to an embodiment of the invention, the botulinum neurotoxin is a botulinum neurotoxin purified from its natural clostridial strain. According to another embodiment, botulinum neurotoxin is a botulinum neurotoxin produced recombinantly in a heterologous host such as *E. coli*. According to the present invention, the Botulinum neurotoxin can be a BoNT of serotype A, B, C, D, E, F or G. According to the present invention, a botulinum neurotoxin can be a modified botulinum neurotoxin as described above. According to the present invention, a recombinant botulinum neurotoxin can be a chimeric botulinum neurotoxin as described above.

[0259] According to a preferred embodiment, the amino acid is used in combination with a surfactant and a buffer comprising sodium, chloride and phosphate ions, and the liquid composition has a pH between 5.5 and 8. Preferably, the surfactant is a non-ionic surfactant, more preferably a

polysorbate, for example PS20, PS60 or PS80. Most preferably, the non-ionic surfactant is PS80. Preferably, the buffer also comprises potassium ions. The buffer can for example be obtained by combining sodium chloride, potassium chloride and sodium phosphate salts. According to a preferred embodiment, the pH is between 6.0 and 7.5, for example 6.3, 6.35, 6.4, 6.45, 6.5, 6.55, 6.6, 6.65, 6.7, 6.75, 6.8, 6.85, 6.9, 6.95, 7.0, 7.05, 7.1, 7.15, 7.2, 7.25, 7.3, 7.35, 7.4, 7.45 or 7.5. Preferably the pH is within one unit from physiological pH (which is around 7.4).

[0260] According to a preferred embodiment of the use according to the invention, the liquid composition is stable for 2 months. For example, it is stable for 2 months at 2 to 8° C. According to one embodiment, it is stable for 3 months at 2 to 8° C., for example at 5° C. According to a preferred embodiment, it is stable for 6 months at 2 to 8° C., for example at 5° C. According to one embodiment, it is stable for 12 months at 2 to 8° C., for example at 5° C. According to one embodiment, it is stable for 18 months at 2 to 8° C., for example at 5° C. According to one embodiment, it is stable for 24 months at 2 to 8° C., for example at 5° C. According to one embodiment, it is stable for 36 months at 2 to 8° C., for example at 5° C. According to one embodiment, it is stable for 3 months at room temperature, for example at 25° C. According to one embodiment, it is stable for 6 months at room temperature, for example at 25° C.

EXAMPLES

[0261] 1. Preparation of Stable Liquid Botulinum Toxin A Formulations

[0262] Liquid botulinum toxin preparations containing 15 ng/mL of highly purified BoNT/A, 15% v/v polysorbate 20, an amino acid selected from tyrosine (Tyr), tryptophan (Trp) and cysteine (Cys) or a mixture of methionine (Met), tyrosine (Tyr), tryptophan (Trp) and cysteine (Cys) (Sigma Aldrich), and Phosphate Buffer Saline (PBS from Calbiochem) (140 mM NaCl, 10 mM sodium phosphate and 3 mM KCl at pH 7.4 at 25° C.) were prepared, filtered using 0.22 μm PVDF (polyvinylidenflourid) filters and stored in siliconized 2 mL glass syringes for 6 days at 40° C., after which a potency test was performed for each preparation.

[0263] For the potency test, the syringes containing the preparations were emptied in 2 mL glass vials (Chromacol, Gold) with lids containing PTFE treated rubber septa (Chromacol) or in 1.7 mL plastic micro centrifuge tubes (Axygen, Maximum Recovery) which both have low protein adsorption properties. The preparations were subsequently diluted using 0.9% NaCl solution with 3% human serum albumin (HSA). For each preparation, 50 μL of sample was injected into the Gastrocnemius muscle of mice on the same day as the dilution was performed. The mice were monitored for 3 days and the degree of paralysis was recorded.

[0264] The results are shown in table 1.

TABLE 1

an accelerated storage test (6 days at 40° C.) of amino acid additions on BoNT/A stability.

Formulation			Potency test: Dilution in 0.9 NaCl with 3% HSA (times)	Potency test: Inj. Dose (ng)	Potency rating		
Buffer	BoNT/A conc.	Amino acid			Day 1	Day 2	Day 3
PBS pH 7.4 15% polysorbate 20	15 ng/mL	Trp 0.25 mg/mL + Cys 0.25 mg/mL + Met 0.25 mg/mL + Tyr 0.25 mg/mL	2 x	0.25	—	—	WN
			20 x	0.025	—	—	WN
		Cys 1 mg/mL	2 x	0.25	—	—	WN
			20 x	0.025	—	—	WN
		Tyr 0.74 mg/mL	2 x	0.25	—	—	Sharp PA whole abdomen
			20 x	0.025	—	—	WN
		Trp 1 mg/mL	2 x	0.25	—	†	
			20 x	0.025	—	—	WN
		—	2 x	0.25	—	—	WN
			20 x	0.025	—	—	WN

Paralysis results in mice: — = not analysed, WN = without note, PA = paralysis and † = death.

[0265] Tyrosine and tryptophan were found to have a protective effect against BoNT/A degradation. Tryptophan was found to have the strongest protective effect. Cysteine, as well as the mixture containing all 4 amino acids did not have a protective effect.

[0266] 2. Preparation of a Stable Liquid Botulinum Toxin B Formulation

[0267] Liquid botulinum toxin preparations containing 350 ng/mL of highly purified BoNT/B, 15% v/v polysorbate 20, an amino acid selected from tyrosine (Tyr), tryptophan (Trp) and cysteine (Cys) or a mixture of methionine (Met), tyrosine (Tyr), tryptophan (Trp) and cysteine (Cys), and Phosphate Buffer Saline (PBS) at pH 7.4 were prepared, filtered using 0.22 μm filters and stored in siliconized 2 mL glass syringes for two weeks at 40° C., after which a potency test was performed for each preparation as described above.

[0268] The results are shown in table 2.

TABLE 2

an accelerated storage test (two weeks at 40° C.) of amino acid additions on BoNT/B stability.

Formulation			Potency test: Dilution in 0.9 NaCl with 3% HSA (times)	Potency test: Inj. Dose (ng)	Potency rating		
Buffer	BoNT/B conc.	Amino acid			Day 1	Day 2	Day 3
PBS pH 7.4 15% polysorbate 20	350 ng/mL	Trp 0.25 mg/mL + Cys 0.25 mg/mL + Met 0.25 mg/mL + Tyr 0.25 mg/mL	10 x	1.75	—	—	PA
		Cys 1 mg/mL	10 x	1.75	—	—	PA
		Tyr 0.575 mg/mL	10 x	1.75	—	†	
		Trp 1 mg/mL	10 x	1.75	—	†	
			35 x	0.5	—	—	PA

Paralysis results in mice: — = not analysed, WN = without note, PA = paralysis and † = death.

[0269] Tyrosine and tryptophan were found to have a protective effect against BoNT/B degradation. Cysteine, as well as the mixture containing all 4 amino acids also had a protective effect but to a weaker extent.

[0270] 3. Evaluation of Different Concentrations of Tryptophan and Polysorbate 20

[0271] Liquid botulinum toxin preparations containing highly purified BoNT/A or BoNT/B and various concentrations of polysorbate 20 (PS 20) and tryptophan and Phosphate Buffer Saline (PBS) at pH 7.4 were prepared, filtered using 0.22 μm filters and stored in siliconized 2 mL glass syringes. Hind limb paralysis potency tests were performed for each preparation as described above.

[0272] The results are shown in table 3.

[0273] 4. Evaluation of Different Salt Concentrations in BoNT/B Preparations

[0274] Liquid botulinum toxin preparations containing 100 ng/mL of highly purified BoNT/B, polysorbate 20, tryptophan from various amino acid suppliers and a buffer selected from PBS pH 7.4 (Calbiochem), 12 nM phosphate buffer pH 7 (Apoteket) and 20 mM sodium acetate (NaAc) pH 5.5 (NaAc from Fluka and acetic acid from Merck) were prepared, filtered using 0.22 μm filters and stored in siliconized 2 mL glass syringes. Hind limb paralysis potency tests were performed for each preparation as described above.

TABLE 3

evaluation of Trp and polysorbate 20 (PS20) concentrations on BoNT/A or BoNT/B stability.

Formulation				POTENCY TESTING: Dilution in 0.9 NaCl with 3% HSA (times)	Inj. Dose (ng)	Potency rating (1-3 d) for samples stored at different temperatures and lengths: 5° C. 6 months 1 d	5° C. 6 months 2 d	5° C. 6 months 3 d	25° C. 5 weeks 1 d	25° C. 5 weeks 2 d	25° C. 5 weeks 3 d	25° C. 4 months 1 d	25° C. 4 months 2 d	25° C. 4 months 3 d
BoNT	BoNT conc. (ng/mL)	Trp (mg/mL)	PS20 (%)											
BoNT/A	15	8	15	1 x	0.75	—	†		—	—	†	—	—	PA
				5 x	0.15	—	—	†	—	—	†	—	—	WN
	15	8	0.25	1 x	0.75	—	†		—	—	†*	—	—	†
				5 x	0.15	—	—	†	—	†*		—	—	WN[2]
	15	1	15	1 x	0.75	—	†		—	†		†		
				5 x	0.15	—	—	†	—	—	†	—	†	PA[3]
BoNT/B	100	8	15	1 x	5	—	—	†	—	—	†	—	—	PA
				10 x	0.5	—	—	PA[1]	—	—	PA	—	—	PA
	100	8	0.25	1 x	5	—	—	†	—	—	†	†		
				10 x	0.5	—	—	PA[4]	—	—	WN[1]	—	—	PA
	100	1	15	1 x	5	—	—	†	—	—	†	†		
				10 x	0.5	—	—	PA[4]		—	PA	—	—	PA

Paralysis results in mice: — = not analysed, WN = without note, PA = paralysis and t = death.

Paralysis degree (PA):

[1]Toes affected;

[2]Slightly numb in hind leg;

[3]Both hind legs paralysed;

[4]Hind leg paralysed; Elution buffer from purification (5): 50 mM sodium acetate pH 4.5 with 0.2% (v:v) polysorbate 20 and 400 mM sodium chloride.

*a mix up of two BoNT/A dilutions of the 25° C. 8 mg/mL Trp 0.25% polysorbate 20 has probably occurred.

[0275] The results are shown in table 4.

TABLE 4

evaluation of different salt concentrations on stability of BoNT/B.

				Potency testing		Potency rating (1-3 d) for samples stored at different length					
BoNT/B		Trp		Dilution in 0.9	Inj.	40° C.					
conc.		manufacturer	PS20	NaCl with 3%	Dose	2 weeks			5 weeks		
(ng/mL)	Buffer	and conc.	(%)	HSA (times)	(ng)	1 d	2 d	3 d	1 d	2 d	3 d
100	PBS	Ajinomoto	15	1 x	5	—	†				
	pH 7.4	4 mg/mL		10 x	0.5	—	—	PA			
100	PBS	Sigma	15	1 x	5	—	†				
	pH 7.4	Aldrich		10 x	0.5	—	—	PA			
		4 mg/mL									
100	PBS	Sigma	15	1 x	5	—	†				
	pH 7.4	Aldrich		10 x	0.5	—	—	PA			
		1 mg/mL									
100	PBS	Sigma	0.25	1 x	5	—	†		—	†	
	pH 7.4	Aldrich		10 x	0.5	—	—	PA	—	—	PA[2]
		4 mg/mL									
100	12 mM	Sigma	15	1 x	5	—	—	WN			
	Phosphate	Aldrich		10 x	0.5	—	—	WN[1]			
	pH 7	4 mg/mL									
100	20 mM	Sigma	15	1 x	5	—	—	PA			
	NaAc	Aldrich		10 x	0.5	—	—	PA			
	pH 5.5	4 mg/mL									

Paralysis results in mice: — = not analysed, WN = without note, PA = paralysis and † = death.
[1]Some loss of function;
[2]Weak paralysis

[0276] The results show that the preparations containing the PBS buffer (containing sodium, chloride, phosphate and potassium ions) appears to play a role in the stability of the botulinum toxin.

[0277] 5. Evaluation of Different Stabilizers

[0278] Liquid botulinum toxin preparations containing 15 ng/mL of highly purified BoNT/A, a polysorbate 20 (PS20) or polysorbate 80 (PS80) or HSA, tryptophan and PBS were prepared, filtered using 0.22 μm filters and stored in siliconized 2 mL glass syringes. Hind limb paralysis potency tests were performed for each preparation as described above.

[0279] The results are shown in table 5.

[0280] 6. Evaluation of Different Formulations

[0281] Liquid botulinum toxin preparations containing 10 ng/mL of highly purified BoNT/A, 0.25% PS80, 1 mg/mL tryptophan and PBS were prepared as described above. The pH was adjusted to 6.6 and 7.0 by adding HCl. Each preparation was stored 5 weeks at 40° C.

[0282] Each preparation was then diluted 10 times and hind limb paralysis potency tests were performed as described above (0.05 ng per injection). In both cases, hind limb paralysis was observed at day 3. The paralysis was stronger with the pH 6.6 preparation.

TABLE 5

evaluation of different surfactants on stability of BoNT/A.

				Potency testing		Potency rating (1-3 d) for samples stored at different length								
BoNT/A				Dilution in 0.9	Inj.	40° C.								
conc.				NaCl with 3%	Dose	6 days			4 weeks			3 months		
(ng/mL)	Buffer	Trp	Stabiliser	HSA (times)	(ng)	1 d	2 d	3 d	1 d	2 d	3 d	1 d	2 d	3 d
15	PBS	1 mg/mL	PS 80	1.7 x	0.45	†			†			—	—	PA[3]
	pH 7.4		0.25%	15 x	0.05	†			†			—	—	WA
15	PBS	1 mg/mL	PS 20	1.7 x	0.45	†			†			—	—	PA[2]
	pH 7.4		0.25%	15 x	0.05	†			—	—	PA	—	—	WN
15	PBS	—	HSA	1.7 x	0.45	†			†			—	†	
	pH 7.4		1 mg/mL	15 x	0.05	†			†			—	—	PA[1]

Paralysis results in mice: — = not analysed, WN = without note, PA = paralysis and † = death.
[1]Severe paralysis both hind legs;
[2]Angles paws;
[3]Severe paralysis

[0283] 7. Evaluation of Different Formulations

[0284] Liquid botulinum toxin preparations containing 0.3 ng/mL of highly purified BoNT/A, a polysorbate selected from PS20 and PS80, 1 mg/mL tryptophan and 12 mM PBS at pH 7.4 were prepared as described above. The pH of each preparation was adjusted to pH 6.6 or 6.9 by adding 1.2 M HCl.

[0285] Polysorbate 20 was tested at one concentration, 0.2% w/v, corresponding to about 20 times its CMC (critical micellar concentration, about 0.01% w/v at 21° C.). Polysorbate 80 was tested 0.04% and 0.2% w/v, corresponding respectively to about 20 and 100 times its CMC (about 0.002% w/v at 21° C.).

TABLE 6

Choice of polysorbate and pH

Composition	PS20 % w/v	PS20 % w/v	pH
PS20-1	0.2	—	6.6
PS20-2	0.2	—	6.9
PS80-1	—	0.04	6.6
PS80-2	—	0.04	6.9
PS80-3	—	0.2	6.6

[0286] For each preparation, a volume of 0.5 mL was filled in 1 mL long glass syringes (BD) and sealed with a fluorocarbon coated plunger.

[0287] The potency was measured by hind limb paralysis test on mice as described above.

[0288] No decrease in potency was observed in any formulation after 6 months storage at 5° C. and after 25° C.

[0289] 8. Evaluation of Different Formulations

[0290] Nineteen different formulations containing highly purified botulinum neurotoxin type A were prepared with varying concentrations of polysorbate 80, tryptophan, sodium phosphate, sodium chloride, potassium chloride and varying pH. Each formulation had a target nominal potency of 500 U/mL. Each formulation was degassed, filtered through 0.2 μm filter and filled into vials. Nitrogen gas was used as a protective atmosphere in the vials. The filling was performed in an anaerobic chamber. Each formulation was filled in 1 mL aliquots in a nitrogen atmosphere in 2 mL glass vials capped with FluroTec® stoppers sealed with aluminium flip off seals and stored upright.

[0291] The stability of the 19 formulations was assessed at 5° C., 25° C. and 37° C. using BoTest® to measure potency.

TABLE 7

Excipient compositions

Exp Name	pH	Sodium phosphate (mM)	Tryptophan (mg/mL)	Polysorbate 80 (v %)	NaCl (mM)	KCl (mM)
N1	6.3	2	0.25	0.01	25	0
N2	7.2	2	0.25	0.01	255	0
N3	6.3	50	0.25	0.01	255	10
N4	7.2	50	0.25	0.01	25	10
N5	6.3	2	3	0.01	255	10
N6	7.2	2	3	0.01	25	10
N7	6.3	50	3	0.01	25	0
N8	7.2	50	3	0.01	255	0
N9	6.3	2	0.25	1	25	10
N10	7.2	2	0.25	1	255	10
N11	6.3	50	0.25	1	255	0
N12	7.2	50	0.25	1	25	0
N13	6.3	2	3	1	255	0
N14	7.2	2	3	1	25	0
N15	6.3	50	3	1	25	10
N16	7.2	50	3	1	255	10
N17	6.75	10	1.625	0.1	140	3

TABLE 8

Packaging components

Article	Article number, Supplier
Clear glass vial of boro silicate Type I plus, 2 mL	1097221, Schott
Grey Flurotec coated bromobutyl stopper Westar RS, 13 mm	1356 4023/50, West
Aluminium flip off seals, 13 mm	5920-6623, West

[0292] For all formulations the solution remained clear and for most parts colourless.

[0293] The excipients concentrations tested in this study seem not to affect the pH of the formulations during the time interval tested. The potency results are presented in table 9.

TABLE 9

Botest potency results

		Baseline potency (U/mL) (Botest)	Remaining potency compared to base line (BoTest)					
			2 months 37° C.		6 months 25° C.		6 months 5° C.	
	pH	0 month	U/mL	%	U/mL	%	U/mL	%
N1	6.3	86	0	0	0	0	39	45
N2	7.2	474	210	44	389	82	485	102
N3	6.3	449	288	64	406	90	512	114
N4	7.2	299	62	21	368	123	406	136
N5	6.3	378	263	70	385	102	422	112
N6	7.2	93	0	0	0	0	52	56
N7	6.3	238	0	0	0	0	239	100
N8	7.2	375	305	81	402	107	450	120
N9	6.3	196	0	0	100	51	294	150
N10	7.2	354	201	57	408	115	411	116
N11	6.3	438	197	45	372	85	492	112
N12	7.2	411	96	23	295	72	417	101

TABLE 9-continued

Botest potency results

		Baseline potency (U/mL) (Botest)	Remaining potency compared to base line (BoTest) 2 months 37° C.		6 months 25° C.		6 months 5° C.	
	pH	0 month	U/mL	%	U/mL	%	U/mL	%
N13	6.3	304	185	61	403	133	416	137
N14	7.2	206	0	0	0	0	183	89
N15	6.3	197	155	79	231	117	214	109
N16	7.2	476	111	48	390	82	685	144
N17	6.75	402	250	62	286	71	488	121

[0294] For several compositions there was no more than 30% loss in potency over 6 months at 5° C. and/or no more than about 40% loss in potency over 3 months at 25° C. and/or no more than about 50% loss in potency over 2 months at 37° C.

[0295] 9. Evaluation of PS 60

[0296] A formulation containing highly purified botulinum neurotoxin type A was prepared with 0.1% (v/v) PS60, 1 mg/mL L-Tryptophan, 10 mM sodium phosphate, 140 mM sodium chloride, 3 mM potassium chloride and water for injection. The pH was adjusted to 6.75 with HCl. The formulation had a target nominal potency of 100 U/mL. The formulation was degassed, filtered through 0.2 μm filters and filled into 2 mL vials aseptically in an anaerobic chamber with nitrogen atmosphere with a fill volume of 1 mL. Nitrogen gas was used as protective atmosphere in the vials. The vials were capped with FluroTec® stoppers sealed with aluminium flip off seals.

TABLE 10

Packaging components

Article	Article number, Supplier
Clear glass vial of boro silicate Type I plus, 2 mL	1097221, Schott
13 mm Inj stopper coated bromobutyl 4023-50 grey	INJ13TB3WRS, Nordic Pack
Blue aluminium flip off seals, 13 mm	5920-1164, Nordic Pack

[0297] The potency over time at 37° C. and 25° C. was measured by the MLD50 test as described herein.

[0298] At 37° C., the remaining potency after 9 weeks was around 50-55% of the initial potency.

[0299] At 25° C., the remaining potency after 3 months was about 80% of the initial potency.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 7

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<212> TYPE: PRT
<213> ORGANISM: Clostridium botulinum

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Val Asp Ile Ala Tyr Ile Lys Ile Pro Asn Ala Gly Gln Met Gln Pro
            20                  25                  30

Val Lys Ala Phe Lys Ile His Asn Lys Ile Trp Val Ile Pro Glu Arg
        35                  40                  45

Asp Thr Phe Thr Asn Pro Glu Glu Gly Asp Leu Asn Pro Pro Pro Glu
    50                  55                  60

Ala Lys Gln Val Pro Val Ser Tyr Tyr Asp Ser Thr Tyr Leu Ser Thr
65                  70                  75                  80

Asp Asn Glu Lys Asp Asn Tyr Leu Lys Gly Val Thr Lys Leu Phe Glu
                85                  90                  95

Arg Ile Tyr Ser Thr Asp Leu Gly Arg Met Leu Leu Thr Ser Ile Val
            100                 105                 110

Arg Gly Ile Pro Phe Trp Gly Gly Ser Thr Ile Asp Thr Glu Leu Lys
        115                 120                 125

Val Ile Asp Thr Asn Cys Ile Asn Val Ile Gln Pro Asp Gly Ser Tyr
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    130                 135                 140

Arg Ser Glu Glu Leu Asn Leu Val Ile Ile Gly Pro Ser Ala Asp Ile
145                 150                 155                 160

Ile Gln Phe Glu Cys Lys Ser Phe Gly His Glu Val Leu Asn Leu Thr
                165                 170                 175

Arg Asn Gly Tyr Gly Ser Thr Gln Tyr Ile Arg Phe Ser Pro Asp Phe
            180                 185                 190

Thr Phe Gly Phe Glu Glu Ser Leu Glu Val Asp Thr Asn Pro Leu Leu
        195                 200                 205

Gly Ala Gly Lys Phe Ala Thr Asp Pro Ala Val Thr Leu Ala His Glu
    210                 215                 220

Leu Ile His Ala Gly His Arg Leu Tyr Gly Ile Ala Ile Asn Pro Asn
225                 230                 235                 240

Arg Val Phe Lys Val Asn Thr Asn Ala Tyr Tyr Glu Met Ser Gly Leu
                245                 250                 255

Glu Val Ser Phe Glu Glu Leu Arg Thr Phe Gly Gly His Asp Ala Lys
            260                 265                 270

Phe Ile Asp Ser Leu Gln Glu Asn Glu Phe Arg Leu Tyr Tyr Tyr Asn
        275                 280                 285

Lys Phe Lys Asp Ile Ala Ser Thr Leu Asn Lys Ala Lys Ser Ile Val
    290                 295                 300

Gly Thr Thr Ala Ser Leu Gln Tyr Met Lys Asn Val Phe Lys Glu Lys
305                 310                 315                 320

Tyr Leu Leu Ser Glu Asp Thr Ser Gly Lys Phe Ser Val Asp Lys Leu
                325                 330                 335

Lys Phe Asp Lys Leu Tyr Lys Met Leu Thr Glu Ile Tyr Thr Glu Asp
            340                 345                 350

Asn Phe Val Lys Phe Phe Lys Val Leu Asn Arg Lys Thr Tyr Leu Asn
        355                 360                 365

Phe Asp Lys Ala Val Phe Lys Ile Asn Ile Val Pro Lys Val Asn Tyr
    370                 375                 380

Thr Ile Tyr Asp Gly Phe Asn Leu Arg Asn Thr Asn Leu Ala Ala Asn
385                 390                 395                 400

Phe Asn Gly Gln Asn Thr Glu Ile Asn Asn Met Asn Phe Thr Lys Leu
                405                 410                 415

Lys Asn Phe Thr Gly Leu Phe Glu Phe Tyr Lys Leu Leu Cys Val Arg
            420                 425                 430

Gly Ile Ile Thr Ser Lys Thr Lys Ser Leu Asp Lys Gly Tyr Asn Lys
        435                 440                 445

Ala Leu Asn Asp Leu Cys Ile Lys Val Asn Asn Trp Asp Leu Phe Phe
    450                 455                 460

Ser Pro Ser Glu Asp Asn Phe Thr Asn Asp Leu Asn Lys Gly Glu Glu
465                 470                 475                 480

Ile Thr Ser Asp Thr Asn Ile Glu Ala Ala Glu Glu Asn Ile Ser Leu
                485                 490                 495

Asp Leu Ile Gln Gln Tyr Tyr Leu Thr Phe Asn Phe Asp Asn Glu Pro
            500                 505                 510

Glu Asn Ile Ser Ile Glu Asn Leu Ser Ser Asp Ile Ile Gly Gln Leu
        515                 520                 525

Glu Leu Met Pro Asn Ile Glu Arg Phe Pro Asn Gly Lys Lys Tyr Glu
    530                 535                 540
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Leu Asp Lys Tyr Thr Met Phe His Tyr Leu Arg Ala Gln Glu Phe Glu
545                 550                 555                 560

His Gly Lys Ser Arg Ile Ala Leu Thr Asn Ser Val Asn Glu Ala Leu
                565                 570                 575

Leu Asn Pro Ser Arg Val Tyr Thr Phe Phe Ser Ser Asp Tyr Val Lys
            580                 585                 590

Lys Val Asn Lys Ala Thr Glu Ala Ala Met Phe Leu Gly Trp Val Glu
        595                 600                 605

Gln Leu Val Tyr Asp Phe Thr Asp Glu Thr Ser Glu Val Ser Thr Thr
    610                 615                 620

Asp Lys Ile Ala Asp Ile Thr Ile Ile Ile Pro Tyr Ile Gly Pro Ala
625                 630                 635                 640

Leu Asn Ile Gly Asn Met Leu Tyr Lys Asp Asp Phe Val Gly Ala Leu
                645                 650                 655

Ile Phe Ser Gly Ala Val Ile Leu Leu Glu Phe Ile Pro Glu Ile Ala
            660                 665                 670

Ile Pro Val Leu Gly Thr Phe Ala Leu Val Ser Tyr Ile Ala Asn Lys
        675                 680                 685

Val Leu Thr Val Gln Thr Ile Asp Asn Ala Leu Ser Lys Arg Asn Glu
    690                 695                 700

Lys Trp Asp Glu Val Tyr Lys Tyr Ile Val Thr Asn Trp Leu Ala Lys
705                 710                 715                 720

Val Asn Thr Gln Ile Asp Leu Ile Arg Lys Lys Met Lys Glu Ala Leu
                725                 730                 735

Glu Asn Gln Ala Glu Ala Thr Lys Ala Ile Ile Asn Tyr Gln Tyr Asn
            740                 745                 750

Gln Tyr Thr Glu Glu Glu Lys Asn Asn Ile Asn Phe Asn Ile Asp Asp
        755                 760                 765

Leu Ser Ser Lys Leu Asn Glu Ser Ile Asn Lys Ala Met Ile Asn Ile
    770                 775                 780

Asn Lys Phe Leu Asn Gln Cys Ser Val Ser Tyr Leu Met Asn Ser Met
785                 790                 795                 800

Ile Pro Tyr Gly Val Lys Arg Leu Glu Asp Phe Asp Ala Ser Leu Lys
                805                 810                 815

Asp Ala Leu Leu Lys Tyr Ile Tyr Asp Asn Arg Gly Thr Leu Ile Gly
            820                 825                 830

Gln Val Asp Arg Leu Lys Asp Lys Val Asn Asn Thr Leu Ser Thr Asp
        835                 840                 845

Ile Pro Phe Gln Leu Ser Lys Tyr Val Asp Asn Gln Arg Leu Leu Ser
    850                 855                 860

Thr Phe Thr Glu Tyr Ile Lys Asn Ile Ile Asn Thr Ser Ile Leu Asn
865                 870                 875                 880

Leu Arg Tyr Glu Ser Asn His Leu Ile Asp Leu Ser Arg Tyr Ala Ser
                885                 890                 895

Lys Ile Asn Ile Gly Ser Lys Val Asn Phe Asp Pro Ile Asp Lys Asn
            900                 905                 910

Gln Ile Gln Leu Phe Asn Leu Glu Ser Ser Lys Ile Glu Val Ile Leu
        915                 920                 925

Lys Asn Ala Ile Val Tyr Asn Ser Met Tyr Glu Asn Phe Ser Thr Ser
    930                 935                 940
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Phe Trp Ile Arg Ile Pro Lys Tyr Phe Asn Ser Ile Ser Leu Asn Asn
945                 950                 955                 960

Glu Tyr Thr Ile Ile Asn Cys Met Glu Asn Asn Ser Gly Trp Lys Val
                965                 970                 975

Ser Leu Asn Tyr Gly Glu Ile Ile Trp Thr Leu Gln Asp Thr Gln Glu
            980                 985                 990

Ile Lys Gln Arg Val Val Phe Lys  Tyr Ser Gln Met Ile  Asn Ile Ser
        995                 1000                1005

Asp Tyr  Ile Asn Arg Trp Ile  Phe Val Thr Ile Thr  Asn Asn Arg
    1010                1015                1020

Leu Asn  Asn Ser Lys Ile Tyr  Ile Asn Gly Arg Leu  Ile Asp Gln
    1025                1030                1035

Lys Pro  Ile Ser Asn Leu Gly  Asn Ile His Ala Ser  Asn Asn Ile
    1040                1045                1050

Met Phe  Lys Leu Asp Gly Cys  Arg Asp Thr His Arg  Tyr Ile Trp
    1055                1060                1065

Ile Lys  Tyr Phe Asn Leu Phe  Asp Lys Glu Leu Asn  Glu Lys Glu
    1070                1075                1080

Ile Lys  Asp Leu Tyr Asp Asn  Gln Ser Asn Ser Gly  Ile Leu Lys
    1085                1090                1095

Asp Phe  Trp Gly Asp Tyr Leu  Gln Tyr Asp Lys Pro  Tyr Tyr Met
    1100                1105                1110

Leu Asn  Leu Tyr Asp Pro Asn  Lys Tyr Val Asp Val  Asn Asn Val
    1115                1120                1125

Gly Ile  Arg Gly Tyr Met Tyr  Leu Lys Gly Pro Arg  Gly Ser Val
    1130                1135                1140

Met Thr  Thr Asn Ile Tyr Leu  Asn Ser Ser Leu Tyr  Arg Gly Thr
    1145                1150                1155

Lys Phe  Ile Ile Lys Lys Tyr  Ala Ser Gly Asn Lys  Asp Asn Ile
    1160                1165                1170

Val Arg  Asn Asn Asp Arg Val  Tyr Ile Asn Val Val  Val Lys Asn
    1175                1180                1185

Lys Glu  Tyr Arg Leu Ala Thr  Asn Ala Ser Gln Ala  Gly Val Glu
    1190                1195                1200

Lys Ile  Leu Ser Ala Leu Glu  Ile Pro Asp Val Gly  Asn Leu Ser
    1205                1210                1215

Gln Val  Val Val Met Lys Ser  Lys Asn Asp Gln Gly  Ile Thr Asn
    1220                1225                1230

Lys Cys  Lys Met Asn Leu Gln  Asp Asn Asn Gly Asn  Asp Ile Gly
    1235                1240                1245

Phe Ile  Gly Phe His Gln Phe  Asn Asn Ile Ala Lys  Leu Val Ala
    1250                1255                1260

Ser Asn  Trp Tyr Asn Arg Gln  Ile Glu Arg Ser Ser  Arg Thr Leu
    1265                1270                1275

Gly Cys  Ser Trp Glu Phe Ile  Pro Val Asp Asp Gly  Trp Gly Glu
    1280                1285                1290

Arg Pro  Leu
    1295


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Asn Asn Ile Ile Met Met Glu Pro Pro Phe Ala Arg Gly Thr Gly Arg
            20                  25                  30

Tyr Tyr Lys Ala Phe Lys Ile Thr Asp Arg Ile Trp Ile Ile Pro Glu
        35                  40                  45

Arg Tyr Thr Phe Gly Tyr Lys Pro Glu Asp Phe Asn Lys Ser Ser Gly
    50                  55                  60

Ile Phe Asn Arg Asp Val Cys Glu Tyr Tyr Asp Pro Asp Tyr Leu Asn
65                  70                  75                  80

Thr Asn Asp Lys Lys Asn Ile Phe Leu Gln Thr Met Ile Lys Leu Phe
                85                  90                  95

Asn Arg Ile Lys Ser Lys Pro Leu Gly Glu Lys Leu Leu Glu Met Ile
            100                 105                 110

Ile Asn Gly Ile Pro Tyr Leu Gly Asp Arg Arg Val Pro Leu Glu Glu
        115                 120                 125

Phe Asn Thr Asn Ile Ala Ser Val Thr Val Asn Lys Leu Ile Ser Asn
    130                 135                 140

Pro Gly Glu Val Glu Arg Lys Lys Gly Ile Phe Ala Asn Leu Ile Ile
145                 150                 155                 160

Phe Gly Pro Gly Pro Val Leu Asn Glu Asn Glu Thr Ile Asp Ile Gly
                165                 170                 175

Ile Gln Asn His Phe Ala Ser Arg Glu Gly Phe Gly Gly Ile Met Gln
            180                 185                 190

Met Lys Phe Cys Pro Glu Tyr Val Ser Val Phe Asn Asn Val Gln Glu
        195                 200                 205

Asn Lys Gly Ala Ser Ile Phe Asn Arg Arg Gly Tyr Phe Ser Asp Pro
    210                 215                 220

Ala Leu Ile Leu Met His Glu Leu Ile His Val Leu His Gly Leu Tyr
225                 230                 235                 240

Gly Ile Lys Val Asp Asp Leu Pro Ile Val Pro Asn Glu Lys Lys Phe
                245                 250                 255

Phe Met Gln Ser Thr Asp Ala Ile Gln Ala Glu Glu Leu Tyr Thr Phe
            260                 265                 270

Gly Gly Gln Asp Pro Ser Ile Ile Thr Pro Ser Thr Asp Lys Ser Ile
        275                 280                 285

Tyr Asp Lys Val Leu Gln Asn Phe Arg Gly Ile Val Asp Arg Leu Asn
    290                 295                 300

Lys Val Leu Val Cys Ile Ser Asp Pro Asn Ile Asn Ile Asn Ile Tyr
305                 310                 315                 320

Lys Asn Lys Phe Lys Asp Lys Tyr Lys Phe Val Glu Asp Ser Glu Gly
                325                 330                 335

Lys Tyr Ser Ile Asp Val Glu Ser Phe Asp Lys Leu Tyr Lys Ser Leu
            340                 345                 350

Met Phe Gly Phe Thr Glu Thr Asn Ile Ala Glu Asn Tyr Lys Ile Lys
        355                 360                 365

Thr Arg Ala Ser Tyr Phe Ser Asp Ser Leu Pro Pro Val Lys Ile Lys
    370                 375                 380
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Asn Leu Leu Asp Asn Glu Ile Tyr Thr Ile Glu Glu Gly Phe Asn Ile
385                 390                 395                 400

Ser Asp Lys Asp Met Glu Lys Glu Tyr Arg Gly Gln Asn Lys Ala Ile
                405                 410                 415

Asn Lys Gln Ala Tyr Glu Glu Ile Ser Lys Glu His Leu Ala Val Tyr
            420                 425                 430

Lys Ile Gln Met Cys Lys Ser Val Lys Ala Pro Gly Ile Cys Ile Asp
        435                 440                 445

Val Asp Asn Glu Asp Leu Phe Phe Ile Ala Asp Lys Asn Ser Phe Ser
    450                 455                 460

Asp Asp Leu Ser Lys Asn Glu Arg Ile Glu Tyr Asn Thr Gln Ser Asn
465                 470                 475                 480

Tyr Ile Glu Asn Asp Phe Pro Ile Asn Glu Leu Ile Leu Asp Thr Asp
                485                 490                 495

Leu Ile Ser Lys Ile Glu Leu Pro Ser Glu Asn Thr Glu Ser Leu Thr
            500                 505                 510

Asp Phe Asn Val Asp Val Pro Val Tyr Glu Lys Gln Pro Ala Ile Lys
        515                 520                 525

Lys Ile Phe Thr Asp Glu Asn Thr Ile Phe Gln Tyr Leu Tyr Ser Gln
    530                 535                 540

Thr Phe Pro Leu Asp Ile Arg Asp Ile Ser Leu Thr Ser Ser Phe Asp
545                 550                 555                 560

Asp Ala Leu Leu Phe Ser Asn Lys Val Tyr Ser Phe Phe Ser Met Asp
                565                 570                 575

Tyr Ile Lys Thr Ala Asn Lys Val Val Glu Ala Gly Leu Phe Ala Gly
            580                 585                 590

Trp Val Lys Gln Ile Val Asn Asp Phe Val Ile Glu Ala Asn Lys Ser
        595                 600                 605

Asn Thr Met Asp Lys Ile Ala Asp Ile Ser Leu Ile Val Pro Tyr Ile
    610                 615                 620

Gly Leu Ala Leu Asn Val Gly Asn Glu Thr Ala Lys Gly Asn Phe Glu
625                 630                 635                 640

Asn Ala Phe Glu Ile Ala Gly Ala Ser Ile Leu Leu Glu Phe Ile Pro
                645                 650                 655

Glu Leu Leu Ile Pro Val Val Gly Ala Phe Leu Leu Glu Ser Tyr Ile
            660                 665                 670

Asp Asn Lys Asn Lys Ile Ile Lys Thr Ile Asp Asn Ala Leu Thr Lys
        675                 680                 685

Arg Asn Glu Lys Trp Ser Asp Met Tyr Gly Leu Ile Val Ala Gln Trp
    690                 695                 700

Leu Ser Thr Val Asn Thr Gln Phe Tyr Thr Ile Lys Glu Gly Met Tyr
705                 710                 715                 720

Lys Ala Leu Asn Tyr Gln Ala Gln Ala Leu Glu Glu Ile Ile Lys Tyr
                725                 730                 735

Arg Tyr Asn Ile Tyr Ser Glu Lys Glu Lys Ser Asn Ile Asn Ile Asp
            740                 745                 750

Phe Asn Asp Ile Asn Ser Lys Leu Asn Glu Gly Ile Asn Gln Ala Ile
        755                 760                 765

Asp Asn Ile Asn Asn Phe Ile Asn Gly Cys Ser Val Ser Tyr Leu Met
    770                 775                 780

Lys Lys Met Ile Pro Leu Ala Val Glu Lys Leu Leu Asp Phe Asp Asn
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785                 790                 795                 800

Thr Leu Lys Lys Asn Leu Leu Asn Tyr Ile Asp Glu Asn Lys Leu Tyr
                805                 810                 815

Leu Ile Gly Ser Ala Glu Tyr Glu Lys Ser Lys Val Asn Lys Tyr Leu
            820                 825                 830

Lys Thr Ile Met Pro Phe Asp Leu Ser Ile Tyr Thr Asn Asp Thr Ile
        835                 840                 845

Leu Ile Glu Met Phe Asn Lys Tyr Asn Ser Glu Ile Leu Asn Asn Ile
    850                 855                 860

Ile Leu Asn Leu Arg Tyr Lys Asp Asn Asn Leu Ile Asp Leu Ser Gly
865                 870                 875                 880

Tyr Gly Ala Lys Val Glu Val Tyr Asp Gly Val Glu Leu Asn Asp Lys
                885                 890                 895

Asn Gln Phe Lys Leu Thr Ser Ser Ala Asn Ser Lys Ile Arg Val Thr
            900                 905                 910

Gln Asn Gln Asn Ile Ile Phe Asn Ser Val Phe Leu Asp Phe Ser Val
        915                 920                 925

Ser Phe Trp Ile Arg Ile Pro Lys Tyr Lys Asn Asp Gly Ile Gln Asn
    930                 935                 940

Tyr Ile His Asn Glu Tyr Thr Ile Ile Asn Cys Met Lys Asn Asn Ser
945                 950                 955                 960

Gly Trp Lys Ile Ser Ile Arg Gly Asn Arg Ile Ile Trp Thr Leu Ile
                965                 970                 975

Asp Ile Asn Gly Lys Thr Lys Ser Val Phe Phe Glu Tyr Asn Ile Arg
            980                 985                 990

Glu Asp Ile Ser Glu Tyr Ile Asn  Arg Trp Phe Phe Val  Thr Ile Thr
        995                 1000                1005

Asn Asn  Leu Asn Asn Ala Lys  Ile Tyr Ile Asn Gly  Lys Leu Glu
    1010                1015                1020

Ser Asn  Thr Asp Ile Lys Asp  Ile Arg Glu Val Ile  Ala Asn Gly
    1025                1030                1035

Glu Ile  Ile Phe Lys Leu Asp  Gly Asp Ile Asp Arg  Thr Gln Phe
    1040                1045                1050

Ile Trp  Met Lys Tyr Phe Ser  Ile Phe Asn Thr Glu  Leu Ser Gln
    1055                1060                1065

Ser Asn  Ile Glu Glu Arg Tyr  Lys Ile Gln Ser Tyr  Ser Glu Tyr
    1070                1075                1080

Leu Lys  Asp Phe Trp Gly Asn  Pro Leu Met Tyr Asn  Lys Glu Tyr
    1085                1090                1095

Tyr Met  Phe Asn Ala Gly Asn  Lys Asn Ser Tyr Ile  Lys Leu Lys
    1100                1105                1110

Lys Asp  Ser Pro Val Gly Glu  Ile Leu Thr Arg Ser  Lys Tyr Asn
    1115                1120                1125

Gln Asn  Ser Lys Tyr Ile Asn  Tyr Arg Asp Leu Tyr  Ile Gly Glu
    1130                1135                1140

Lys Phe  Ile Ile Arg Arg Lys  Ser Asn Ser Gln Ser  Ile Asn Asp
    1145                1150                1155

Asp Ile  Val Arg Lys Glu Asp  Tyr Ile Tyr Leu Asp  Phe Phe Asn
    1160                1165                1170

Leu Asn  Gln Glu Trp Arg Val  Tyr Thr Tyr Lys Tyr  Phe Lys Lys
    1175                1180                1185
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Glu Glu  Glu Lys Leu Phe Leu  Ala Pro Ile Ser Asp  Ser Asp Glu
    1190                1195                1200

Phe Tyr  Asn Thr Ile Gln Ile  Lys Glu Tyr Asp Glu  Gln Pro Thr
    1205                1210                1215

Tyr Ser  Cys Gln Leu Leu Phe  Lys Lys Asp Glu Glu  Ser Thr Asp
    1220                1225                1230

Glu Ile  Gly Leu Ile Gly Ile  His Arg Phe Tyr Glu  Ser Gly Ile
    1235                1240                1245

Val Phe  Glu Glu Tyr Lys Asp  Tyr Phe Cys Ile Ser  Lys Trp Tyr
    1250                1255                1260

Leu Lys  Glu Val Lys Arg Lys  Pro Tyr Asn Leu Lys  Leu Gly Cys
    1265                1270                1275

Asn Trp  Gln Phe Ile Pro Lys  Asp Glu Gly Trp Thr  Glu
    1280                1285                1290


<210> SEQ ID NO 3
<211> LENGTH: 1291
<212> TYPE: PRT
<213> ORGANISM: Clostridium botulinum

<400> SEQUENCE: 3

Met Pro Ile Thr Ile Asn Asn Phe Asn Tyr Ser Asp Pro Val Asp Asn
1               5                   10                  15

Lys Asn Ile Leu Tyr Leu Asp Thr His Leu Asn Thr Leu Ala Asn Glu
            20                  25                  30

Pro Glu Lys Ala Phe Arg Ile Thr Gly Asn Ile Trp Val Ile Pro Asp
        35                  40                  45

Arg Phe Ser Arg Asn Ser Asn Pro Asn Leu Asn Lys Pro Pro Arg Val
    50                  55                  60

Thr Ser Pro Lys Ser Gly Tyr Tyr Asp Pro Asn Tyr Leu Ser Thr Asp
65                  70                  75                  80

Ser Asp Lys Asp Pro Phe Leu Lys Glu Ile Ile Lys Leu Phe Lys Arg
                85                  90                  95

Ile Asn Ser Arg Glu Ile Gly Glu Glu Leu Ile Tyr Arg Leu Ser Thr
            100                 105                 110

Asp Ile Pro Phe Pro Gly Asn Asn Asn Thr Pro Ile Asn Thr Phe Asp
        115                 120                 125

Phe Asp Val Asp Phe Asn Ser Val Asp Val Lys Thr Arg Gln Gly Asn
    130                 135                 140

Asn Trp Val Lys Thr Gly Ser Ile Asn Pro Ser Val Ile Ile Thr Gly
145                 150                 155                 160

Pro Arg Glu Asn Ile Ile Asp Pro Glu Thr Ser Thr Phe Lys Leu Thr
                165                 170                 175

Asn Asn Thr Phe Ala Ala Gln Glu Gly Phe Gly Ala Leu Ser Ile Ile
            180                 185                 190

Ser Ile Ser Pro Arg Phe Met Leu Thr Tyr Ser Asn Ala Thr Asn Asp
        195                 200                 205

Val Gly Glu Gly Arg Phe Ser Lys Ser Glu Phe Cys Met Asp Pro Ile
    210                 215                 220

Leu Ile Leu Met His Glu Leu Asn His Ala Met His Asn Leu Tyr Gly
225                 230                 235                 240

Ile Ala Ile Pro Asn Asp Gln Thr Ile Ser Ser Val Thr Ser Asn Ile
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               245                 250                 255

Phe Tyr Ser Gln Tyr Asn Val Lys Leu Glu Tyr Ala Glu Ile Tyr Ala
            260                 265                 270

Phe Gly Gly Pro Thr Ile Asp Leu Ile Pro Lys Ser Ala Arg Lys Tyr
        275                 280                 285

Phe Glu Glu Lys Ala Leu Asp Tyr Tyr Arg Ser Ile Ala Lys Arg Leu
    290                 295                 300

Asn Ser Ile Thr Thr Ala Asn Pro Ser Ser Phe Asn Lys Tyr Ile Gly
305                 310                 315                 320

Glu Tyr Lys Gln Lys Leu Ile Arg Lys Tyr Arg Phe Val Val Glu Ser
                325                 330                 335

Ser Gly Glu Val Thr Val Asn Arg Asn Lys Phe Val Glu Leu Tyr Asn
            340                 345                 350

Glu Leu Thr Gln Ile Phe Thr Glu Phe Asn Tyr Ala Lys Ile Tyr Asn
        355                 360                 365

Val Gln Asn Arg Lys Ile Tyr Leu Ser Asn Val Tyr Thr Pro Val Thr
    370                 375                 380

Ala Asn Ile Leu Asp Asp Asn Val Tyr Asp Ile Gln Asn Gly Phe Asn
385                 390                 395                 400

Ile Pro Lys Ser Asn Leu Asn Val Leu Phe Met Gly Gln Asn Leu Ser
                405                 410                 415

Arg Asn Pro Ala Leu Arg Lys Val Asn Pro Glu Asn Met Leu Tyr Leu
            420                 425                 430

Phe Thr Lys Phe Cys His Lys Ala Ile Asp Gly Arg Ser Leu Tyr Asn
        435                 440                 445

Lys Thr Leu Asp Cys Arg Glu Leu Leu Val Lys Asn Thr Asp Leu Pro
    450                 455                 460

Phe Ile Gly Asp Ile Ser Asp Val Lys Thr Asp Ile Phe Leu Arg Lys
465                 470                 475                 480

Asp Ile Asn Glu Glu Thr Glu Val Ile Tyr Tyr Pro Asp Asn Val Ser
                485                 490                 495

Val Asp Gln Val Ile Leu Ser Lys Asn Thr Ser Glu His Gly Gln Leu
            500                 505                 510

Asp Leu Leu Tyr Pro Ser Ile Asp Ser Glu Ser Glu Ile Leu Pro Gly
        515                 520                 525

Glu Asn Gln Val Phe Tyr Asp Asn Arg Thr Gln Asn Val Asp Tyr Leu
    530                 535                 540

Asn Ser Tyr Tyr Tyr Leu Glu Ser Gln Lys Leu Ser Asp Asn Val Glu
545                 550                 555                 560

Asp Phe Thr Phe Thr Arg Ser Ile Glu Glu Ala Leu Asp Asn Ser Ala
                565                 570                 575

Lys Val Tyr Thr Tyr Phe Pro Thr Leu Ala Asn Lys Val Asn Ala Gly
            580                 585                 590

Val Gln Gly Gly Leu Phe Leu Met Trp Ala Asn Asp Val Val Glu Asp
        595                 600                 605

Phe Thr Thr Asn Ile Leu Arg Lys Asp Thr Leu Asp Lys Ile Ser Asp
    610                 615                 620

Val Ser Ala Ile Ile Pro Tyr Ile Gly Pro Ala Leu Asn Ile Ser Asn
625                 630                 635                 640

Ser Val Arg Arg Gly Asn Phe Thr Glu Ala Phe Ala Val Thr Gly Val
                645                 650                 655
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Thr Ile Leu Leu Glu Ala Phe Pro Glu Phe Thr Ile Pro Ala Leu Gly
            660                 665                 670

Ala Phe Val Ile Tyr Ser Lys Val Gln Glu Arg Asn Glu Ile Ile Lys
        675                 680                 685

Thr Ile Asp Asn Cys Leu Glu Gln Arg Ile Lys Arg Trp Lys Asp Ser
    690                 695                 700

Tyr Glu Trp Met Met Gly Thr Trp Leu Ser Arg Ile Ile Thr Gln Phe
705                 710                 715                 720

Asn Asn Ile Ser Tyr Gln Met Tyr Asp Ser Leu Asn Tyr Gln Ala Gly
                725                 730                 735

Ala Ile Lys Ala Lys Ile Asp Leu Glu Tyr Lys Lys Tyr Ser Gly Ser
            740                 745                 750

Asp Lys Glu Asn Ile Lys Ser Gln Val Glu Asn Leu Lys Asn Ser Leu
        755                 760                 765

Asp Val Lys Ile Ser Glu Ala Met Asn Asn Ile Asn Lys Phe Ile Arg
    770                 775                 780

Glu Cys Ser Val Thr Tyr Leu Phe Lys Asn Met Leu Pro Lys Val Ile
785                 790                 795                 800

Asp Glu Leu Asn Glu Phe Asp Arg Asn Thr Lys Ala Lys Leu Ile Asn
                805                 810                 815

Leu Ile Asp Ser His Asn Ile Ile Leu Val Gly Glu Val Asp Lys Leu
            820                 825                 830

Lys Ala Lys Val Asn Asn Ser Phe Gln Asn Thr Ile Pro Phe Asn Ile
        835                 840                 845

Phe Ser Tyr Thr Asn Asn Ser Leu Leu Lys Asp Ile Ile Asn Glu Tyr
    850                 855                 860

Phe Asn Asn Ile Asn Asp Ser Lys Ile Leu Ser Leu Gln Asn Arg Lys
865                 870                 875                 880

Asn Thr Leu Val Asp Thr Ser Gly Tyr Asn Ala Glu Val Ser Glu Glu
                885                 890                 895

Gly Asp Val Gln Leu Asn Pro Ile Phe Pro Phe Asp Phe Lys Leu Gly
            900                 905                 910

Ser Ser Gly Glu Asp Arg Gly Lys Val Ile Val Thr Gln Asn Glu Asn
        915                 920                 925

Ile Val Tyr Asn Ser Met Tyr Glu Ser Phe Ser Ile Ser Phe Trp Ile
    930                 935                 940

Arg Ile Asn Lys Trp Val Ser Asn Leu Pro Gly Tyr Thr Ile Ile Asp
945                 950                 955                 960

Ser Val Lys Asn Asn Ser Gly Trp Ser Ile Gly Ile Ile Ser Asn Phe
                965                 970                 975

Leu Val Phe Thr Leu Lys Gln Asn Glu Asp Ser Glu Gln Ser Ile Asn
            980                 985                 990

Phe Ser Tyr Asp Ile Ser Asn Asn  Ala Pro Gly Tyr Asn  Lys Trp Phe
        995                 1000                1005

Phe Val  Thr Val Thr Asn Asn  Met Met Gly Asn Met  Lys Ile Tyr
    1010                1015                1020

Ile Asn  Gly Lys Leu Ile Asp  Thr Ile Lys Val Lys  Glu Leu Thr
    1025                1030                1035

Gly Ile  Asn Phe Ser Lys Thr  Ile Thr Phe Glu Ile  Asn Lys Ile
    1040                1045                1050
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Pro Asp  Thr Gly Leu Ile Thr  Ser Asp Ser Asp Asn  Ile Asn Met
    1055                 1060                 1065

Trp Ile  Arg Asp Phe Tyr Ile  Phe Ala Lys Glu Leu  Asp Gly Lys
    1070                 1075                 1080

Asp Ile  Asn Ile Leu Phe Asn  Ser Leu Gln Tyr Thr  Asn Val Val
    1085                 1090                 1095

Lys Asp  Tyr Trp Gly Asn Asp  Leu Arg Tyr Asn Lys  Glu Tyr Tyr
    1100                 1105                 1110

Met Val  Asn Ile Asp Tyr Leu  Asn Arg Tyr Met Tyr  Ala Asn Ser
    1115                 1120                 1125

Arg Gln  Ile Val Phe Asn Thr  Arg Arg Asn Asn Asn  Asp Phe Asn
    1130                 1135                 1140

Glu Gly  Tyr Lys Ile Ile Ile  Lys Arg Ile Arg Gly  Asn Thr Asn
    1145                 1150                 1155

Asp Thr  Arg Val Arg Gly Gly  Asp Ile Leu Tyr Phe  Asp Met Thr
    1160                 1165                 1170

Ile Asn  Asn Lys Ala Tyr Asn  Leu Phe Met Lys Asn  Glu Thr Met
    1175                 1180                 1185

Tyr Ala  Asp Asn His Ser Thr  Glu Asp Ile Tyr Ala  Ile Gly Leu
    1190                 1195                 1200

Arg Glu  Gln Thr Lys Asp Ile  Asn Asp Asn Ile Ile  Phe Gln Ile
    1205                 1210                 1215

Gln Pro  Met Asn Asn Thr Tyr  Tyr Tyr Ala Ser Gln  Ile Phe Lys
    1220                 1225                 1230

Ser Asn  Phe Asn Gly Glu Asn  Ile Ser Gly Ile Cys  Ser Ile Gly
    1235                 1240                 1245

Thr Tyr  Arg Phe Arg Leu Gly  Gly Asp Trp Tyr Arg  His Asn Tyr
    1250                 1255                 1260

Leu Val  Pro Thr Val Lys Gln  Gly Asn Tyr Ala Ser  Leu Leu Glu
    1265                 1270                 1275

Ser Thr  Ser Thr His Trp Gly  Phe Val Pro Val Ser  Glu
    1280                 1285                 1290


<210> SEQ ID NO 4
<211> LENGTH: 1276
<212> TYPE: PRT
<213> ORGANISM: Clostridium botulinum

<400> SEQUENCE: 4

Met Thr Trp Pro Val Lys Asp Phe Asn Tyr Ser Asp Pro Val Asn Asp
1               5                   10                  15

Asn Asp Ile Leu Tyr Leu Arg Ile Pro Gln Asn Lys Leu Ile Thr Thr
            20                  25                  30

Pro Val Lys Ala Phe Met Ile Thr Gln Asn Ile Trp Val Ile Pro Glu
        35                  40                  45

Arg Phe Ser Ser Asp Thr Asn Pro Ser Leu Ser Lys Pro Pro Arg Pro
    50                  55                  60

Thr Ser Lys Tyr Gln Ser Tyr Tyr Asp Pro Ser Tyr Leu Ser Thr Asp
65                  70                  75                  80

Glu Gln Lys Asp Thr Phe Leu Lys Gly Ile Ile Lys Leu Phe Lys Arg
                85                  90                  95

Ile Asn Glu Arg Asp Ile Gly Lys Lys Leu Ile Asn Tyr Leu Val Val
            100                 105                 110
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Gly Ser Pro Phe Met Gly Asp Ser Ser Thr Pro Glu Asp Thr Phe Asp
        115                 120                 125

Phe Thr Arg His Thr Thr Asn Ile Ala Val Glu Lys Phe Glu Asn Gly
    130                 135                 140

Ser Trp Lys Val Thr Asn Ile Ile Thr Pro Ser Val Leu Ile Phe Gly
145                 150                 155                 160

Pro Leu Pro Asn Ile Leu Asp Tyr Thr Ala Ser Leu Thr Leu Gln Gly
                165                 170                 175

Gln Gln Ser Asn Pro Ser Phe Glu Gly Phe Gly Thr Leu Ser Ile Leu
            180                 185                 190

Lys Val Ala Pro Glu Phe Leu Leu Thr Phe Ser Asp Val Thr Ser Asn
        195                 200                 205

Gln Ser Ser Ala Val Leu Gly Lys Ser Ile Phe Cys Met Asp Pro Val
    210                 215                 220

Ile Ala Leu Met His Glu Leu Thr His Ser Leu His Gln Leu Tyr Gly
225                 230                 235                 240

Ile Asn Ile Pro Ser Asp Lys Arg Ile Arg Pro Gln Val Ser Glu Gly
                245                 250                 255

Phe Phe Ser Gln Asp Gly Pro Asn Val Gln Phe Glu Glu Leu Tyr Thr
            260                 265                 270

Phe Gly Gly Leu Asp Val Glu Ile Ile Pro Gln Ile Glu Arg Ser Gln
        275                 280                 285

Leu Arg Glu Lys Ala Leu Gly His Tyr Lys Asp Ile Ala Lys Arg Leu
    290                 295                 300

Asn Asn Ile Asn Lys Thr Ile Pro Ser Ser Trp Ile Ser Asn Ile Asp
305                 310                 315                 320

Lys Tyr Lys Lys Ile Phe Ser Glu Lys Tyr Asn Phe Asp Lys Asp Asn
                325                 330                 335

Thr Gly Asn Phe Val Val Asn Ile Asp Lys Phe Asn Ser Leu Tyr Ser
            340                 345                 350

Asp Leu Thr Asn Val Met Ser Glu Val Val Tyr Ser Ser Gln Tyr Asn
        355                 360                 365

Val Lys Asn Arg Thr His Tyr Phe Ser Arg His Tyr Leu Pro Val Phe
    370                 375                 380

Ala Asn Ile Leu Asp Asp Asn Ile Tyr Thr Ile Arg Asp Gly Phe Asn
385                 390                 395                 400

Leu Thr Asn Lys Gly Phe Asn Ile Glu Asn Ser Gly Gln Asn Ile Glu
                405                 410                 415

Arg Asn Pro Ala Leu Gln Lys Leu Ser Ser Glu Ser Val Val Asp Leu
            420                 425                 430

Phe Thr Lys Val Cys Leu Arg Leu Thr Lys Asn Ser Arg Asp Asp Ser
        435                 440                 445

Thr Cys Ile Lys Val Lys Asn Asn Arg Leu Pro Tyr Val Ala Asp Lys
    450                 455                 460

Asp Ser Ile Ser Gln Glu Ile Phe Glu Asn Lys Ile Ile Thr Asp Glu
465                 470                 475                 480

Thr Asn Val Gln Asn Tyr Ser Asp Lys Phe Ser Leu Asp Glu Ser Ile
                485                 490                 495

Leu Asp Gly Gln Val Pro Ile Asn Pro Glu Ile Val Asp Pro Leu Leu
            500                 505                 510
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Pro Asn Val Asn Met Glu Pro Leu Asn Leu Pro Gly Glu Glu Ile Val
        515                 520                 525

Phe Tyr Asp Asp Ile Thr Lys Tyr Val Asp Tyr Leu Asn Ser Tyr Tyr
    530                 535                 540

Tyr Leu Glu Ser Gln Lys Leu Ser Asn Asn Val Glu Asn Ile Thr Leu
545                 550                 555                 560

Thr Thr Ser Val Glu Glu Ala Leu Gly Tyr Ser Asn Lys Ile Tyr Thr
                565                 570                 575

Phe Leu Pro Ser Leu Ala Glu Lys Val Asn Lys Gly Val Gln Ala Gly
            580                 585                 590

Leu Phe Leu Asn Trp Ala Asn Glu Val Val Glu Asp Phe Thr Thr Asn
        595                 600                 605

Ile Met Lys Lys Asp Thr Leu Asp Lys Ile Ser Asp Val Ser Val Ile
    610                 615                 620

Ile Pro Tyr Ile Gly Pro Ala Leu Asn Ile Gly Asn Ser Ala Leu Arg
625                 630                 635                 640

Gly Asn Phe Asn Gln Ala Phe Ala Thr Ala Gly Val Ala Phe Leu Leu
                645                 650                 655

Glu Gly Phe Pro Glu Phe Thr Ile Pro Ala Leu Gly Val Phe Thr Phe
            660                 665                 670

Tyr Ser Ser Ile Gln Glu Arg Glu Lys Ile Ile Lys Thr Ile Glu Asn
        675                 680                 685

Cys Leu Glu Gln Arg Val Lys Arg Trp Lys Asp Ser Tyr Gln Trp Met
    690                 695                 700

Val Ser Asn Trp Leu Ser Arg Ile Thr Thr Gln Phe Asn His Ile Asn
705                 710                 715                 720

Tyr Gln Met Tyr Asp Ser Leu Ser Tyr Gln Ala Asp Ala Ile Lys Ala
                725                 730                 735

Lys Ile Asp Leu Glu Tyr Lys Lys Tyr Ser Gly Ser Asp Lys Glu Asn
            740                 745                 750

Ile Lys Ser Gln Val Glu Asn Leu Lys Asn Ser Leu Asp Val Lys Ile
        755                 760                 765

Ser Glu Ala Met Asn Asn Ile Asn Lys Phe Ile Arg Glu Cys Ser Val
    770                 775                 780

Thr Tyr Leu Phe Lys Asn Met Leu Pro Lys Val Ile Asp Glu Leu Asn
785                 790                 795                 800

Lys Phe Asp Leu Arg Thr Lys Thr Glu Leu Ile Asn Leu Ile Asp Ser
                805                 810                 815

His Asn Ile Ile Leu Val Gly Glu Val Asp Arg Leu Lys Ala Lys Val
            820                 825                 830

Asn Glu Ser Phe Glu Asn Thr Met Pro Phe Asn Ile Phe Ser Tyr Thr
        835                 840                 845

Asn Asn Ser Leu Leu Lys Asp Ile Ile Asn Glu Tyr Phe Asn Ser Ile
    850                 855                 860

Asn Asp Ser Lys Ile Leu Ser Leu Gln Asn Lys Lys Asn Ala Leu Val
865                 870                 875                 880

Asp Thr Ser Gly Tyr Asn Ala Glu Val Arg Val Gly Asp Asn Val Gln
                885                 890                 895

Leu Asn Thr Ile Tyr Thr Asn Asp Phe Lys Leu Ser Ser Ser Gly Asp
            900                 905                 910

Lys Ile Ile Val Asn Leu Asn Asn Asn Ile Leu Tyr Ser Ala Ile Tyr
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        915                 920                 925

Glu Asn Ser Ser Val Ser Phe Trp Ile Lys Ile Ser Lys Asp Leu Thr
    930                 935                 940

Asn Ser His Asn Glu Tyr Thr Ile Ile Asn Ser Ile Glu Gln Asn Ser
945                 950                 955                 960

Gly Trp Lys Leu Cys Ile Arg Asn Gly Asn Ile Glu Trp Ile Leu Gln
                965                 970                 975

Asp Val Asn Arg Lys Tyr Lys Ser Leu Ile Phe Asp Tyr Ser Glu Ser
            980                 985                 990

Leu Ser His Thr Gly Tyr Thr Asn  Lys Trp Phe Phe Val  Thr Ile Thr
        995                 1000                1005

Asn Asn  Ile Met Gly Tyr Met  Lys Leu Tyr Ile Asn  Gly Glu Leu
    1010                1015                1020

Lys Gln  Ser Gln Lys Ile Glu  Asp Leu Asp Glu Val  Lys Leu Asp
    1025                1030                1035

Lys Thr  Ile Val Phe Gly Ile  Asp Glu Asn Ile Asp  Glu Asn Gln
    1040                1045                1050

Met Leu  Trp Ile Arg Asp Phe  Asn Ile Phe Ser Lys  Glu Leu Ser
    1055                1060                1065

Asn Glu  Asp Ile Asn Ile Val  Tyr Glu Gly Gln Ile  Leu Arg Asn
    1070                1075                1080

Val Ile  Lys Asp Tyr Trp Gly  Asn Pro Leu Lys Phe  Asp Thr Glu
    1085                1090                1095

Tyr Tyr  Ile Ile Asn Asp Asn  Tyr Ile Asp Arg Tyr  Ile Ala Pro
    1100                1105                1110

Glu Ser  Asn Val Leu Val Leu  Val Gln Tyr Pro Asp  Arg Ser Lys
    1115                1120                1125

Leu Tyr  Thr Gly Asn Pro Ile  Thr Ile Lys Ser Val  Ser Asp Lys
    1130                1135                1140

Asn Pro  Tyr Ser Arg Ile Leu  Asn Gly Asp Asn Ile  Ile Leu His
    1145                1150                1155

Met Leu  Tyr Asn Ser Arg Lys  Tyr Met Ile Ile Arg  Asp Thr Asp
    1160                1165                1170

Thr Ile  Tyr Ala Thr Gln Gly  Gly Glu Cys Ser Gln  Asn Cys Val
    1175                1180                1185

Tyr Ala  Leu Lys Leu Gln Ser  Asn Leu Gly Asn Tyr  Gly Ile Gly
    1190                1195                1200

Ile Phe  Ser Ile Lys Asn Ile  Val Ser Lys Asn Lys  Tyr Cys Ser
    1205                1210                1215

Gln Ile  Phe Ser Ser Phe Arg  Glu Asn Thr Met Leu  Leu Ala Asp
    1220                1225                1230

Ile Tyr  Lys Pro Trp Arg Phe  Ser Phe Lys Asn Ala  Tyr Thr Pro
    1235                1240                1245

Val Ala  Val Thr Asn Tyr Glu  Thr Lys Leu Leu Ser  Thr Ser Ser
    1250                1255                1260

Phe Trp  Lys Phe Ile Ser Arg  Asp Pro Gly Trp Val  Glu
    1265                1270                1275


<210> SEQ ID NO 5
<211> LENGTH: 1252
<212> TYPE: PRT
<213> ORGANISM: Clostridium botulinum
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<400> SEQUENCE: 5

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Met Pro Lys Ile Asn Ser Phe Asn Tyr Asn Asp Pro Val Asn Asp Arg
1               5                   10                  15

Thr Ile Leu Tyr Ile Lys Pro Gly Gly Cys Gln Glu Phe Tyr Lys Ser
            20                  25                  30

Phe Asn Ile Met Lys Asn Ile Trp Ile Ile Pro Glu Arg Asn Val Ile
        35                  40                  45

Gly Thr Thr Pro Gln Asp Phe His Pro Pro Thr Ser Leu Lys Asn Gly
    50                  55                  60

Asp Ser Ser Tyr Tyr Asp Pro Asn Tyr Leu Gln Ser Asp Glu Glu Lys
65                  70                  75                  80

Asp Arg Phe Leu Lys Ile Val Thr Lys Ile Phe Asn Arg Ile Asn Asn
                85                  90                  95

Asn Leu Ser Gly Gly Ile Leu Leu Glu Glu Leu Ser Lys Ala Asn Pro
            100                 105                 110

Tyr Leu Gly Asn Asp Asn Thr Pro Asp Asn Gln Phe His Ile Gly Asp
        115                 120                 125

Ala Ser Ala Val Glu Ile Lys Phe Ser Asn Gly Ser Gln Asp Ile Leu
    130                 135                 140

Leu Pro Asn Val Ile Ile Met Gly Ala Glu Pro Asp Leu Phe Glu Thr
145                 150                 155                 160

Asn Ser Ser Asn Ile Ser Leu Arg Asn Asn Tyr Met Pro Ser Asn His
                165                 170                 175

Gly Phe Gly Ser Ile Ala Ile Val Thr Phe Ser Pro Glu Tyr Ser Phe
            180                 185                 190

Arg Phe Asn Asp Asn Ser Met Asn Glu Phe Ile Gln Asp Pro Ala Leu
        195                 200                 205

Thr Leu Met His Glu Leu Ile His Ser Leu His Gly Leu Tyr Gly Ala
    210                 215                 220

Lys Gly Ile Thr Thr Lys Tyr Thr Ile Thr Gln Lys Gln Asn Pro Leu
225                 230                 235                 240

Ile Thr Asn Ile Arg Gly Thr Asn Ile Glu Glu Phe Leu Thr Phe Gly
                245                 250                 255

Gly Thr Asp Leu Asn Ile Ile Thr Ser Ala Gln Ser Asn Asp Ile Tyr
            260                 265                 270

Thr Asn Leu Leu Ala Asp Tyr Lys Lys Ile Ala Ser Lys Leu Ser Lys
        275                 280                 285

Val Gln Val Ser Asn Pro Leu Leu Asn Pro Tyr Lys Asp Val Phe Glu
    290                 295                 300

Ala Lys Tyr Gly Leu Asp Lys Asp Ala Ser Gly Ile Tyr Ser Val Asn
305                 310                 315                 320

Ile Asn Lys Phe Asn Asp Ile Phe Lys Lys Leu Tyr Ser Phe Thr Glu
                325                 330                 335

Phe Asp Leu Ala Thr Lys Phe Gln Val Lys Cys Arg Gln Thr Tyr Ile
            340                 345                 350

Gly Gln Tyr Lys Tyr Phe Lys Leu Ser Asn Leu Leu Asn Asp Ser Ile
        355                 360                 365

Tyr Asn Ile Ser Glu Gly Tyr Asn Ile Asn Asn Leu Lys Val Asn Phe
    370                 375                 380

Arg Gly Gln Asn Ala Asn Leu Asn Pro Arg Ile Ile Thr Pro Ile Thr
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385                 390                 395                 400

Gly Arg Gly Leu Val Lys Lys Ile Ile Arg Phe Cys Lys Asn Ile Val
                405                 410                 415

Ser Val Lys Gly Ile Arg Lys Ser Ile Cys Ile Glu Ile Asn Asn Gly
            420                 425                 430

Glu Leu Phe Phe Val Ala Ser Glu Asn Ser Tyr Asn Asp Asp Asn Ile
        435                 440                 445

Asn Thr Pro Lys Glu Ile Asp Asp Thr Val Thr Ser Asn Asn Asn Tyr
    450                 455                 460

Glu Asn Asp Leu Asp Gln Val Ile Leu Asn Phe Asn Ser Glu Ser Ala
465                 470                 475                 480

Pro Gly Leu Ser Asp Glu Lys Leu Asn Leu Thr Ile Gln Asn Asp Ala
                485                 490                 495

Tyr Ile Pro Lys Tyr Asp Ser Asn Gly Thr Ser Asp Ile Glu Gln His
            500                 505                 510

Asp Val Asn Glu Leu Asn Val Phe Phe Tyr Leu Asp Ala Gln Lys Val
        515                 520                 525

Pro Glu Gly Glu Asn Asn Val Asn Leu Thr Ser Ser Ile Asp Thr Ala
    530                 535                 540

Leu Leu Glu Gln Pro Lys Ile Tyr Thr Phe Phe Ser Ser Glu Phe Ile
545                 550                 555                 560

Asn Asn Val Asn Lys Pro Val Gln Ala Ala Leu Phe Val Ser Trp Ile
                565                 570                 575

Gln Gln Val Leu Val Asp Phe Thr Thr Glu Ala Asn Gln Lys Ser Thr
            580                 585                 590

Val Asp Lys Ile Ala Asp Ile Ser Ile Val Val Pro Tyr Ile Gly Leu
        595                 600                 605

Ala Leu Asn Ile Gly Asn Glu Ala Gln Lys Gly Asn Phe Lys Asp Ala
    610                 615                 620

Leu Glu Leu Leu Gly Ala Gly Ile Leu Leu Glu Phe Glu Pro Glu Leu
625                 630                 635                 640

Leu Ile Pro Thr Ile Leu Val Phe Thr Ile Lys Ser Phe Leu Gly Ser
                645                 650                 655

Ser Asp Asn Lys Asn Lys Val Ile Lys Ala Ile Asn Asn Ala Leu Lys
            660                 665                 670

Glu Arg Asp Glu Lys Trp Lys Glu Val Tyr Ser Phe Ile Val Ser Asn
        675                 680                 685

Trp Met Thr Lys Ile Asn Thr Gln Phe Asn Lys Arg Lys Glu Gln Met
    690                 695                 700

Tyr Gln Ala Leu Gln Asn Gln Val Asn Ala Ile Lys Thr Ile Ile Glu
705                 710                 715                 720

Ser Lys Tyr Asn Ser Tyr Thr Leu Glu Glu Lys Asn Glu Leu Thr Asn
                725                 730                 735

Lys Tyr Asp Ile Lys Gln Ile Glu Asn Glu Leu Asn Gln Lys Val Ser
            740                 745                 750

Ile Ala Met Asn Asn Ile Asp Arg Phe Leu Thr Glu Ser Ser Ile Ser
        755                 760                 765

Tyr Leu Met Lys Leu Ile Asn Glu Val Lys Ile Asn Lys Leu Arg Glu
    770                 775                 780

Tyr Asp Glu Asn Val Lys Thr Tyr Leu Leu Asn Tyr Ile Ile Gln His
785                 790                 795                 800
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Gly Ser Ile Leu Gly Glu Ser Gln Gln Glu Leu Asn Ser Met Val Thr
               805                 810                 815

Asp Thr Leu Asn Asn Ser Ile Pro Phe Lys Leu Ser Ser Tyr Thr Asp
           820                 825                 830

Asp Lys Ile Leu Ile Ser Tyr Phe Asn Lys Phe Phe Lys Arg Ile Lys
       835                 840                 845

Ser Ser Ser Val Leu Asn Met Arg Tyr Lys Asn Asp Lys Tyr Val Asp
   850                 855                 860

Thr Ser Gly Tyr Asp Ser Asn Ile Asn Ile Asn Gly Asp Val Tyr Lys
865                 870                 875                 880

Tyr Pro Thr Asn Lys Asn Gln Phe Gly Ile Tyr Asn Asp Lys Leu Ser
               885                 890                 895

Glu Val Asn Ile Ser Gln Asn Asp Tyr Ile Ile Tyr Asp Asn Lys Tyr
           900                 905                 910

Lys Asn Phe Ser Ile Ser Phe Trp Val Arg Ile Pro Asn Tyr Asp Asn
       915                 920                 925

Lys Ile Val Asn Val Asn Asn Glu Tyr Thr Ile Ile Asn Cys Met Arg
   930                 935                 940

Asp Asn Asn Ser Gly Trp Lys Val Ser Leu Asn His Asn Glu Ile Ile
945                 950                 955                 960

Trp Thr Leu Gln Asp Asn Ala Gly Ile Asn Gln Lys Leu Ala Phe Asn
               965                 970                 975

Tyr Gly Asn Ala Asn Gly Ile Ser Asp Tyr Ile Asn Lys Trp Ile Phe
           980                 985                 990

Val Thr Ile Thr Asn Asp Arg Leu  Gly Asp Ser Lys Leu  Tyr Ile Asn
       995                 1000                1005

Gly Asn  Leu Ile Asp Gln Lys  Ser Ile Leu Asn Leu  Gly Asn Ile
   1010                1015                1020

His Val  Ser Asp Asn Ile Leu  Phe Lys Ile Val Asn  Cys Ser Tyr
   1025                1030                1035

Thr Arg  Tyr Ile Gly Ile Arg  Tyr Phe Asn Ile Phe  Asp Lys Glu
   1040                1045                1050

Leu Asp  Glu Thr Glu Ile Gln  Thr Leu Tyr Ser Asn  Glu Pro Asn
   1055                1060                1065

Thr Asn  Ile Leu Lys Asp Phe  Trp Gly Asn Tyr Leu  Leu Tyr Asp
   1070                1075                1080

Lys Glu  Tyr Tyr Leu Leu Asn  Val Leu Lys Pro Asn  Asn Phe Ile
   1085                1090                1095

Asp Arg  Arg Lys Asp Ser Thr  Leu Ser Ile Asn Asn  Ile Arg Ser
   1100                1105                1110

Thr Ile  Leu Leu Ala Asn Arg  Leu Tyr Ser Gly Ile  Lys Val Lys
   1115                1120                1125

Ile Gln  Arg Val Asn Asn Ser  Ser Thr Asn Asp Asn  Leu Val Arg
   1130                1135                1140

Lys Asn  Asp Gln Val Tyr Ile  Asn Phe Val Ala Ser  Lys Thr His
   1145                1150                1155

Leu Phe  Pro Leu Tyr Ala Asp  Thr Ala Thr Thr Asn  Lys Glu Lys
   1160                1165                1170

Thr Ile  Lys Ile Ser Ser Ser  Gly Asn Arg Phe Asn  Gln Val Val
   1175                1180                1185
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Val Met  Asn Ser Val Gly Asn  Asn Cys Thr Met Asn  Phe Lys Asn
    1190                 1195                 1200

Asn Asn  Gly Asn Asn Ile Gly  Leu Leu Gly Phe Lys  Ala Asp Thr
    1205                 1210                 1215

Val Val  Ala Ser Thr Trp Tyr  Tyr Thr His Met Arg  Asp His Thr
    1220                 1225                 1230

Asn Ser  Asn Gly Cys Phe Trp  Asn Phe Ile Ser Glu  Glu His Gly
    1235                 1240                 1245

Trp Gln  Glu Lys
    1250


<210> SEQ ID NO 6
<211> LENGTH: 1274
<212> TYPE: PRT
<213> ORGANISM: Clostridium botulinum

<400> SEQUENCE: 6

Met Pro Val Ala Ile Asn Ser Phe Asn Tyr Asn Asp Pro Val Asn Asp
1               5                   10                  15

Asp Thr Ile Leu Tyr Met Gln Ile Pro Tyr Glu Glu Lys Ser Lys Lys
            20                  25                  30

Tyr Tyr Lys Ala Phe Glu Ile Met Arg Asn Val Trp Ile Ile Pro Glu
        35                  40                  45

Arg Asn Thr Ile Gly Thr Asn Pro Ser Asp Phe Asp Pro Pro Ala Ser
    50                  55                  60

Leu Lys Asn Gly Ser Ser Ala Tyr Tyr Asp Pro Asn Tyr Leu Thr Thr
65                  70                  75                  80

Asp Ala Glu Lys Asp Arg Tyr Leu Lys Thr Thr Ile Lys Leu Phe Lys
                85                  90                  95

Arg Ile Asn Ser Asn Pro Ala Gly Lys Val Leu Leu Gln Glu Ile Ser
            100                 105                 110

Tyr Ala Lys Pro Tyr Leu Gly Asn Asp His Thr Pro Ile Asp Glu Phe
        115                 120                 125

Ser Pro Val Thr Arg Thr Thr Ser Val Asn Ile Lys Leu Ser Thr Asn
    130                 135                 140

Val Glu Ser Ser Met Leu Leu Asn Leu Leu Val Leu Gly Ala Gly Pro
145                 150                 155                 160

Asp Ile Phe Glu Ser Cys Cys Tyr Pro Val Arg Lys Leu Ile Asp Pro
                165                 170                 175

Asp Val Val Tyr Asp Pro Ser Asn Tyr Gly Phe Gly Ser Ile Asn Ile
            180                 185                 190

Val Thr Phe Ser Pro Glu Tyr Glu Tyr Thr Phe Asn Asp Ile Ser Gly
        195                 200                 205

Gly His Asn Ser Ser Thr Glu Ser Phe Ile Ala Asp Pro Ala Ile Ser
    210                 215                 220

Leu Ala His Glu Leu Ile His Ala Leu His Gly Leu Tyr Gly Ala Arg
225                 230                 235                 240

Gly Val Thr Tyr Glu Glu Thr Ile Glu Val Lys Gln Ala Pro Leu Met
                245                 250                 255

Ile Ala Glu Lys Pro Ile Arg Leu Glu Glu Phe Leu Thr Phe Gly Gly
            260                 265                 270

Gln Asp Leu Asn Ile Ile Thr Ser Ala Met Lys Glu Lys Ile Tyr Asn
        275                 280                 285
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Asn Leu Leu Ala Asn Tyr Glu Lys Ile Ala Thr Arg Leu Ser Glu Val
    290                 295                 300

Asn Ser Ala Pro Pro Glu Tyr Asp Ile Asn Glu Tyr Lys Asp Tyr Phe
305                 310                 315                 320

Gln Trp Lys Tyr Gly Leu Asp Lys Asn Ala Asp Gly Ser Tyr Thr Val
                325                 330                 335

Asn Glu Asn Lys Phe Asn Glu Ile Tyr Lys Lys Leu Tyr Ser Phe Thr
            340                 345                 350

Glu Ser Asp Leu Ala Asn Lys Phe Lys Val Lys Cys Arg Asn Thr Tyr
        355                 360                 365

Phe Ile Lys Tyr Glu Phe Leu Lys Val Pro Asn Leu Leu Asp Asp Asp
    370                 375                 380

Ile Tyr Thr Val Ser Glu Gly Phe Asn Ile Gly Asn Leu Ala Val Asn
385                 390                 395                 400

Asn Arg Gly Gln Ser Ile Lys Leu Asn Pro Lys Ile Ile Asp Ser Ile
                405                 410                 415

Pro Asp Lys Gly Leu Val Glu Lys Ile Val Lys Phe Cys Lys Ser Val
            420                 425                 430

Ile Pro Arg Lys Gly Thr Lys Ala Pro Pro Arg Leu Cys Ile Arg Val
        435                 440                 445

Asn Asn Ser Glu Leu Phe Phe Val Ala Ser Glu Ser Ser Tyr Asn Glu
    450                 455                 460

Asn Asp Ile Asn Thr Pro Lys Glu Ile Asp Asp Thr Thr Asn Leu Asn
465                 470                 475                 480

Asn Asn Tyr Arg Asn Asn Leu Asp Glu Val Ile Leu Asp Tyr Asn Ser
                485                 490                 495

Gln Thr Ile Pro Gln Ile Ser Asn Arg Thr Leu Asn Thr Leu Val Gln
            500                 505                 510

Asp Asn Ser Tyr Val Pro Arg Tyr Asp Ser Asn Gly Thr Ser Glu Ile
        515                 520                 525

Glu Glu Tyr Asp Val Val Asp Phe Asn Val Phe Phe Tyr Leu His Ala
    530                 535                 540

Gln Lys Val Pro Glu Gly Glu Thr Asn Ile Ser Leu Thr Ser Ser Ile
545                 550                 555                 560

Asp Thr Ala Leu Leu Glu Glu Ser Lys Asp Ile Phe Phe Ser Ser Glu
                565                 570                 575

Phe Ile Asp Thr Ile Asn Lys Pro Val Asn Ala Ala Leu Phe Ile Asp
            580                 585                 590

Trp Ile Ser Lys Val Ile Arg Asp Phe Thr Thr Glu Ala Thr Gln Lys
        595                 600                 605

Ser Thr Val Asp Lys Ile Ala Asp Ile Ser Leu Ile Val Pro Tyr Val
    610                 615                 620

Gly Leu Ala Leu Asn Ile Ile Ile Glu Ala Glu Lys Gly Asn Phe Glu
625                 630                 635                 640

Glu Ala Phe Glu Leu Leu Gly Val Gly Ile Leu Leu Glu Phe Val Pro
                645                 650                 655

Glu Leu Thr Ile Pro Val Ile Leu Val Phe Thr Ile Lys Ser Tyr Ile
            660                 665                 670

Asp Ser Tyr Glu Asn Lys Asn Lys Ala Ile Lys Ala Ile Asn Asn Ser
        675                 680                 685
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Leu Ile Glu Arg Glu Ala Lys Trp Lys Glu Ile Tyr Ser Trp Ile Val
    690                 695                 700

Ser Asn Trp Leu Thr Arg Ile Asn Thr Gln Phe Asn Lys Arg Lys Glu
705                 710                 715                 720

Gln Met Tyr Gln Ala Leu Gln Asn Gln Val Asp Ala Ile Lys Thr Ala
                725                 730                 735

Ile Glu Tyr Lys Tyr Asn Asn Tyr Thr Ser Asp Glu Lys Asn Arg Leu
            740                 745                 750

Glu Ser Glu Tyr Asn Ile Asn Asn Ile Glu Glu Glu Leu Asn Lys Lys
        755                 760                 765

Val Ser Leu Ala Met Lys Asn Ile Glu Arg Phe Met Thr Glu Ser Ser
    770                 775                 780

Ile Ser Tyr Leu Met Lys Leu Ile Asn Glu Ala Lys Val Gly Lys Leu
785                 790                 795                 800

Lys Lys Tyr Asp Asn His Val Lys Ser Asp Leu Leu Asn Tyr Ile Leu
                805                 810                 815

Asp His Arg Ser Ile Leu Gly Glu Gln Thr Asn Glu Leu Ser Asp Leu
            820                 825                 830

Val Thr Ser Thr Leu Asn Ser Ser Ile Pro Phe Glu Leu Ser Ser Tyr
        835                 840                 845

Thr Asn Asp Lys Ile Leu Ile Ile Tyr Phe Asn Arg Leu Tyr Lys Lys
    850                 855                 860

Ile Lys Asp Ser Ser Ile Leu Asp Met Arg Tyr Glu Asn Asn Lys Phe
865                 870                 875                 880

Ile Asp Ile Ser Gly Tyr Gly Ser Asn Ile Ser Ile Asn Gly Asn Val
                885                 890                 895

Tyr Ile Tyr Ser Thr Asn Arg Asn Gln Phe Gly Ile Tyr Asn Ser Arg
            900                 905                 910

Leu Ser Glu Val Asn Ile Ala Gln Asn Asn Asp Ile Ile Tyr Asn Ser
        915                 920                 925

Arg Tyr Gln Asn Phe Ser Ile Ser Phe Trp Val Arg Ile Pro Lys His
    930                 935                 940

Tyr Lys Pro Met Asn His Asn Arg Glu Tyr Thr Ile Ile Asn Cys Met
945                 950                 955                 960

Gly Asn Asn Asn Ser Gly Trp Lys Ile Ser Leu Arg Thr Val Arg Asp
                965                 970                 975

Cys Glu Ile Ile Trp Thr Leu Gln Asp Thr Ser Gly Asn Lys Glu Asn
            980                 985                 990

Leu Ile Phe Arg Tyr Glu Glu Leu  Asn Arg Ile Ser Asn  Tyr Ile Asn
        995                 1000                1005

Lys Trp  Ile Phe Val Thr Ile  Thr Asn Asn Arg Leu  Gly Asn Ser
    1010                1015                1020

Arg Ile  Tyr Ile Asn Gly Asn  Leu Ile Val Glu Lys  Ser Ile Ser
    1025                1030                1035

Asn Leu  Gly Asp Ile His Val  Ser Asp Asn Ile Leu  Phe Lys Ile
    1040                1045                1050

Val Gly  Cys Asp Asp Glu Thr  Tyr Val Gly Ile Arg  Tyr Phe Lys
    1055                1060                1065

Val Phe  Asn Thr Glu Leu Asp  Lys Thr Glu Ile Glu  Thr Leu Tyr
    1070                1075                1080

Ser Asn  Glu Pro Asp Pro Ser  Ile Leu Lys Asn Tyr  Trp Gly Asn
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    1085                1090                1095

Tyr Leu  Leu Tyr Asn Lys Lys  Tyr Tyr Leu Phe Asn  Leu Leu Arg
    1100                1105                1110

Lys Asp  Lys Tyr Ile Thr Leu  Asn Ser Gly Ile Leu  Asn Ile Asn
    1115                1120                1125

Gln Gln  Arg Gly Val Thr Glu  Gly Ser Val Phe Leu  Asn Tyr Lys
    1130                1135                1140

Leu Tyr  Glu Gly Val Glu Val  Ile Ile Arg Lys Asn  Gly Pro Ile
    1145                1150                1155

Asp Ile  Ser Asn Thr Asp Asn  Phe Val Arg Lys Asn  Asp Leu Ala
    1160                1165                1170

Tyr Ile  Asn Val Val Asp Arg  Gly Val Glu Tyr Arg  Leu Tyr Ala
    1175                1180                1185

Asp Thr  Lys Ser Glu Lys Glu  Lys Ile Ile Arg Thr  Ser Asn Leu
    1190                1195                1200

Asn Asp  Ser Leu Gly Gln Ile  Ile Val Met Asp Ser  Ile Gly Asn
    1205                1210                1215

Asn Cys  Thr Met Asn Phe Gln  Asn Asn Asn Gly Ser  Asn Ile Gly
    1220                1225                1230

Leu Leu  Gly Phe His Ser Asn  Asn Leu Val Ala Ser  Ser Trp Tyr
    1235                1240                1245

Tyr Asn  Asn Ile Arg Arg Asn  Thr Ser Ser Asn Gly  Cys Phe Trp
    1250                1255                1260

Ser Ser  Ile Ser Lys Glu Asn  Gly Trp Lys Glu
    1265                1270


<210> SEQ ID NO 7
<211> LENGTH: 1297
<212> TYPE: PRT
<213> ORGANISM: Clostridium botulinum

<400> SEQUENCE: 7

Met Pro Val Asn Ile Lys Asn Phe Asn Tyr Asn Asp Pro Ile Asn Asn
1               5                   10                  15

Asp Asp Ile Ile Met Met Glu Pro Phe Asn Asp Pro Gly Pro Gly Thr
            20                  25                  30

Tyr Tyr Lys Ala Phe Arg Ile Ile Asp Arg Ile Trp Ile Val Pro Glu
        35                  40                  45

Arg Phe Thr Tyr Gly Phe Gln Pro Asp Gln Phe Asn Ala Ser Thr Gly
    50                  55                  60

Val Phe Ser Lys Asp Val Tyr Glu Tyr Tyr Asp Pro Thr Tyr Leu Lys
65                  70                  75                  80

Thr Asp Ala Glu Lys Asp Lys Phe Leu Lys Thr Met Ile Lys Leu Phe
                85                  90                  95

Asn Arg Ile Asn Ser Lys Pro Ser Gly Gln Arg Leu Leu Asp Met Ile
            100                 105                 110

Val Asp Ala Ile Pro Tyr Leu Gly Asn Ala Ser Thr Pro Pro Asp Lys
        115                 120                 125

Phe Ala Ala Asn Val Ala Asn Val Ser Ile Asn Lys Lys Ile Ile Gln
    130                 135                 140

Pro Gly Ala Glu Asp Gln Ile Lys Gly Leu Met Thr Asn Leu Ile Ile
145                 150                 155                 160
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Phe Gly Pro Gly Pro Val Leu Ser Asp Asn Phe Thr Asp Ser Met Ile
                165                 170                 175

Met Asn Gly His Ser Pro Ile Ser Glu Gly Phe Gly Ala Arg Met Met
            180                 185                 190

Ile Arg Phe Cys Pro Ser Cys Leu Asn Val Phe Asn Asn Val Gln Glu
        195                 200                 205

Asn Lys Asp Thr Ser Ile Phe Ser Arg Arg Ala Tyr Phe Ala Asp Pro
    210                 215                 220

Ala Leu Thr Leu Met His Glu Leu Ile His Val Leu His Gly Leu Tyr
225                 230                 235                 240

Gly Ile Lys Ile Ser Asn Leu Pro Ile Thr Pro Asn Thr Lys Glu Phe
                245                 250                 255

Phe Met Gln His Ser Asp Pro Val Gln Ala Glu Glu Leu Tyr Thr Phe
            260                 265                 270

Gly Gly His Asp Pro Ser Val Ile Ser Pro Ser Thr Asp Met Asn Ile
        275                 280                 285

Tyr Asn Lys Ala Leu Gln Asn Phe Gln Asp Ile Ala Asn Arg Leu Asn
    290                 295                 300

Ile Val Ser Ser Ala Gln Gly Ser Gly Ile Asp Ile Ser Leu Tyr Lys
305                 310                 315                 320

Gln Ile Tyr Lys Asn Lys Tyr Asp Phe Val Glu Asp Pro Asn Gly Lys
                325                 330                 335

Tyr Ser Val Asp Lys Asp Lys Phe Asp Lys Leu Tyr Lys Ala Leu Met
            340                 345                 350

Phe Gly Phe Thr Glu Thr Asn Leu Ala Gly Glu Tyr Gly Ile Lys Thr
        355                 360                 365

Arg Tyr Ser Tyr Phe Ser Glu Tyr Leu Pro Pro Ile Lys Thr Glu Lys
    370                 375                 380

Leu Leu Asp Asn Thr Ile Tyr Thr Gln Asn Glu Gly Phe Asn Ile Ala
385                 390                 395                 400

Ser Lys Asn Leu Lys Thr Glu Phe Asn Gly Gln Asn Lys Ala Val Asn
                405                 410                 415

Lys Glu Ala Tyr Glu Glu Ile Ser Leu Glu His Leu Val Ile Tyr Arg
            420                 425                 430

Ile Ala Met Cys Lys Pro Val Met Tyr Lys Asn Thr Gly Lys Ser Glu
        435                 440                 445

Gln Cys Ile Ile Val Asn Asn Glu Asp Leu Phe Phe Ile Ala Asn Lys
    450                 455                 460

Asp Ser Phe Ser Lys Asp Leu Ala Lys Ala Glu Thr Ile Ala Tyr Asn
465                 470                 475                 480

Thr Gln Asn Asn Thr Ile Glu Asn Asn Phe Ser Ile Asp Gln Leu Ile
                485                 490                 495

Leu Asp Asn Asp Leu Ser Ser Gly Ile Asp Leu Pro Asn Glu Asn Thr
            500                 505                 510

Glu Pro Phe Thr Asn Phe Asp Asp Ile Asp Ile Pro Val Tyr Ile Lys
        515                 520                 525

Gln Ser Ala Leu Lys Lys Ile Phe Val Asp Gly Asp Ser Leu Phe Glu
    530                 535                 540

Tyr Leu His Ala Gln Thr Phe Pro Ser Asn Ile Glu Asn Leu Gln Leu
545                 550                 555                 560

Thr Asn Ser Leu Asn Asp Ala Leu Arg Asn Asn Asn Lys Val Tyr Thr
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               565                 570                 575

Phe Phe Ser Thr Asn Leu Val Glu Lys Ala Asn Thr Val Val Gly Ala
            580                 585                 590

Ser Leu Phe Val Asn Trp Val Lys Gly Val Ile Asp Asp Phe Thr Ser
        595                 600                 605

Glu Ser Thr Gln Lys Ser Thr Ile Asp Lys Val Ser Asp Val Ser Ile
    610                 615                 620

Ile Ile Pro Tyr Ile Gly Pro Ala Leu Asn Val Gly Asn Glu Thr Ala
625                 630                 635                 640

Lys Glu Asn Phe Lys Asn Ala Phe Glu Ile Gly Gly Ala Ala Ile Leu
                645                 650                 655

Met Glu Phe Ile Pro Glu Leu Ile Val Pro Ile Val Gly Phe Phe Thr
            660                 665                 670

Leu Glu Ser Tyr Val Gly Asn Lys Gly His Ile Ile Met Thr Ile Ser
        675                 680                 685

Asn Ala Leu Lys Lys Arg Asp Gln Lys Trp Thr Asp Met Tyr Gly Leu
    690                 695                 700

Ile Val Ser Gln Trp Leu Ser Thr Val Asn Thr Gln Phe Tyr Thr Ile
705                 710                 715                 720

Lys Glu Arg Met Tyr Asn Ala Leu Asn Asn Gln Ser Gln Ala Ile Glu
                725                 730                 735

Lys Ile Ile Glu Asp Gln Tyr Asn Arg Tyr Ser Glu Glu Asp Lys Met
            740                 745                 750

Asn Ile Asn Ile Asp Phe Asn Asp Ile Asp Phe Lys Leu Asn Gln Ser
        755                 760                 765

Ile Asn Leu Ala Ile Asn Asn Ile Asp Asp Phe Ile Asn Gln Cys Ser
    770                 775                 780

Ile Ser Tyr Leu Met Asn Arg Met Ile Pro Leu Ala Val Lys Lys Leu
785                 790                 795                 800

Lys Asp Phe Asp Asp Asn Leu Lys Arg Asp Leu Leu Glu Tyr Ile Asp
                805                 810                 815

Thr Asn Glu Leu Tyr Leu Leu Asp Glu Val Asn Ile Leu Lys Ser Lys
            820                 825                 830

Val Asn Arg His Leu Lys Asp Ser Ile Pro Phe Asp Leu Ser Leu Tyr
        835                 840                 845

Thr Lys Asp Thr Ile Leu Ile Gln Val Phe Asn Asn Tyr Ile Ser Asn
    850                 855                 860

Ile Ser Ser Asn Ala Ile Leu Ser Leu Ser Tyr Arg Gly Gly Arg Leu
865                 870                 875                 880

Ile Asp Ser Ser Gly Tyr Gly Ala Thr Met Asn Val Gly Ser Asp Val
                885                 890                 895

Ile Phe Asn Asp Ile Gly Asn Gly Gln Phe Lys Leu Asn Asn Ser Glu
            900                 905                 910

Asn Ser Asn Ile Thr Ala His Gln Ser Lys Phe Val Val Tyr Asp Ser
        915                 920                 925

Met Phe Asp Asn Phe Ser Ile Asn Phe Trp Val Arg Thr Pro Lys Tyr
    930                 935                 940

Asn Asn Asn Asp Ile Gln Thr Tyr Leu Gln Asn Glu Tyr Thr Ile Ile
945                 950                 955                 960

Ser Cys Ile Lys Asn Asp Ser Gly Trp Lys Val Ser Ile Lys Gly Asn
                965                 970                 975
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Arg Ile Ile Trp Thr Leu Ile Asp Val Asn Ala Lys Ser Lys Ser Ile
            980                 985                 990

Phe Phe Glu Tyr Ser Ile Lys Asp  Asn Ile Ser Asp Tyr  Ile Asn Lys
        995                 1000                1005

Trp Phe  Ser Ile Thr Ile Thr  Asn Asp Arg Leu Gly  Asn Ala Asn
    1010                1015                1020

Ile Tyr  Ile Asn Gly Ser Leu  Lys Lys Ser Glu Lys  Ile Leu Asn
    1025                1030                1035

Leu Asp  Arg Ile Asn Ser Ser  Asn Asp Ile Asp Phe  Lys Leu Ile
    1040                1045                1050

Asn Cys  Thr Asp Thr Thr Lys  Phe Val Trp Ile Lys  Asp Phe Asn
    1055                1060                1065

Ile Phe  Gly Arg Glu Leu Asn  Ala Thr Glu Val Ser  Ser Leu Tyr
    1070                1075                1080

Trp Ile  Gln Ser Ser Thr Asn  Thr Leu Lys Asp Phe  Trp Gly Asn
    1085                1090                1095

Pro Leu  Arg Tyr Asp Thr Gln  Tyr Tyr Leu Phe Asn  Gln Gly Met
    1100                1105                1110

Gln Asn  Ile Tyr Ile Lys Tyr  Phe Ser Lys Ala Ser  Met Gly Glu
    1115                1120                1125

Thr Ala  Pro Arg Thr Asn Phe  Asn Asn Ala Ala Ile  Asn Tyr Gln
    1130                1135                1140

Asn Leu  Tyr Leu Gly Leu Arg  Phe Ile Ile Lys Lys  Ala Ser Asn
    1145                1150                1155

Ser Arg  Asn Ile Asn Asn Asp  Asn Ile Val Arg Glu  Gly Asp Tyr
    1160                1165                1170

Ile Tyr  Leu Asn Ile Asp Asn  Ile Ser Asp Glu Ser  Tyr Arg Val
    1175                1180                1185

Tyr Val  Leu Val Asn Ser Lys  Glu Ile Gln Thr Gln  Leu Phe Leu
    1190                1195                1200

Ala Pro  Ile Asn Asp Asp Pro  Thr Phe Tyr Asp Val  Leu Gln Ile
    1205                1210                1215

Lys Lys  Tyr Tyr Glu Lys Thr  Thr Tyr Asn Cys Gln  Ile Leu Cys
    1220                1225                1230

Glu Lys  Asp Thr Lys Thr Phe  Gly Leu Phe Gly Ile  Gly Lys Phe
    1235                1240                1245

Val Lys  Asp Tyr Gly Tyr Val  Trp Asp Thr Tyr Asp  Asn Tyr Phe
    1250                1255                1260

Cys Ile  Ser Gln Trp Tyr Leu  Arg Arg Ile Ser Glu  Asn Ile Asn
    1265                1270                1275

Lys Leu  Arg Leu Gly Cys Asn  Trp Gln Phe Ile Pro  Val Asp Glu
    1280                1285                1290

Gly Trp  Thr Glu
    1295
```

1. A liquid composition comprising a proteinaceous neurotoxin, a surfactant, an amino acid selected from tryptophan and tyrosine, a buffer comprising sodium, chloride and phosphate ions,

wherein said liquid composition has a pH between 5.5 and 8,

wherein said composition is free of animal derived proteins, and

wherein said liquid composition is stable over time.

2. A liquid composition according to claim **1**, wherein said surfactant is a non-ionic surfactant.

3. A liquid composition according to claim **2**, wherein said non-ionic surfactant is a polysorbate, preferably Polysorbate 20, Polysorbate 60 or Polysorbate 80.

4. A liquid composition according to claim **1**, wherein said amino acid is tryptophan, preferably L-tryptophan.

5. A liquid composition according to claim **1**, wherein said buffer further comprises potassium ions.

6. A liquid composition according to claim **1**, wherein said composition has a pH between 6.0 and 7.5.

7. A liquid composition according to claim **1**, wherein no more than 30% loss in extracellular proteolytic activity occurs over 2, 3, 6, 12, 18, 24 or 36 months at 5° C.

8. A liquid composition according to claim **1**, wherein said proteinaceous neurotoxin is a botulinum neurotoxin, selected from a natural botulinum neurotoxin in complex form, a high purity natural botulinum neurotoxin and a recombinant botulinum neurotoxin.

9. A liquid composition according to claim **8**, wherein said botulinum neurotoxin is a recombinant botulinum neurotoxin selected from a botulinum neurotoxin A, B, C, D, E, F or G, a modified botulinum neurotoxin and a chimeric botulinum neurotoxin.

10. A liquid composition according to claim **1**, wherein said liquid composition comprises:

4 to 10000 LD50 units of botulinum neurotoxin per mL,

0.001 to 15% v/v polysorbate,

0.1 to 5 mg/mL tryptophan,

10 to 500 mM NaCl,

1 to 50 mM KCl,

1 to 100 mM Sodium phosphate,

has a pH between 5.5 and 8, and is stable for 6 months at 5° C.

11. A liquid composition according to claim **10**, wherein said liquid composition comprises:

10 to 2000 LD50 units of botulinum neurotoxin per mL,

0.05 to 0.2% v/v polysorbate 80,

0.1 to 5 mg/mL tryptophan,

25 to 300 mM NaCl,

1 to 10 mM KCl,

2 to 50 mM Sodium phosphate,

has a pH between 6.0 and 7.5, and is stable for 12 months at 5° C.

12. A liquid composition according to claim **1** for use in therapy.

13. A liquid composition according to claim **12** for use in treating or preventing muscular disorders, neuromuscular disorders, neurological disorders, ophthalmological disorders, pain disorders, psychological disorders, articular disorders, inflammatory disorders, endocrine disorders or urological disorders.

14. Use of liquid composition according to claim **1** in aesthetic medicine, for example for treating or preventing skin wrinkles, in particular facial wrinkles such as facial frown lines, wrinkles of the contour of the eye, glabellar frown lines, downturned mouth, wrinkles of the neck (platysmal bands), wrinkles of the chin (mentalis, peau d' orange, dimpled chin), forehead lines, "scratched skin" wrinkles, nasal lift treatment or sleep lines.

15. Use of an amino acid selected from tryptophan and tyrosine to protect a proteinaceous neurotoxin from degradation in a liquid composition which is free of animal derived proteins.

16. Use according to claim **15**, wherein said amino acid is tryptophan.

17. Use according to claim **15**, wherein said proteinaceous neurotoxin is a botulinum neurotoxin.

18. Use according to claim **15**, wherein said amino acid is used in combination with a surfactant and a buffer comprising sodium, chloride and phosphate ions, and said liquid composition has a pH between 5.5 and 8.

* * * * *