(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
28 July 2011 (28.07.2011)

PCT

(10) International Publication Number
WO 2011/091370 A1

(51) International Patent Classification:
C07K 14/33 (2006.01) *C12N 15/70* (2006.01)
C12P 21/02 (2006.01) *C12N 15/866* (2006.01)
C12N 15/62 (2006.01) *C12P 21/06* (2006.01)
CI2N 9/50 (2006.01)

(21) International Application Number:
PCT/US201 1/022272

(22) International Filing Date:
24 January 201 1 (24.01 .201 1)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/286,963 25 January 2010 (25.01 .2010) US

(71) Applicant *(for all designated States except US):* **ALLERGAN, INC.** [US/US]; 2525 Dupont Drive, Irvine, California 92612 (US).

(72) Inventors; and

(75) Inventors/ Applicants *(for US only):* **GHANSHANI, Sanjiv** [US/US]; 11 Morningside, Irvine, California 92603 (US). **LE, Linh Q.** [US/US]; 12955 Crawford Drive, Tustin, California 92782 (US). **LIU, Yi** [US/US]; 29 Vassar Aisle, Irvine, California 92612 (US). **STEWARD, Lance E.** [US/US]; 35 Sweet Rain, Irvine, California 92614 (US).

(74) Agents: **ABEL, Kenton B.** et al; Allergan, Inc., 2525 Dupont Drive, Irvine, California 92612 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available):* AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available):* ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(H))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(Hi))*

Published:

— *with international search report (Art. 21(3))*

[Continued on next page]

(54) Title: METHODS OF INTRACELLULAR CONVERSION OF SINGLE-CHAIN PROTEINS INTO THEIR DI-CHAIN FORM

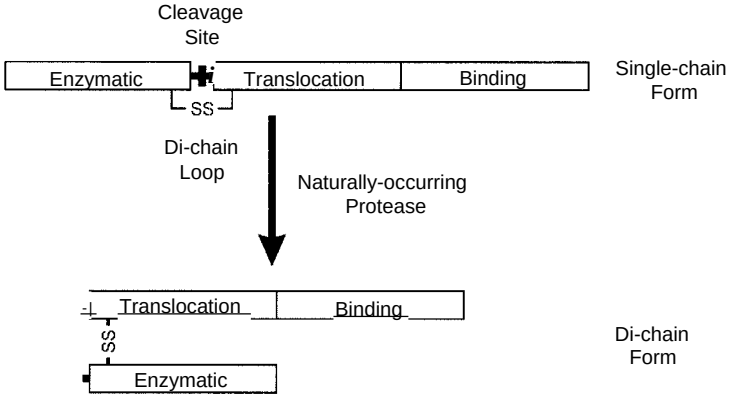


FIG. 1.

(57) Abstract: The present specification discloses expression constructs comprising single-chain proteins comprising a di-chain loop region comprising an exogenous protease cleavage site and a protease that can cleave the exogenous protease cleavage site located within the di-chain loop, cell compositions comprising such expression construct, and intracellular methods of converting the single-chain protein into its di-chain form.

WO 2011/091370 A1

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

Methods of Intracellular Conversion of Single-Chain Proteins into their Di-chain Form

[01] This patent application claims priority pursuant to 35 U.S.C. § 119(e) to U. S. Provisional Patent Application Serial No. 61/286,963 filed January 25, 201 0, entirely incorporated by reference.

[02] The ability of Clostridial toxins, such as, e.g., Botulinum neurotoxins (BoNTs), BoNT/A, BoNT/B, BoNT/C1 , BoNT/D, BoNT/E, BoNT/F and BoNT/G, and Tetanus neurotoxin (TeNT), to inhibit neuronal transmission are being exploited in a wide variety of therapeutic and cosmetic applications, see e.g., William J. Lipham, COSMETIC AND CLINICAL APPLICATIONS OF BOTULINUM TOXIN (Slack, Inc., 2004). Clostridial toxins commercially available as pharmaceutical compositions include, BoNT/A preparations, such as, e.g., BOTOX® (Allergan, Inc., Irvine, CA), DYSPORT®/RELOXIN®, (Beaufour Ipsen, Porton Down, England), NEURONOX® (Medy-Tox, Inc., Ochang-myeon, South Korea) BTX-A (Lanzhou Institute Biological Products, China) and XEOMIN® (Merz Pharmaceuticals, GmbH., Frankfurt, Germany); and BoNT/B preparations, such as, e.g., MYOBLOC™/NEUROBLOC ™ (Elan Pharmaceuticals, San Francisco, CA). As an example, BOTOX® is currently approved in one or more countries for the following indications: achalasia, adult spasticity, anal fissure, back pain, blepharospasm, bruxism, cervical dystonia, essential tremor, glabellar lines or hyperkinetic facial lines, headache, hemifacial spasm, hyperactivity of bladder, hyperhidrosis, juvenile cerebral palsy, multiple sclerosis, myoclonic disorders, nasal labial lines, spasmodic dysphonia, strabismus and VII nerve disorder.

[03] The therapeutic utility of Clostrdial toxins has been expanded beyond their current myo-relaxant applications to treat sensory nerve-based ailments, such as, e.g., various kinds of chronic pain, neurogenic inflammation and urogentital disorders, as well as non-neuronal-based disorders, such as, e.g., pancreatitis. One approach that is currently being exploited to expand Clostridial toxin-based therapies involves modifying a Clostridial toxin so that the modified toxin has an altered cell targeting capability for a non-Clostridial toxin target cell. This re-targeted capability is achieved by replacing a naturally-occurring targeting domain of a Clostridial toxin with a targeting domain showing a selective binding activity for a non-Clostridial toxin receptor present in a non-Clostridial toxin target cell. Such modifications to a targeting domain result in a modified toxin that is able to selectively bind to a non-Clostridial toxin receptor (target receptor) present on a non-Clostridial toxin target cell (re-targeted). A re¬targeted Clostridial toxin with a targeting activity for a non-Clostridial toxin target cell can bind to a receptor present on the non-Clostridial toxin target cell, translocate into the cytoplasm, and exert its proteolytic effect on the SNARE complex of the non-Clostridial toxin target cell.

[04] Non-limiting examples of re-targeted Clostridial toxins with a targeting activity for a non-Clostridial toxin target cell are described in, e.g., Keith A. Foster et al., *Clostridial Toxin Derivatives Able To Modify Peripheral Sensory Afferent Functions,* U.S. Patent 5,989,545; Clifford C. Shone et al., *Recombinant Toxin Fragments,* U.S. Patent 6,46 1,61 7; Stephan Donovan, *Clostridial Toxin Derivatives and Methods For Treating Pain,* U.S. Patent 6,500,436; Conrad P. Quinn et al., *Methods and Compounds for the Treatment of Mucus Hypersecretion,* U.S. Patent 6,632,440; Lance E. Steward et al., *Methods And Compositions For The Treatment Of Pancreatitis,* U.S. Patent 6,843,998; J. Oliver Dolly et al., *Activatable Recombinant Neurotoxins,* U.S. Patent 7,41 9,676; Lance E. Steward et al., *Multivalent Clostridial Toxin Derivatives and Methods of Their Use,* U.S. Patent 7,51 4,088; Keith A. Foster et al., *Inhibition of Secretion from Non-neural Cells,* U.S. Patent Publication 2003/01 80289; and Keith A. Foster et al., *Re-targeted Toxin Conjugates,* International Patent Publication WO 2005/023309. The ability to re-target the

therapeutic effects associated with Clostridial toxins has greatly extended the number of medicinal applications able to use a Clostridial toxin therapy. As a non-limiting example, modified Clostridial toxins retargeted to sensory neurons are useful in treating various kinds of chronic pain, such as, e.g., hyperalgesia and allodynia, neuropathic pain and inflammatory pain, see, e.g., Foster, *supra,* (1999); and Donovan, *supra,* (2002); and Stephan Donovan, *Method For Treating Neurogenic Inflammation Pain with Botulinum Toxin and Substance P Components,* U.S. Patent 7,022,329. As another non-limiting example, modified Clostridial toxins retargeted to pancreatic cells are useful in treating pancreatitis, see, e.g., Steward, *supra,* (2005).

[05] Clostridial toxins, whether naturally occurring or modified, are processed into a di-chain form in order to achieve full activity. Naturally-occurring Clostridial toxins are each translated as a single-chain polypeptide of approximately 150 kDa that is subsequently cleaved by proteolytic scission within a disulfide loop by a naturally-occurring protease (FIG. 1). This cleavage occurs within the discrete di-chain loop region created between two cysteine residues that form a disulfide bridge. This posttranslational processing yields a di-chain molecule comprising an approximately 50 kDa light chain (LC), comprising the enzymatic domain, and an approximately 100 kDa heavy chain (HC), comprising the translocation and cell binding domains, the LC and HC being held together by the single disulfide bond and non-covalent interactions (FIG. 1). Recombinantly-produced Clostridial toxins generally substitute the naturally-occurring di-chain loop protease cleavage site with an exogenous protease cleavage site (FIG. 2). *See e.g.,* Dolly, J.O. et al., *Activatable Clostridial Toxins,* U.S. Patent 7,419,676, which is hereby incorporated by reference. Although re-targeted Clostridial toxins vary in their overall molecular weight because of the size of the targeting moiety, the activation process and its reliance on exogenous cleavage sites is essentially the same as that for recombinantly-produced Clostridial toxins. *See e.g.,* Steward, L.E. et al., *Activatable Clostridial Toxins,* U.S. Patent Publication 2009/0005313; Steward, L.E. et al., *Modified Clostridial Toxins with Enhanced Translocation Capabilities and Altered Targeting Activity For Non-Clostridial Toxin Target Cells,* U.S. Patent Application 11/776,075; Steward, L.E. et al., *Modified Clostridial Toxins with Enhanced Translocation Capabilities and Altered Targeting Activity for Clostridial Toxin Target Cells,* U.S. Patent Publication 2008/0241881, each of which is hereby incorporated by reference.

[06] To date, the conversion of the single-chain form of a recombinantly produced Clostridial toxin or modified Clostridial toxin into its di-chain form required an in vitro activation process. First, the bacterial cells used to recombinantly produce these toxins lack the naturally-occurring protease present in the Clostridial strains that produce the native toxins. Second, there has been no great need for bacterial cells to produce activated toxins recombinantly because of safety concerns raised in handling activated toxins. *See e.g.,* Dolly, U.S. 7,419,676, *supra,* (2008). However, if these concerns could be overcome, the production of recombinantly produced activated toxins would streamline the manufacturing process of recombinantly produced Clostridial toxins or modified Clostridial toxins. For example, currently the manufacture of recombinantly produced Clostridial toxins or modified Clostridial toxins involves the following purification steps: 1) immobilized metal affinity chromatography, 2) buffer exchange dialysis, 3) protease cleavage reaction , 4), ion exchange chromatography and and 5) addition of PEG and flash freezing for storage at -80 °C. The use of a bacterial cell that can protealytically cleave the recombinant Clostridial toxin intracellular^ while it is still expressing the toxin can reduce the number of purification

steps to the following: 1) immobilized metal affinity chromatography, 2) buffer exchange dialysis, 3) ion exchange chromatography, and 4) addition of PEG and flash freezing for storage at -80 °C.

[07] The present specification discloses a method of converting a single-chain protein comprising a di-chain loop region into its di-chain form that does not rely on an in vitro process for converting the single-chain form of the toxin into its di-chain form. This is accomplished by the use of cells that express both the protein and the protease necessary to convert it to active di-chain.

[08] Thus, aspects of the present specification provide, a dual expression construct that includes an open reading frame encoding a single-chain protein comprising a di-chain loop region comprising an exogenous protease cleavage site and an open reading frame encoding a protease that can proteolytically cleave the exogenous protease cleavage site located in the di-chain loop region. In further aspects, the single-chain protein comprising a di-chain loop region comprising an exogenous protease cleavage site can be , e.g. , a Clostridial toxin comprising a di-chain loop region comprising an exogenous protease cleavage site, a modified Clostridial toxin comprising a di-chain loop region comprising an exogenous protease cleavage site, or a single-chain protein comprising a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a non-Clostridial toxin binding domain, and a di-chain loop region comprising an exogenous protease cleavage site. Polynucleotides, as well as the Clostridial toxins comprising a di-chain loop region comprising an exogenous protease cleavage site that they encode, are described in, e.g. , Dolly, J.O. et al., *Activatable Clostridial Toxins,* U.S. Patent 7,132,259; Dolly, J.O. et al., *Activatable Clostridial Toxins,* U.S. Patent 7,419,676, each of which is hereby incorporated by reference in its entirety. Polynucleotides, as well as the proteins comprising a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a non-Clostridial toxin binding domain, and a di-chain loop region comprising an exogenous protease cleavage site that they encode, are described in, e.g. , Steward, L.E. et al. , *Multivalent Clostridial Toxins,* U.S. Patent Publication 2009/0048431 ; Steward, L.E. et al., *Activatable Clostridial Toxins,* U.S. Patent Publication 2009/0069238; Steward, L.E. et al., *Modified Clostridial Toxins with Enhanced Translocation Capabilities and Altered Targeting Activity For Non-Clostridial Toxin Target Cells,* U.S. Patent Application 11/776,075; Steward , L.E. et al., *Modified Clostridial Toxins with Enhanced Translocation Capabilities and Altered Targeting Activity for Clostridial Toxin Target Cells,* U.S. Patent Publication 2008/0241 881 ; Foster, K.A. et al. , *Fusion Proteins,* U.S. Patent Publication 2009/0035822; Foster, K.A. et al. , *Non-Cytotoxic Protein Conjugates,* U.S. Patent Publication 2009/01 6234 1; Steward, L.E. et al., *Activatable Clostridial Toxins,* U.S. Patent Publication 2008/0032931 ; Foster, K.A. et al. , *Non-Cytotoxic Protein Conjugates,* U.S. Patent Publication 2008/01 87960; Steward , L.E. et al. , *Degradable Clostridial Toxins,* U.S. Patent Publication 2008/021 3830; Steward, L.E. et al. , *Modified Clostridial Toxins With Enhanced Translocation Capabilities and Altered Targeting Activity For Clostridial Toxin Target Cells,* U.S. Patent Publication 2008/0241 881 ; Dolly, J.O. et al., *Activatable Clostridial Toxins,* U.S. Patent 7,419,676; and a companion patent application Ghanshani, et al. , *Modified Clostridial Toxins Comprising an Integrated Protease Cleavage Site-Binding Domain,* Attorney Docket No. 18468 PROV (BOT), each of which is hereby incorporated by reference in its entirety.

[09] Other aspects of the present specification provide a cell comprising a dual expression construct that includes an open reading frame encoding a single-chain protein comprising a di-chain loop region

comprising an exogenous protease cleavage site and an open reading frame encoding a protease that can proteolytically cleave the exogenous protease cleavage site located in the di-chain loop region. In further aspects, the single-chain protein comprising a di-chain loop region comprising an exogenous protease cleavage site can be, e.g., a Clostridial toxin comprising a di-chain loop region comprising an exogenous protease cleavage site, a modified Clostridial toxin comprising a di-chain loop region comprising an exogenous protease cleavage site, or a single-chain protein comprising a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a non-Clostridial toxin binding domain, and a di-chain loop region comprising an exogenous protease cleavage site as disclosed in the present specification.

[010] Yet other aspects of the present specification provide an intracellular method of converting a single-chain protein into its di-chain form, the method comprising the steps of a) growing a cell comprising a dual expression construct at a first temperature for a certain period of time in order to achieve maximal cell density, the dual expression construct comprising; i) an open reading frame encoding a single-chain protein comprising a di-chain loop region comprising an exogenous protease cleavage site; and ii) an open reading frame encoding a protease; wherein the protease can cleave the exogenous protease cleavage site located within the di-chain loop; b) growing the cell at a second temperature for a certain period of time in order to achieve maximal induction of protein expression from the open reading frame encoding the single-chain protein, wherein growth at step (b) induces expression of the single-chain protein and the protease from the dual expression construct; and wherein the produced protease cleaves the single-chain protein at the exogenous protease cleavage site located within the di-chain loop region, thereby converting the single-chain protein into its di-chain form.

[011] Still other aspects of the present specification provide an intracellular method of converting a single-chain Clostridial toxin into its di-chain form, the method comprising the steps of: a) growing a cell comprising a dual expression construct at 37 °C for about 2 to about 3.5 hours, the dual expression construct comprising; i) an open reading frame encoding a single-chain Clostridial toxin, the single-chain Clostridial toxin comprising an enzymatic domain, a translocation domain, a binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; and ii) an open reading frame encoding a protease; wherein the protease can cleave the exogenous protease cleavage site located within the di-chain loop; b) growing the cell at 22 °C for about 16 to about 18 hours, wherein growth at step (b) induces expression of the single-chain Clostridial toxin and the protease from the dual expression construct; and wherein the produced protease cleaves the single-chain Clostridial toxin at the exogenous protease cleavage site located within the di-chain loop region, thereby converting the single-chain Clostridial toxin into its di-chain form.

[012] Further aspects of the present specification provide an intracellular method of converting a single-chain protein into its di-chain form, the method comprising the steps of a) growing a cell comprising a dual expression construct at 37 °C for about 2 to about 8 hours, the dual expression construct comprising; i) an open reading frame encoding a single-chain protein, the single-chain protein comprising an enzymatic domain, a translocation domain, and an integrated TEV protease cleavage site-opioid binding domain; and ii) an open reading frame encoding a TEV protease; b) growing the cell at about 12 to about 16 °C for about 16 to about 18 hours, wherein growth at step (b) induces expression of the single-chain protein and the TEV protease from the dual expression construct; and wherein the

produced TEV protease cleaves the single-chain protein at the TEV protease cleavage site located within the integrated TEV cleavage site opioid binding domain, thereby converting the single-chain protein into its di-chain form.

[013] Further aspects of the present specification provide an intracellular method of converting a single-chain protein into its di-chain form, the method comprising the steps of a) growing a cell comprising a dual expression construct at 37 °C for about 2 to about 8 hours, the dual expression construct comprising; i) an open reading frame encoding a single-chain protein, the single-chain protein comprising an enzymatic domain, a translocation domain, and an integrated TEV protease cleavage site-opioid binding domain; and ii) an open reading frame encoding a TEV protease; b) growing the cell at about 20 to about 24 °C for about 16 to about 18 hours, wherein growth at step (b) induces expression of the single-chain protein and the TEV protease from the dual expression construct; and wherein the produced TEV protease cleaves the single-chain protein at the TEV protease cleavage site located within the integrated TEV cleavage site opioid binding domain, thereby converting the single-chain protein into its di-chain form.

[014] Yet further aspects of the present specification provide an intracellular method of converting a single-chain protein into its di-chain form, the method comprising the steps of a) growing a cell comprising a dual expression construct at 37 °C for about 2 to about 8 hours, the dual expression construct comprising; i) an open reading frame encoding a single-chain protein, the single-chain protein comprising an enzymatic domain, a translocation domain, a non-Clostridial toxin binding domain and a di-chain loop region comprising a TEV protease cleavage site; and ii) an open reading frame encoding a TEV protease; b) growing the cell at about 12 to about 16 °C for about 16 to about 18 hours, wherein growth at step (b) induces expression of the single-chain protein and the TEV protease from the dual expression construct; and wherein the produced TEV protease cleaves the single-chain protein at the TEV protease cleavage site located within the di-chain loop region, thereby converting the single-chain protein into its di-chain form.

[015] Yet further aspects of the present specification provide an intracellular method of converting a single-chain protein into its di-chain form, the method comprising the steps of a) growing a cell comprising a dual expression construct at 37 °C for about 2 to about 8 hours, the dual expression construct comprising; i) an open reading frame encoding a single-chain protein, the single-chain protein comprising an enzymatic domain, a translocation domain, a non-Clostridial toxin binding domain and a di-chain loop region comprising a TEV protease cleavage site; and ii) an open reading frame encoding a TEV protease; b) growing the cell at about 20 to about 24 °C for about 16 to about 18 hours, wherein growth at step (b) induces expression of the single-chain protein and the TEV protease from the dual expression construct; and wherein the produced TEV protease cleaves the single-chain protein at the TEV protease cleavage site located within the di-chain loop region, thereby converting the single-chain protein into its di-chain form.

[016] Other aspects of the present specification provide, an expression construct comprising an open reading frame encoding a single-chain protein comprising a di-chain loop region comprising an exogenous protease cleavage site. In further aspects, the single-chain protein comprising a di-chain loop region comprising an exogenous protease cleavage site can be, e.g., a Clostridial toxin comprising a di-chain loop region comprising an exogenous protease cleavage site, a modified Clostridial toxin

comprising a di-chain loop region comprising an exogenous protease cleavage site, or a single-chain protein comprising a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a non-Clostridial toxin binding domain, and a di-chain loop region comprising an exogenous protease cleavage site as disclosed in the present specification.

[017] Other aspects of the present specification provide, an expression construct comprising an open reading frame encoding a protease that can proteolytically cleave the exogenous protease cleavage site located in the di-chain loop region of a single-chain protein comprising a di-chain loop region comprising an exogenous protease cleavage site.

[018] Other aspects of the present specification provide a cell comprising an expression construct comprising an open reading frame encoding a single-chain protein comprising a di-chain loop region comprising an exogenous protease cleavage site and another expression construct comprising an open reading frame encoding a protease that can proteolytically cleave the exogenous protease cleavage site located in the di-chain loop region of a single-chain protein comprising a di-chain loop region comprising an exogenous protease cleavage site. In further aspects, the single-chain protein comprising a di-chain loop region comprising an exogenous protease cleavage site can be, e.g., a Clostridial toxin comprising a di-chain loop region comprising an exogenous protease cleavage site, a modified Clostridial toxin comprising a di-chain loop region comprising an exogenous protease cleavage site, or a single-chain protein comprising a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a non-Clostridial toxin binding domain, and a di-chain loop region comprising an exogenous protease cleavage site as disclosed in the present specification.

[019] Yet other aspects of the present specification provide an intracellular method of converting a single-chain protein into its di-chain form, the method comprising the steps of a) growing a cell comprising i) an expression construct comprising an open reading frame encoding a single-chain protein comprising a di-chain loop region comprising an exogenous protease cleavage site and ii) another expression construct comprising an open reading frame encoding a protease that can proteolytically cleave the exogenous protease cleavage site located in the di-chain loop region of a single-chain protein comprising a di-chain loop region comprising an exogenous protease cleavage site; b) growing the cell at a second temperature for a certain period of time in order to achieve maximal induction of protein expression from the open reading frame encoding the single-chain protein, wherein growth at step (b) induces expression of the single-chain protein and the protease from the expression constructs; and wherein the produced protease cleaves the single-chain protein at the exogenous protease cleavage site located within the di-chain loop region, thereby converting the single-chain protein into its di-chain form.

[020] Still other aspects of the present specification provide an intracellular method of converting a single-chain Clostridial toxin into its di-chain form, the method comprising the steps of: a) growing at 37 °C for about 2 to about 3.5 hours a cell, the cell comprising i) an expression construct comprising an open reading frame encoding a single-chain Clostridial toxin comprising an enzymatic domain, a translocation domain, a binding domain, and a di-chain loop region comprising an exogenous protease cleavage site and ii) another expression construct comprising an open reading frame encoding a protease that can proteolytically cleave the exogenous protease cleavage site located in the di-chain loop region of a single-chain protein comprising a di-chain loop region comprising an exogenous protease cleavage site; b) growing the cell at 22 °C for about 16 to about 18 hours, wherein growth at step (b) induces expression

of the single-chain Clostridial toxin and the protease from the expression constructs; and wherein the produced protease cleaves the single-chain Clostridial toxin at the exogenous protease cleavage site located within the di-chain loop region, thereby converting the single-chain Clostridial toxin into its di-chain form.

[021] Further aspects of the present specification provide an intracellular method of converting a single-chain protein into its di-chain form, the method comprising the steps of a) growing at 37 °C for about 2 to about 8 hours a cell, the cell comprising i) an expression construct comprising an open reading frame encoding a single-chain protein comprising an enzymatic domain, a translocation domain, and an integrated TEV protease cleavage site-opioid binding domain and ii) another expression construct comprising an open reading frame encoding TEV protease; b) growing the cell at about 12 to about 16 °C for about 16 to about 18 hours, wherein growth at step (b) induces expression of the single-chain protein and the TEV protease from the expression constructs; and wherein the produced TEV protease cleaves the single-chain protein at the TEV protease cleavage site located within the integrated TEV cleavage site opioid binding domain, thereby converting the single-chain protein into its di-chain form.

[022] Further aspects of the present specification provide an intracellular method of converting a single-chain protein into its di-chain form, the method comprising the steps of a) growing at 37 °C for about 2 to about 8 hours a cell, the cell comprising i) an expression construct comprising an open reading frame encoding a single-chain protein comprising an enzymatic domain, a translocation domain, and an integrated TEV protease cleavage site-opioid binding domain and ii) another expression construct comprising an open reading frame encoding TEV protease; b) growing the cell at about 20 to about 24 °C for about 16 to about 18 hours, wherein growth at step (b) induces expression of the single-chain protein and the TEV protease from the expression constructs; and wherein the produced TEV protease cleaves the single-chain protein at the TEV protease cleavage site located within the integrated TEV cleavage site opioid binding domain, thereby converting the single-chain protein into its di-chain form.

[023] Yet further aspects of the present specification provide an intracellular method of converting a single-chain protein into its di-chain form, the method comprising the steps of a) growing at 37 °C for about 2 to about 8 hours a cell, the cell comprising i) an expression construct comprising an open reading frame encoding a single-chain protein comprising an enzymatic domain, a translocation domain, a non-Clostridial toxin binding domain and a di-chain loop region comprising a TEV protease cleavage site and ii) another expression construct comprising an open reading frame encoding TEV protease; b) growing the cell at about 12 to about 16 °C for about 16 to about 18 hours, wherein growth at step (b) induces expression of the single-chain protein and the TEV protease from the expression constructs; and wherein the produced TEV protease cleaves the single-chain protein at the TEV protease cleavage site located within the integrated TEV cleavage site opioid binding domain, thereby converting the single-chain protein into its di-chain form.

[024] Yet further aspects of the present specification provide an intracellular method of converting a single-chain protein into its di-chain form, the method comprising the steps of a) growing at 37 °C for about 2 to about 8 hours a cell, the cell comprising i) an expression construct comprising an open reading frame encoding a single-chain protein comprising an enzymatic domain, a translocation domain, a non-Clostridial toxin binding domain and a di-chain loop region comprising a TEV protease cleavage site and ii) another expression construct comprising an open reading frame encoding TEV protease; b) growing

the cell at about 20 to about 24 °C for about 16 to about 18 hours, wherein growth at step (b) induces expression of the single-chain protein and the TEV protease from the expression constructs; and wherein the produced TEV protease cleaves the single-chain protein at the TEV protease cleavage site located within the integrated TEV cleavage site opioid binding domain, thereby converting the single-chain protein into its di-chain form.

BRIEF DESCRIPTION OF THE DRAWINGS

[025] **FIG. 1** shows the domain organization of naturally-occurring Clostridial toxins. The single chain form depicts the amino to carboxyl linear organization comprising an enzymatic domain, a translocation domain, and a H_c binding domain. The di-chain loop region located between the translocation and enzymatic domains is depicted by the double SS bracket. This region comprises an endogenous di-chain loop protease cleavage site that upon proteolytic cleavage with a naturally-occurring protease, such as, e.g., an endogenous Clostridial toxin protease or a naturally-occurring protease produced in the environment, converts the single chain form of the toxin into the di-chain form.

[026] **FIG. 2** shows a schematic of the current paradigm of neurotransmitter release and Clostridial toxin intoxication in a central and peripheral neuron. FIG. 2A shows a schematic for the neurotransmitter release mechanism of a central and peripheral neuron. The release process can be described as comprising two steps: 1) vesicle docking, where the vesicle-bound SNARE protein of a vesicle containing neurotransmitter molecules associates with the membrane-bound SNARE proteins located at the plasma membrane; and 2) neurotransmitter release, where the vesicle fuses with the plasma membrane and the neurotransmitter molecules are exocytosed. FIG. 2B shows a schematic of the intoxication mechanism for tetanus and botulinum toxin activity in a central and peripheral neuron. This intoxication process can be described as comprising four steps: 1) receptor binding, where a Clostridial toxin binds to a Clostridial receptor system and initiates the intoxication process; 2) complex internalization, where after toxin binding, a vesicle containing the toxin/receptor system complex is endocytosed into the cell; 3) light chain translocation, where multiple events are thought to occur, including, e.g., changes in the internal pH of the vesicle, formation of a channel pore comprising the H_N domain of the Clostridial toxin heavy chain, separation of the Clostridial toxin light chain from the heavy chain, and release of the active light chain and 4) enzymatic target modification, where the activate light chain of Clostridial toxin proteolytically cleaves its target SNARE substrate, such as, e.g., SNAP-25, VAMP or Syntaxin, thereby preventing vesicle docking and neurotransmitter release.

[027] Clostridia toxins produced by *Clostridium botulinum, Clostridium tetani, Clostridium baratii* and *Clostridium butyricum* are the most widely used in therapeutic and cosmetic treatments of humans and other mammals. Strains of C. *botulinum* produce seven antigenically-distinct types of Botulinum toxins (BoNTs), which have been identified by investigating botulism outbreaks in man (BoNT/A, *IB, IE* and *IF),* animals (BoNT/C1 and *ID),* or isolated from soil (BoNT/G). BoNTs possess approximately 35% amino acid identity with each other and share the same functional domain organization and overall structural architecture. It is recognized by those of skill in the art that within each type of Clostridial toxin there can be subtypes that differ somewhat in their amino acid sequence, and also in the nucleic acids encoding these proteins. For example, there are presently four BoNT/A subtypes, BoNT/A1 , BoNT/A2, BoNT/A3 and BoNT/A4, with specific subtypes showing approximately 89% amino acid identity when compared to another BoNT/A subtype. While all seven BoNT serotypes have similar structure and pharmacological

properties, each also displays heterogeneous bacteriological characteristics. In contrast, tetanus toxin (TeNT) is produced by a uniform group of *C. tetani.* Two other Clostridia species, *C. baratii* and C. *butyricum,* produce toxins, BaNT and BuNT, which are similar to BoNT/F and BoNT/E, respectively.

[028] Each mature di-chain molecule comprises three functionally distinct domains: 1) an enzymatic domain located in the LC that includes a metalloprotease region containing a zinc-dependent endopeptidase activity which specifically targets core components of the neurotransmitter release apparatus; 2) a translocation domain (H_N) contained within the amino-terminal half of the HC that facilitates release of the LC from intracellular vesicles into the cytoplasm of the target cell; and 3) a binding domain (H_C) found within the carboxyl-terminal half of the HC that determines the binding activity and binding specificity of the toxin to the receptor complex located at the surface of the target cell. The H_c domain comprises two distinct structural features of roughly equal size that indicate function and are designated the H_CN and H_Cc subdomains. Table 1 gives approximate boundary regions for each domain found in exemplary Clostridial toxins.

[029] Table 1. Clostridial Toxin Reference Sequences and Regions

Toxin	SEQ ID NO:	LC	H_N	H_C
BoNT/A	1	M1-K448	A449-K871	N872-L1296
BoNT/B	2	M1-K441	A442-S858	E859-E1291
BoNT/C1	3	M1-K449	T450-N866	N867-E1291
BoNT/D	4	M1-R445	D446-N862	S863-E1276
BoNT/E	5	M1-R422	K423-K845	R846-K1252
BoNT/F	6	M1-K439	A440-K864	K865-E1274
BoNT/G	7	M1-K446	S447-S863	N864-E1297
TeNT	8	M1-A457	S458-V879	I880-D1315
BaNT	9	M1-K431	N432-I857	I858-E1268
BuNT	10	M1-R422	K423-I847	K848-K1251

[030] The binding, translocation, and enzymatic activity of these three functional domains are all necessary for toxicity. While all details of this process are not yet precisely known, the overall cellular intoxication mechanism whereby Clostridial toxins enter a neuron and inhibit neurotransmitter release is similar, regardless of serotype or subtype. Although the applicants have no wish to be limited by the following description, the intoxication mechanism can be described as comprising at least four steps: 1) receptor binding, 2) complex internalization, 3) light chain translocation, and 4) enzymatic target modification (FIG. 3). The process is initiated when the H_c domain of a Clostridial toxin binds to a toxin-specific receptor system located on the plasma membrane surface of a target cell. The binding specificity of a receptor complex is thought to be achieved, in part, by specific combinations of gangliosides and protein receptors that appear to distinctly comprise each Clostridial toxin receptor complex. Once bound, the toxin/receptor complexes are internalized by endocytosis and the internalized vesicles are sorted to specific intracellular routes. The translocation step appears to be triggered by the acidification of the vesicle compartment. This process seems to initiate two important pH-dependent structural

rearrangements that increase hydrophobicity and promote formation of the di-chain form of the toxin. Once activated, light chain endopeptidase of the toxin is released from the intracellular vesicle into the cytosol where it appears to specifically target one of three known core components of the neurotransmitter release apparatus. These core proteins, vesicle-associated membrane protein (VAMP)/synaptobrevin, synaptosomal-associated protein of 25 kDa (SNAP-25) and Syntaxin, are necessary for synaptic vesicle docking and fusion at the nerve terminal and constitute members of the soluble /V-ethylmaleimide-sensitive factor-attachment protein-receptor (SNARE) family. BoNT/A and BoNT/E cleave SNAP-25 in the carboxyl-terminal region, releasing a nine or twenty-six amino acid segment, respectively, and BoNT/C1 also cleaves SNAP-25 near the carboxyl-terminus. The botulinum serotypes BoNT/B, BoNT/D, BoNT/F and BoNT/G, and tetanus toxin, act on the conserved central portion of VAMP, and release the amino-terminal portion of VAMP into the cytosol. BoNT/C1 cleaves syntaxin at a single site near the cytosolic membrane surface. The selective proteolysis of synaptic SNAREs accounts for the block of neurotransmitter release caused by Clostridial toxins *in vivo.* The SNARE protein targets of Clostridial toxins are common to exocytosis in a variety of non-neuronal types; in these cells, as in neurons, light chain peptidase activity inhibits exocytosis, see, e.g., Yann Humeau et al., *How Botulinum and Tetanus Neurotoxins Block Neurotransmitter Release,* 82(5) Biochimie. 427-446 (2000); Kathryn Turton et al., *Botulinum and Tetanus Neurotoxins: Structure, Function and Therapeutic Utility,* 27(1 1) Trends Biochem. Sci. 552-558. (2002); Giovanna Lalli et al., *The Journey of Tetanus and Botulinum Neurotoxins in Neurons,* 11(9) Trends Microbiol. 431-437, (2003).

[031] In an aspect of the invention, a modified Clostridial toxin comprises, in part, a single-chain modified Clostridial toxin and a di-chain modified Clostridial toxin. As discussed above, a Clostridial toxin, whether naturally-occurring or non-naturally-occurring, are initially synthesized as a single-chain polypeptide. This single-chain form is subsequently cleaved at a protease cleavage site located within a discrete di-chain loop region created between two cysteine residues that form a disulfide bridge by a protease. This posttranslational processing yields a di-chain molecule comprising a light chain (LC) and a heavy chain. As used herein, the term "di-chain loop region" refers to loop region of a naturally-occurring or non-naturally-occurring Clostridial toxin formed by a disulfide bridge located between the LC domain and the HC domain. As used herein, the term "single-chain modified Clostridial toxin" refers to any modified Clostridial toxin disclosed in the present specification that is in its single-chain form, *i.e.,* the toxin has not been cleaved at the protease cleavage site located within the di-chain loop region by its cognate protease. As used herein, the term "di-chain modified Clostridial toxin" refers to any modified Clostridial toxin disclosed in the present specification that is in its di-chain form, *i.e.,* the toxin has been cleaved at the protease cleavage site located within the di-chain loop region by its cognate protease.

[032] Aspects of the present invention provide, in part, polynucleotide molecules. As used herein, the term "polynucleotide molecule" is synonymous with "nucleic acid molecule" and means a polymeric form of nucleotides, such as, *e.g.,* ribonucleotides and deoxyribonucleotides, of any length. Useful polynucleotide molecules, include, without limitation, naturally-occurring and non-naturally-occurring DNA molecules and naturally-occurring and non-naturally-occurring RNA molecules. Non-limiting examples of naturally-occurring and non-naturally-occurring DNA molecules include single-stranded DNA molecules, double-stranded DNA molecules, genomic DNA molecules, cDNA molecules, vector constructs, such as, *e.g.,* plasmid constructs, phagemid constructs, bacteriophage constructs, retroviral constructs and

artificial chromosome constructs. Non-limiting examples of naturally-occurring and non-naturally-occurring RNA molecules include single-stranded RNA, double stranded RNA and mRNA.

[033] Well-established molecular biology techniques that may be necessary to make a polynucleotide molecule encoding a modified Clostridial toxin disclosed in the present specification include, but not limited to, procedures involving polymerase chain reaction (PCR) amplification, restriction enzyme reactions, agarose gel electrophoresis, nucleic acid ligation, bacterial transformation, nucleic acid purification, nucleic acid sequencing and recombination-based techniques are routine and well within the scope of one skilled in the art and from the teaching herein. Non-limiting examples of specific protocols necessary to make a polynucleotide molecule encoding a modified Clostridial toxin are described in e.g., MOLECULAR CLONING A LABORATORY MANUAL, *supra,* (2001); and CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (Frederick M. Ausubel et al., eds. John Wiley & Sons, 2004). Additionally, a variety of commercially available products useful for making a polynucleotide molecule encoding a modified Clostridial toxin are widely available. These protocols are routine procedures well within the scope of one skilled in the art and from the teaching herein.

[034] The methods disclosed in the present specification include, in part, an open reading frame. As used herein, the term "open reading frame" is synonymous with "ORF" and means any polynucleotide molecule that encodes a protein, or a portion of a protein. An open reading frame usually begins with a start codon (represented as, e.g. AUG for an RNA molecule and ATG in a DNA molecule in the standard code) and is read in codon-triplets until the frame ends with a STOP codon (represented as, e.g. UAA, UGA or UAG for an RNA molecule and TAA, TGA or TAG in a DNA molecule in the standard code). As used herein, the term "codon" means a sequence of three nucleotides in a polynucleotide molecule that specifies a particular amino acid during protein synthesis; also called a triplet or codon-triplet.

[035] The methods disclosed in the present specification include, in part, an expression construct. An expression construct comprises a polynucleotide molecule including an open reading frame disclosed in the present specification operably-linked to an expression vector useful for expressing the polynucleotide molecule in a cell or cell-free extract. A wide variety of expression vectors can be employed for expressing a polynucleotide molecule disclosed in the present specification, including, without limitation, a viral expression vector; a prokaryotic expression vector; eukaryotic expression vectors, such as, e.g., a yeast expression vector, an insect expression vector and a mammalian expression vector; and a cell-free *(in vitro)* expression vector. It is further understood that expression vectors useful to practice aspects of these methods may include those which express the polynucleotide molecule under control of a constitutive, tissue-specific, cell-specific or inducible promoter element, enhancer element or both. Non-limiting examples of expression vectors, along with well-established reagents and conditions for making and using an expression construct from such expression vectors are readily available from commercial vendors that include, without limitation, BD Biosciences-Clontech, Palo Alto, CA; BD Biosciences Pharmingen, San Diego, CA; Invitrogen, Inc, Carlsbad, CA; EMD Biosciences-Novagen, Madison, WI; QIAGEN, Inc., Valencia, CA; and Stratagene, La Jolla, CA. The selection, making and use of an appropriate expression vector are routine procedures well within the scope of one skilled in the art and from the teachings herein.

[036] The expression constructs disclosed in the present specification can comprise an open reading frame encoding a protein including a di-chain loop region comprising an exogenous protease cleavage

site, wherein cleavage of the exogenous protease cleavage site converts the single-chain protein into its di-chain form. In aspects of this embodiment, a viral expression vector is operably-linked to a polynucleotide molecule encoding a protein comprising an exogenous protease cleavage site located within the di-chain loop; a prokaryotic expression vector is operably-linked to a polynucleotide molecule encoding a protein comprising an exogenous protease cleavage site located within the di-chain loop; a yeast expression vector is operably-linked to a polynucleotide molecule encoding a protein comprising an exogenous protease cleavage site located within the di-chain loop; an insect expression vector is operably-linked to a polynucleotide molecule encoding a protein comprising an exogenous protease cleavage site located within the di-chain loop; and a mammalian expression vector is operably-linked to a polynucleotide molecule encoding a protein comprising an exogenous protease cleavage site located within the di-chain loop. In other aspects of this embodiment, an expression construct - suitable for expressing a polynucleotide molecule disclosed in the present specification can be expressed using a cell-free extract. In an aspect of this embodiment, a cell-free expression vector is operably linked to a polynucleotide molecule encoding a protein comprising an exogenous protease cleavage site located within the di-chain loop.

[037] In an embodiment, an expression construct disclosed in the present specification can comprise an open reading frame encoding a Clostridial toxin comprising a di-chain loop region comprising an exogenous protease cleavage site. In aspects of this embodiment, an expression construct disclosed in the present specification can comprise an open reading frame encoding a Clostridial toxin comprising a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a Clostridial toxin binding domain, and a di-chain loop region comprising an exogenous protease cleavage site. In aspects of this embodiment, the single-chain Clostridial toxin comprises a linear amino-to-carboxyl order of 1) the Clostridial enzymatic domain, the di-chain loop region comprising an exogenous protease cleavage site, the Clostridial translocation domain and the Clostridial binding domain; 2) the Clostridial enzymatic domain, the di-chain loop region comprising an exogenous protease cleavage site, the Clostridial binding domain and the Clostridial translocation domain; 3) the Clostridial binding domain, the Clostridial toxin translocation domain, the di-chain loop region comprising an exogenous protease cleavage site and the Clostridial toxin enzymatic domain; 4) the Clostridial binding domain, the Clostridial toxin enzymatic domain, the di-chain loop region comprising an exogenous protease cleavage site and the Clostridial toxin translocation domain; 5) the Clostridial toxin translocation domain, the di-chain loop region comprising an exogenous protease cleavage site, the Clostridial toxin enzymatic domain and the Clostridial binding domain; or 6) the Clostridial toxin translocation domain, the di-chain loop region comprising an exogenous protease cleavage site, the Clostridial binding domain and the Clostridial toxin enzymatic domain.

[038] In other aspects of this embodiment, an expression construct comprises an open reading frame encoding 1) a BoNT/A toxin enzymatic domain, a BoNT/A translocation domain, a BoNT/A binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 2) a BoNT/B enzymatic domain, a BoNT/B translocation domain, a BoNT/B binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 3) a BoNT/C1 enzymatic domain, a BoNT/C1 translocation domain, a BoNT/C1 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 4) a BoNT/D enzymatic domain, a BoNT/D translocation domain, a BoNT/D

binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 5) a BoNT/E enzymatic domain, a BoNT/E translocation domain, a BoNT/E binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 6) a BoNT/F enzymatic domain, a BoNT/F translocation domain, a BoNT/F binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 7) a BoNT/G enzymatic domain, a BoNT/G translocation domain, a BoNT/G binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 8) a TeNT enzymatic domain, a TeNT translocation domain, a TeNT binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 9) a BaNT enzymatic domain, a BaNT translocation domain, a BaNT binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 10) a BuNT enzymatic domain, a BuNT translocation domain, a BuNT binding domain, and a di-chain loop region comprising an exogenous protease cleavage site.

[039] In further other aspects of this embodiment, an expression construct comprises an open reading frame encoding 1) a BoNT/A toxin enzymatic domain, a BoNT/A translocation domain, a BoNT/A binding domain, and a di-chain loop region comprising a TEV protease cleavage site; 2) a BoNT/B enzymatic domain, a BoNT/B translocation domain, a BoNT/B binding domain, and a di-chain loop region comprising a TEV protease cleavage site; 3) a BoNT/C1 enzymatic domain, a BoNT/C1 translocation domain, a BoNT/C1 binding domain, and a di-chain loop region comprising a TEV protease cleavage site; 4) a BoNT/D enzymatic domain, a BoNT/D translocation domain, a BoNT/D binding domain, and a di-chain loop region comprising a TEV protease cleavage site; 5) a BoNT/E enzymatic domain, a BoNT/E translocation domain, a BoNT/E binding domain, and a di-chain loop region comprising a TEV protease cleavage site; 6) a BoNT/F enzymatic domain, a BoNT/F translocation domain, a BoNT/F binding domain, and a di-chain loop region comprising a TEV protease cleavage site; 7) a BoNT/G enzymatic domain, a BoNT/G translocation domain, a BoNT/G binding domain, and a di-chain loop region comprising a TEV protease cleavage site; 8) a TeNT enzymatic domain, a TeNT translocation domain, a TeNT binding domain, and a di-chain loop region comprising a TEV protease cleavage site; 9) a BaNT enzymatic domain, a BaNT translocation domain, a BaNT binding domain, and a di-chain loop region comprising a TEV protease cleavage site; 10) a BuNT enzymatic domain, a BuNT translocation domain, a BuNT binding domain, and a di-chain loop region comprising a TEV protease cleavage site.

[040] Examples of such Clostridial toxins comprising a di-chain loop region comprising an exogenous protease cleavage sit are described in, e.g., J. Oliver Dolly, et al., *Activatable Recombinant Neurotoxins,* U.S. Patent 7,132,529; J. Oliver Dolly, et al., *Activatable Recombinant Neurotoxins,* U.S. Patent 7,419,676; Lance Steward, et al., *Leucine-Based Motifs and Clostridial Neurotoxins,* U.S. Patent 6,903,187; Lance Steward, et al., *Leucine-Based Motifs and Clostridial Neurotoxins,* U.S. Patent 7,393,925; Wei-Jen Lin, et al., *Neurotoxins with Enhanced Target Specificity,* U.S. Patent 7,273,722; Lance Steward, et al., *Modified Botulinum Neurotoxins,* U.S. Patent 7,491,799; Lance E. Steward, et al., *Optimized Expression of Active Botulinum Toxin Type E,* U.S. Patent Publication 2008/0138893; Ester Fernandez-Salas, et al., *Optimized Expression of Active Botulinum Toxin Type A,* U.S. Patent Publication 2008/0057575; each of which is hereby incorporated by reference in its entirety.

[041] In another embodiment, an expression construct disclosed in the present specification can comprise an open reading frame encoding a protein comprising a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a non-Clostridial toxin binding domain, and a di-chain loop region

comprising an exogenous protease cleavage site. In aspects of this embodiment, the single-chain protein comprises a linear amino-to-carboxyl order of 1) the Clostridial enzymatic domain, the di-chain loop region comprising an exogenous protease cleavage site, the Clostridial translocation domain and the non-Clostridial binding domain; 2) the Clostridial enzymatic domain, the di-chain loop region comprising an exogenous protease cleavage site, the non-Clostridial binding domain and the Clostridial translocation domain; 3) the non-Clostridial binding domain, the Clostridial toxin translocation domain, the di-chain loop region comprising an exogenous protease cleavage site and the Clostridial toxin enzymatic domain; 4) the non-Clostridial binding domain, the Clostridial toxin enzymatic domain, the di-chain loop region comprising an exogenous protease cleavage site and the Clostridial toxin translocation domain; 5) the Clostridial toxin translocation domain, the di-chain loop region comprising an exogenous protease cleavage site, the Clostridial toxin enzymatic domain and the non-Clostridial binding domain; or 6) the Clostridial toxin translocation domain, the di-chain loop region comprising an exogenous protease cleavage site, the non-Clostridial binding domain and the Clostridial toxin enzymatic domain.

[042] In other aspects of this embodiment, an expression construct comprises an open reading frame encoding a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, an opioid binding domain, and a di-chain loop region comprising an exogenous protease cleavage site. In further aspects of this embodiment, an expression construct comprises an open reading frame encoding 1) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, an enkephalin binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 2) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a bovine adrenomedullary-22 (BAM22) peptide binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 3) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, an endomorphin binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 4) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, an endorphin binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 5) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a dynorphin binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 6) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a nociceptin binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 7) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a hemorphin binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; or 8) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a rimorphin binding domain, and a di-chain loop region comprising an exogenous protease cleavage site.

[043] In other aspects of this embodiment, an expression construct comprises an open reading frame encoding a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a melanocortin peptide binding domain, and a di-chain loop region comprising an exogenous protease cleavage site. In further aspects of this embodiment, an expression construct comprises an open reading frame encoding 1) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, an melanocyte stimulating hormone binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 2) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, an adrenocorticotropin binding domain, and a di-chain loop region comprising an exogenous protease

cleavage site; or 3) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a lipotropin binding domain, and a di-chain loop region comprising an exogenous protease cleavage site.

[044] In other aspects of this embodiment, an expression construct comprises an open reading frame encoding a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a galanin peptide binding domain, and a di-chain loop region comprising an exogenous protease cleavage site. In further aspects of this embodiment, an expression construct comprises an open reading frame encoding 1) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a galanin binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; or 2) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a galanin message-associated peptide (GMAP) binding domain, and a di-chain loop region comprising an exogenous protease cleavage site.

[045] In other aspects of this embodiment, an expression construct comprises an open reading frame encoding a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a granin peptide binding domain, and a di-chain loop region comprising an exogenous protease cleavage site. In further aspects of this embodiment, an expression construct comprises an open reading frame encoding 1) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a chromogranin A binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 2) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a chromogranin B binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; or 3) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a chromogranin C binding domain, and a di-chain loop region comprising an exogenous protease cleavage site.

[046] In other aspects of this embodiment, an expression construct comprises an open reading frame encoding a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a tachykinin peptide binding domain, and a di-chain loop region comprising an exogenous protease cleavage site. In further aspects of this embodiment, an expression construct comprises an open reading frame encoding 1) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a Substance P binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 2) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a neuropeptide K binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 3) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a neuropeptide gamma binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 4) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a neurokinin A binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 5) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a hemokinin binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; or 6) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a endokinin binding domain, and a di-chain loop region comprising an exogenous protease cleavage site.

[047] In other aspects of this embodiment, an expression construct comprises an open reading frame encoding a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a Neuropeptide Y related peptide binding domain, and a di-chain loop region comprising an exogenous protease cleavage site. In further aspects of this embodiment, an expression construct comprises an open reading frame encoding 1) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a neuropeptide

Y (NPY) binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 2) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a Peptide YY (PYY) binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 3) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a Pancreatic peptide (PP) binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; or 4) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a Pancreatic icosapeptide (PIP) binding domain, and a di-chain loop region comprising an exogenous protease cleavage site.

[048] In other aspects of this embodiment, an expression construct comprises an open reading frame encoding a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a neurohormone peptide binding domain, and a di-chain loop region comprising an exogenous protease cleavage site. In further aspects of this embodiment, an expression construct comprises an open reading frame encoding 1) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a corticotropin-releasing hormone (CCRH) binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 2) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a parathyroid hormone (PTH) binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 3) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a thyrotropin-releasing hormone (TRH) binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; or 4) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a somatostatin binding domain, and a di-chain loop region comprising an exogenous protease cleavage site.

[049] In other aspects of this embodiment, an expression construct comprises an open reading frame encoding a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a cytokine peptide binding domain, and a di-chain loop region comprising an exogenous protease cleavage site. In further aspects of this embodiment, an expression construct comprises an open reading frame encoding 1) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a ciliary neurotrophic factor (CNTF) binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 2) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a glycophorin-A (GPA) binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 3) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a leukemia inhibitory factor (LIF) binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 4) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, an interleukin (IL) binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 5) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, an onostatin M binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 6) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a cardiotrophin-1 (CT-1) binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 7) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a cardiotrophin-like cytokine (CLC) binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 8) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a neuroleukin binding domain, and a di-chain loop region comprising an exogenous protease cleavage site.

[050] In other aspects of this embodiment, an expression construct comprises an open reading frame encoding a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a kinin peptide binding domain, and a di-chain loop region comprising an exogenous protease cleavage site. In further aspects of this embodiment, an expression construct comprises an open reading frame encoding 1) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a bradykinin binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 2) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a kallidin binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 3) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a desArg9 bradykinin binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; or 4) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a desArg l O bradykinin binding domain, and a di-chain loop region comprising an exogenous protease cleavage site.

[051] In other aspects of this embodiment, an expression construct comprises an open reading frame encoding a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a Fibroblast growth factor (FGF) peptide binding domain, and a di-chain loop region comprising an exogenous protease cleavage site. In further aspects of this embodiment, an expression construct comprises an open reading frame encoding 1) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a FGF-1 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 2) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a FGF-2 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 3) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a FGF-4 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 4) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a FGF-8 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 5) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a FGF-9 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 6) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a FGF-1 7 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; or 4) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a FGF-1 8 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site.

[052] In other aspects of this embodiment, an expression construct comprises an open reading frame encoding a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a neurotrophin peptide binding domain, and a di-chain loop region comprising an exogenous protease cleavage site. In further aspects of this embodiment, an expression construct comprises an open reading frame encoding 1) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a nerve growth factor (NGF) binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 2) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a brain derived neurotrophic factor (BDNF) binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 3) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a neurotrophin-3 (NT-3) binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 4) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a neurotrophin-4/5 (NT-4/5)

binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; or 5) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a head activator peptide (HA) binding domain, and a di-chain loop region comprising an exogenous protease cleavage site.

[053] In other aspects of this embodiment, an expression construct comprises an open reading frame encoding a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a tumor necrosis factor (TNF)peptide binding domain, and a di-chain loop region comprising an exogenous protease cleavage site.

[054] In other aspects of this embodiment, an expression construct comprises an open reading frame encoding a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a Glial derived growth factor (GDNF) peptide binding domain, and a di-chain loop region comprising an exogenous protease cleavage site. In further aspects of this embodiment, an expression construct comprises an open reading frame encoding 1) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a neurturin binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 2) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a persephrin binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; or 3) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, an artemin binding domain, and a di-chain loop region comprising an exogenous protease cleavage site.

[055] In other aspects of this embodiment, an expression construct comprises an open reading frame encoding a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a Transformation growth factor β (TΘPβ) peptide binding domain, and a di-chain loop region comprising an exogenous protease cleavage site. In further aspects of this embodiment, an expression construct comprises an open reading frame encoding 1) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a TΘPβI binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 2) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a TΘPβ2 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 3) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a TΘPβ3 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; or 4) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a TΘPβ4 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site.

[056] In other aspects of this embodiment, an expression construct comprises an open reading frame encoding a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a Bone morphogenetic protein β (BMP) peptide binding domain, and a di-chain loop region comprising an exogenous protease cleavage site. In further aspects of this embodiment, an expression construct comprises an open reading frame encoding 1) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a BMP2 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 2) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a BMP3 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 3) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a BMP4 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 4) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a BMP5 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 5) a Clostridial toxin enzymatic domain, a Clostridial

toxin translocation domain, a BMP6 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 6) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a BMP7 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 7) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a BMP8 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; or 8) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a BMP10 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site.

[057] In other aspects of this embodiment, an expression construct comprises an open reading frame encoding a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a Growth differentiation factor β (GDF) peptide binding domain, and a di-chain loop region comprising an exogenous protease cleavage site. In further aspects of this embodiment, an expression construct comprises an open reading frame encoding 1) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a GDF1 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 2) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a GDF2 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 3) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a GDF3 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 4) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a GDF5 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 5) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a GDF6 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 6) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a GDF7 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 7) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a GDF8 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 8) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a GDF10 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 9) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a GDF11 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; or 10) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a GDF15 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site.

[058] In other aspects of this embodiment, an expression construct comprises an open reading frame encoding a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, an activin peptide binding domain, and a di-chain loop region comprising an exogenous protease cleavage site. In further aspects of this embodiment, an expression construct comprises an open reading frame encoding 1) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, an activin A binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 2) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, an activin B binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 3) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, an activin C binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 4) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, an activin E binding domain, and a di-chain loop region comprising an exogenous

protease cleavage site; or 5) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, an inhibin A binding domain, and a di-chain loop region comprising an exogenous protease cleavage site.

[059] In other aspects of this embodiment, an expression construct comprises an open reading frame encoding a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a Vascular endothelial growth factor (VEGF) peptide binding domain, and a di-chain loop region comprising an exogenous protease cleavage site.

[060] In other aspects of this embodiment, an expression construct comprises an open reading frame encoding a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, an insulin growth factor (IGF) peptide binding domain, and a di-chain loop region comprising an exogenous protease cleavage site. In further aspects of this embodiment, an expression construct comprises an open reading frame encoding 1) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, an IGF-1 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; or 2) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, an IGF-2 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site.

[061] In other aspects of this embodiment, an expression construct comprises an open reading frame encoding a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, an Epidermal growth factor (EGF) peptide binding domain, and a di-chain loop region comprising an exogenous protease cleavage site.

[062] In other aspects of this embodiment, an expression construct comprises an open reading frame encoding a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a Glucagon like hormone peptide binding domain, and a di-chain loop region comprising an exogenous protease cleavage site. In further aspects of this embodiment, an expression construct comprises an open reading frame encoding 1) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a secretin binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; or 2) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a glucagon-like peptide binding domain, and a di-chain loop region comprising an exogenous protease cleavage site.

[063] In other aspects of this embodiment, an expression construct comprises an open reading frame encoding a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a Pituitary adenylate cyclase activating peptide (PACAP) peptide binding domain, and a di-chain loop region comprising an exogenous protease cleavage site.

[064] In other aspects of this embodiment, an expression construct comprises an open reading frame encoding a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a Growth hormone-releasing hormone (GHRH) peptide binding domain, and a di-chain loop region comprising an exogenous protease cleavage site.

[065] In other aspects of this embodiment, an expression construct comprises an open reading frame encoding a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a Growth hormone-releasing hormone (GHRH) peptide binding domain, and a di-chain loop region comprising an exogenous protease cleavage site.

[066] In other aspects of this embodiment, an expression construct comprises an open reading frame encoding a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a Vasoactive

intestinal peptide (VIP) peptide binding domain, and a di-chain loop region comprising an exogenous protease cleavage site. In further aspects of this embodiment, an expression construct comprises an open reading frame encoding 1) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a VIP1 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; or 2) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a VIP2 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site.

[067] In other aspects of this embodiment, an expression construct comprises an open reading frame encoding a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a Gastric inhibitory polypeptide (GIP) peptide binding domain, and a di-chain loop region comprising an exogenous protease cleavage site.

[068] In other aspects of this embodiment, an expression construct comprises an open reading frame encoding a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a Calcitonin-related peptidesvisceral gut peptide binding domain, and a di-chain loop region comprising an exogenous protease cleavage site. In further aspects of this embodiment, an expression construct comprises an open reading frame encoding 1) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a gastrin binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 2) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a gastrin-releasing peptide binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; or 3) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a cholecystokinin (CCK) binding domain, and a di-chain loop region comprising an exogenous protease cleavage site.

[069] In other aspects of this embodiment, an expression construct comprises an open reading frame encoding a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a protease activated receptor (PAR) peptide binding domain, and a di-chain loop region comprising an exogenous protease cleavage site. In further aspects of this embodiment, an expression construct comprises an open reading frame encoding 1) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a PARI binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 2) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a PAR2 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; 3) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a PAR3 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; or 4) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a PAR3 binding domain, and a di-chain loop region comprising an exogenous protease cleavage site.

[070] Examples, of such proteins comprising a di-chain loop region comprising an exogenous protease cleavage site are described in, e.g., J. Oliver Dolly, et al., *Activatable Recombinant Neurotoxins,* U.S. Patent 7,132,529; J. Oliver Dolly, et al., *Activatable Recombinant Neurotoxins,* U.S. Patent 7,419,676; Lance E. Steward et al., *Multivalent Clostridial Toxin Derivatives and Methods of Their Use,* U.S. Patent 7,514,088; Keith A. Foster et al., *Re-targeted Toxin Conjugates,* International Patent Publication WO 2005/023309; Lance E. Steward, et al., *Activatable Recombinant Neurotoxins,* U.S. Patent Publication 2008/0032930; Lance E. Steward, et al., *Activatable Recombinant Neurotoxins,* U.S. Patent Publication 2008/0032931 ; Lance E. Steward, et al., *Activatable Recombinant Neurotoxins,* U.S. Patent Publication

2008/01 6 1226; Lance E. Steward, et al., *Activatable Recombinant Neurotoxins,* U.S. Patent Publication 2008/0221 0 12; Lance E. Steward, et al., *Activatable Recombinant Neurotoxins,* U.S. Patent Publication 2009/0004224; Lance E. Steward, et al., *Activatable Recombinant Neurotoxins,* U.S. Patent Publication 2009/000531 3; Lance E. Steward, et al., *Activatable Recombinant Neurotoxins,* U.S. Patent Publication 2009/00 18081; Lance E. Steward, et al., *Activatable Recombinant Neurotoxins,* U.S. Patent Publication 2009/0069238; and Lance E. Steward et al., *Multivalent Clostridial Toxin Derivatives and Methods of Their Use,* U.S. Patent Publication 2009/004843 1, each of which is hereby incorporated by reference in its entirety.

[071] In another embodiment, an expression construct comprises an open reading frame encoding a protein comprising a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, and an integrated protease cleavage site-binding domain. In aspects of this embodiment, the single-chain protein comprises a linear amino-to-carboxyl order of 1) an integrated protease cleavage site-binding domain, a Clostridial toxin translocation domain and a Clostridial toxin enzymatic domain; 2) an integrated protease cleavage site-binding domain, a Clostridial toxin enzymatic domain, and a Clostridial toxin translocation domain; 3) a Clostridial toxin enzymatic domain, an integrated protease cleavage site-binding domain, and a Clostridial toxin translocation domain; 4) a Clostridial toxin translocation domain, an integrated protease cleavage site-binding domain, and a Clostridial toxin enzymatic domain; 5) a Clostridial toxin translocation domain, a Clostridial toxin enzymatic domain, and an integrated protease cleavage site-binding domain; and 6) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, and an integrated protease cleavage site-binding domain.

[072] In other aspects of this embodiment, an expression construct comprises an open reading frame encoding a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, an integrated TEV protease cleavage site-opioid binding domain. In further aspects of this embodiment, an expression construct comprises an open reading frame encoding 1) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, an integrated TEV protease cleavage site-enkephalin binding domain; 2) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, an integrated TEV protease cleavage site-bovine adrenomedullary-22 (BAM22) binding domain; 3) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, an integrated TEV protease cleavage site-endomorphin binding domain; 4) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, an integrated TEV protease cleavage site-endorphin binding domain; 5) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, an integrated TEV protease cleavage site-dynorphin binding domain; 6) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, an integrated TEV protease cleavage site-nociceptin binding domain; **7)** a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, an integrated TEV protease cleavage site-hemorphin binding domain; or 8) a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, an integrated TEV protease cleavage site-rimorphin binding domain

[073] Examples, of such proteins comprising integrated protease cleavage site-binding domain are described in, *e.g.,* companion patent application Sanjiv Ghanshani, et al., Modified Clostridial Toxins Comprising an Integrated Protease Cleavage Site-Binding Domain, which is hereby incorporated by reference in its entirety.

[074] The expression constructs disclosed in the present specification can comprise an open reading frame encoding a protease. In aspects of this embodiment, a viral expression vector is operably-linked to a polynucleotide molecule encoding a protease; a prokaryotic expression vector is operably-linked to a polynucleotide molecule encoding a protease; a yeast expression vector is operably-linked to a polynucleotide molecule encoding a protease; an insect expression vector is operably-linked to a polynucleotide molecule encoding a protease; and a mammalian expression vector is operably-linked to a polynucleotide molecule encoding a protease. In other aspects of this embodiment, an expression construct is suitable for expressing a polynucleotide molecule disclosed in the present specification can be expressed using a cell-free extract. In an aspect of this embodiment, a cell-free extract expression vector is operably linked to a polynucleotide molecule encoding a protease.

[075] In aspect of this embodiment, an expression construct comprising an open reading frame encodes an enterokinase, a human rhinovirus 3C protease, a human enterovirus 3C protease, a tobacco etch virus (TEV) protease, a Tobacco Vein Mottling Virus (TVMV) protease, a subtilisin protease, or a Caspase 3 protease. Examples of Enterokinase proteases and the polynucleotide molecules that encode them are described in, e.g., Edward R. LaVallie, *Cloning of Enterokinase and Method of Use,* U.S. Patent 5,665,566; Edward R. LaVallie, *Cloning of Enterokinase and Method of Use,* U.S. Patent 6,746,859, each of which is hereby incorporated by reference in its entirety. Examples of subtilisin proteases and the polynucleotide molecules that encode them are described in, e.g., Donn N. Rubingh, et al., *Subtillisin Protease Variants having Amino Acid Deletions and Substitutions in Defined Epitope Regions,* U.S. Patent 6,586,224, which is hereby incorporated by reference in its entirety.

[076] In another aspect of this embodiment, an enterokinase is SEQ ID NO: 11. In another aspect of this embodiment, an enterokinase comprises amino acids 239-1035 of SEQ ID NO: 11. In yet another aspect of this embodiment, an enterokinase is a naturally occurring enterokinase variant, such as, e.g., an enterokinase isoform. In still another aspect of this embodiment, an enterokinase is a non-naturally occurring enterokinase variant, such as, e.g., a conservative enterokinase variant, a non-conservative enterokinase variant, an enterokinase chimeric, an active enterokinase fragment, or any combination thereof. In another aspect of this embodiment, an Enterokinase is one disclosed in U.S. Patent 5,665,566 or U.S. Patent 6,746,859. In another aspect of this embodiment, an enterokinase, a naturally occurring enterokinase variant, or a non-naturally occurring enterokinase variant is obtained from a species of mammal such as, e.g., a human, a cow, or a rodent.

[077] In other aspects of this embodiment, an enterokinase comprises a polypeptide having an amino acid identity of, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95% to SEQ ID NO: 11; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90% or at most 95% to SEQ ID NO: 11. In yet other aspects of this embodiment, an enterokinase comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 11; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 11. In still other aspects of this embodiment, an enterokinase comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 11; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 11.

[078] In another aspect of this embodiment, a human rhinovirus 3C protease is SEQ ID NO: 12. In yet another aspect of this embodiment, a human rhinovirus 3C protease is a naturally occurring human rhinovirus 3C protease variant, such as, e.g., a human rhinovirus 3C protease isoform. In still another aspect of this embodiment, a human rhinovirus 3C protease is a non-naturally occurring human rhinovirus 3C protease variant, such as, e.g., a conservative human rhinovirus 3C protease variant, a non-conservative human rhinovirus 3C protease variant, a human rhinovirus 3C protease chimeric, an active human rhinovirus 3C protease fragment, or any combination thereof. In another aspect of this embodiment, a human rhinovirus 3C protease, a naturally occurring human rhinovirus 3C protease variant, or a non-naturally occurring human rhinovirus 3C protease variant is obtained from a species of Rhinovirus.

[079] In other aspects of this embodiment, a human rhinovirus 3C protease comprises a polypeptide having an amino acid identity of, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95% to SEQ ID NO: 12; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90% or at most 95% to SEQ ID NO: 12. In yet other aspects of this embodiment, a human rhinovirus 3C protease comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 12; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 12. In still other aspects of this embodiment, a human rhinovirus 3C protease comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 12; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 12.

[080] In another aspect of this embodiment, a human enterovirus 3C protease is SEQ ID NO: 13. In yet another aspect of this embodiment, a human enterovirus 3C protease is a naturally occurring human enterovirus 3C protease variant, such as, e.g., a human enterovirus 3C protease isoform. In still another aspect of this embodiment, a human enterovirus 3C protease is a non-naturally occurring human enterovirus 3C protease variant, such as, e.g., a conservative human enterovirus 3C protease variant, a non-conservative human enterovirus 3C protease variant, a human enterovirus 3C protease chimeric, an active human enterovirus 3C protease fragment, or any combination thereof. In another aspect of this embodiment, a human enterovirus 3C protease, a naturally occurring human enterovirus 3C protease variant, or a non-naturally occurring human enterovirus 3C protease variant is obtained from a species of Enterovirus.

[081] In other aspects of this embodiment, a human enterovirus 3C protease comprises a polypeptide having an amino acid identity of, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95% to SEQ ID NO: 13; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90% or at most 95% to SEQ ID NO: 13. In yet other aspects of this embodiment, a human enterovirus 3C protease comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 13; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 13. In still other aspects of this embodiment, a human enterovirus 3C protease comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30,

40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 13; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 13.

[082] In another aspect of this embodiment, a TEV protease is SEQ ID NO: 14. In another aspect of this embodiment, a TEV protease comprises amino acids 2038-2270 of SEQ IS NO: 14. In another aspect of this embodiment, a TEV protease comprises SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, or SEQ ID NO: 23. In yet another aspect of this embodiment, a TEV protease is a naturally occurring TEV protease variant, such as, e.g., a TEV protease isoform. In still another aspect of this embodiment, a TEV protease is a non-naturally occurring TEV protease variant, such as, e.g., a conservative TEV protease variant, a non-conservative TEV protease variant, a TEV protease chimeric, an active TEV protease fragment, or any combination thereof. In another aspect of this embodiment, a TEV protease, a naturally occurring TEV protease variant, or a non-naturally occurring TEV protease variant is obtained from a species of Potyvirus.

[083] In other aspects of this embodiment, a TEV protease comprises a polypeptide having an amino acid identity of, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95% to SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, or SEQ ID NO: 23; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90% or at most 95% to SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, or SEQ ID NO: 23. In yet other aspects of this embodiment, a TEV protease comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, or SEQ ID NO: 23; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, or SEQ ID NO: 23. In still other aspects of this embodiment, a TEV protease comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, or SEQ ID NO: 23; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, or SEQ ID NO: 23.

[084] In another aspect of this embodiment, a TVMV protease is SEQ ID NO: 24. In another aspect of this embodiment, a TEV protease comprises amino acids 2002-2236 of SEQ IS NO: 24. In yet another aspect of this embodiment, a TVMV protease is a naturally occurring TVMV protease variant, such as, e.g., a TVMV protease isoform. In still another aspect of this embodiment, a TVMV protease is a non-naturally occurring TVMV protease variant, such as, e.g., a conservative TVMV protease variant, a non-conservative TVMV protease variant, a TVMV protease chimeric, an active TVMV protease fragment, or

any combination thereof. In another aspect of this embodiment, a TVMV protease, a naturally occurring TVMV protease variant, or a non-naturally occurring TVMV protease variant is obtained from a species of Potyvirus.

[085] In other aspects of this embodiment, a TVMV protease comprises a polypeptide having an amino acid identity of, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95% to SEQ ID NO: 24 or amino acids 2002-2236 of SEQ IS NO: 24; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90% or at most 95% to SEQ ID NO: 24 or amino acids 2002-2236 of SEQ IS NO: 24. In yet other aspects of this embodiment, a TVMV protease comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 24 or amino acids 2002-2236 of SEQ IS NO: 24; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 24 or amino acids 2002-2236 of SEQ IS NO: 24. In still other aspects of this embodiment, a TVMV protease comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 24 or amino acids 2002-2236 of SEQ IS NO: 24; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 24 or amino acids 2002-2236 of SEQ IS NO: 24.

[086] In another aspect of this embodiment, a subtilisin protease is SEQ ID NO: 25. In another aspect of this embodiment, a subtilisin protease comprises amino acids 107-365 of SEQ IS NO: 25. In yet another aspect of this embodiment, a subtilisin protease is a naturally occurring subtilisin protease variant, such as, e.g., a subtilisin protease isoform. In still another aspect of this embodiment, a subtilisin protease is a non-naturally occurring subtilisin protease variant, such as, e.g., a conservative subtilisin protease variant, a non-conservative subtilisin protease variant, a subtilisin protease chimeric, an active subtilisin protease fragment, or any combination thereof. In another aspect of this embodiment, a subtilisin protease, a naturally occurring subtilisin protease variant, or a non-naturally occurring subtilisin protease variant is obtained from a species of Bacillus.

[087] In other aspects of this embodiment, a subtilisin protease comprises a polypeptide having an amino acid identity of, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95% to SEQ ID NO: 25 or amino acids 107-365 of SEQ IS NO: 25; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90% or at most 95% to SEQ ID NO: 25 or amino acids 107-365 of SEQ IS NO: 25. In yet other aspects of this embodiment, a subtilisin protease comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 25 or amino acids 107-365 of SEQ IS NO: 25; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 25 or amino acids 107-365 of SEQ IS NO: 25. In still other aspects of this embodiment, a subtilisin protease comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 25 or amino acids 107-365 of SEQ IS NO: 25; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 25 or amino acids 107-365 of SEQ IS NO: 25.

[088] In another aspect of this embodiment, a Caspase 3 protease is SEQ ID NO: 26. In yet another aspect of this embodiment, a Caspase 3 protease is a naturally occurring Caspase 3 protease variant, such as, e.g., a Caspase 3 protease isoform. In still another aspect of this embodiment, a Caspase 3 protease is a non-naturally occurring Caspase 3 protease variant, such as, e.g., a conservative Caspase 3 protease variant, a non-conservative Caspase 3 protease variant, a Caspase 3 protease chimeric, an active Caspase 3 protease fragment, or any combination thereof. In another aspect of this embodiment, a Caspase 3 protease, a naturally occurring Caspase 3 protease variant, or a non-naturally occurring Caspase 3 protease variant is obtained from a species of mammal such as, e.g., a human, a cow, or a rodent.

[089] In other aspects of this embodiment, a Caspase 3 protease comprises a polypeptide having an amino acid identity of, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95% to SEQ ID NO: 26; or at most 70%, at most 75%, at most 80%, at most 85%, at most 90% or at most 95% to SEQ ID NO: 26. In yet other aspects of this embodiment, a Caspase 3 protease comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 26; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 non-contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 26. In still other aspects of this embodiment, a Caspase 3 protease comprises a polypeptide having, e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 26; or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, or 100 contiguous amino acid deletions, additions, and/or substitutions relative to SEQ ID NO: 26.

[090] The methods disclosed in the present specification include, in part, a dual expression construct. A dual expression construct comprises two polynucleotide molecules, each including an open reading frame disclosed in the present specification operably-linked to an expression vector useful for expressing both polynucleotide molecules in a cell or cell-free extract. A wide variety of dual expression vectors can be employed for expressing a polynucleotide molecule disclosed in the present specification, including, without limitation, a viral dual expression vector; a prokaryotic dual expression vector; an eukaryotic dual expression vector, such as, e.g., a yeast dual expression vector, an insect dual expression vector and a mammalian dual expression vector; and a cell-free extract dual expression vector. It is further understood that dual expression vectors useful to practice aspects of these methods may include those which express the polynucleotide molecules under the control of a constitutive, tissue-specific, cell-specific or inducible promoter element, enhancer element or both. Non-limiting examples of dual expression vectors, along with well-established reagents and conditions for making and using an expression construct from such expression vectors are readily available from commercial vendors that include, without limitation, EMD Biosciences-Novagen, Madison, WI. The selection, making and use of an appropriate dual expression vector are routine procedures well within the scope of one skilled in the art and from the teachings herein.

[091] The dual expression constructs disclosed in the present specification can comprise an open reading frame encoding a protein including a di-chain loop region comprising an exogenous protease cleavage site and another open reading frame encoding a protease that can cleave the exogenous

protease cleavage site located within the di-chain loop, thereby converting the single-chain protein into its di-chain form.

[092] Thus, in an embodiment, a dual expression construct comprises an open reading frame encoding a protein comprising a di-chain loop region comprising an exogenous protease cleavage site as disclosed in the present specification and another open reading frame encoding a protease that can cleave the exogenous protease cleavage site located within the di-chain loop as disclosed in the present specification.

[093] In an aspect of this embodiment, a dual expression construct can comprise one open reading frame encoding a Clostridial toxin including a di-chain loop region comprising a TEV protease cleavage site and another open reading frame encoding a TEV protease. In another aspect of this embodiment, a dual expression construct can comprise one open reading frame encoding a Clostridial toxin including a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a Clostridial toxin binding domain, and a di-chain loop region comprising a TEV protease cleavage site and another open reading frame encoding a TEV protease. In yet another aspect of this embodiment, a dual expression construct can comprise one open reading frame encoding a Clostridial toxin including a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a Clostridial toxin binding domain, a di-chain loop region, and a TEV protease cleavage site, wherein the TEV protease cleavage site is located within the di-chain loop region and another open reading frame encoding a TEV protease.

[094] In an aspect of this embodiment, a dual expression construct comprises an open reading frame encoding a protein comprising a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a non-Clostridial toxin binding domain, and a di-chain loop region comprising an exogenous protease cleavage site and another open reading frame encoding a protease that can cleave the exogenous protease cleavage site located within the di-chain loop region. In another aspect of this embodiment, a dual expression construct can comprise one open reading frame encoding a protein comprising a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a non-Clostridial toxin binding domain, and a di-chain loop region comprising a TEV protease cleavage site and another open reading frame encoding a TEV protease. In yet another aspect of this embodiment, a dual expression construct can comprise one open reading frame encoding a protein comprising a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a non-Clostridial toxin binding domain, a di-chain loop region, and a TEV protease cleavage site, wherein the TEV protease cleavage site is located within the di-chain loop region and another open reading frame encoding a TEV protease.

[095] In an aspect of this embodiment, a dual expression construct comprises an open reading frame encoding a protein comprising a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, and an integrated protease cleavage site-binding domain. In another aspect of this embodiment, a dual expression construct can comprise one open reading frame encoding a protein comprising a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, an integrated TEV protease cleavage site-binding domain and another open reading frame encoding a TEV protease. In yet another aspect of this embodiment, a dual expression construct can comprise one open reading frame encoding a protein comprising a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, and an integrated TEV protease cleavage site-binding domain, wherein the TEV protease cleavage site is located within the di-chain loop region and another open reading frame encoding a TEV protease.

[096] The location of one of the open reading frames contained within the dual expression construct can be in any order relative to the location of the other open reading frame, with the proviso that transcription from both open reading frames can still occur. When a dual expression construct is made, transcriptional initiation from the first promoter region typically transcribes both open reading frames, whereas, transcriptional initiation from the second promoter region typically transcribes only one of the open reading frames. Thus, depending on the location of the open reading frame relative to the first and second promoter regions, twice as many transcripts can be made from one of the open reading frames.

[097] Thus, in one embodiment, the open reading frame encoding a protease is under the control of the first promoter region whereas the open reading frame encoding a protein comprising a di-chain loop region comprising an exogenous protease cleavage site is under the control of both the first promoter and second promoter regions. In an aspect of this embodiment, the open reading frame encoding a TEV protease is under the control of the first promoter region whereas the open reading frame encoding a Clostridial toxin comprising a TEV protease cleavage site located within the di-chain loop region is under the control of both the first promoter and second promoter regions. In another aspect of this embodiment, the open reading frame encoding a TEV protease is under the control of the first promoter region whereas the open reading frame encoding a protein comprising a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a non-Clostridial toxin binding domain, and a di-chain loop region comprising a TEV protease cleavage site is under the control of both the first promoter and second promoter regions. In yet another aspect of this embodiment, the open reading frame encoding a TEV protease is under the control of the first promoter region whereas the open reading frame encoding a protein comprising a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, and an integrated TEV protease cleavage site-binding domain is under the control of both the first promoter and second promoter regions.

[098] In another embodiment, the open reading frame encoding a protein comprising a di-chain loop region comprising an exogenous protease cleavage site is under the control of the first promoter region whereas the open reading frame encoding a protease is under the control of both the first promoter and second promoter regions. In an aspect of this embodiment, the open reading frame encoding a Clostridial toxin comprising a di-chain loop region comprising a TEV protease cleavage site is under the control of the first promoter region whereas the open reading frame encoding a TEV protease is under the control of both the first promoter and second promoter regions. In another aspect of this embodiment, the open reading frame encoding a protein comprising a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, a non-Clostridial toxin binding domain, and a di-chain loop region comprising a TEV protease cleavage site is under the control of the first promoter region whereas the open reading frame encoding a TEV protease is under the control of both the first promoter and second promoter regions. In yet another aspect of this embodiment, the open reading frame encoding a protein comprising a Clostridial toxin enzymatic domain, a Clostridial toxin translocation domain, and an integrated TEV protease cleavage site-binding domain is under the control of the first promoter region whereas the open reading frame encoding a TEV protease is under the control of both the first promoter and second promoter regions.

[099] The 5'-3' orientation of one of the open reading frames contained within the dual expression construct can be in any direction relative to the 5'-3' orientation of the other open reading frame, with the

proviso that transcription from both open reading frames can still occur. In one embodiment, the 5'-3' orientation of one of the open reading frames is in the same direction as the 5'-3' orientation of the other open reading frame. In another embodiment, the 5'-3' orientation of one of the open reading frames is in the opposite direction as the 5'-3' orientation of the other open reading frame. In an aspect of this embodiment, the 5'-3' orientation of one of the open reading frames is convergent relative to the 5'-3' orientation of the other open reading frame. In another aspect of this embodiment, the 5'-3' orientation of one of the open reading frames is divergent relative to the 5'-3' orientation of the other open reading frame.

[0100] The methods disclosed in the present specification include, in part, a protein comprising a di-chain loop region comprising an exogenous protease cleavage site. As used herein, the term "di-chain loop region" means the amino acid sequence of a Clostridial toxin containing a protease cleavage site used to convert the single-chain form of a Clostridial toxin into the di-chain form. Non-limiting examples of a Clostridial toxin di-chain loop region, include, a di-chain loop region of BoNT/A comprising amino acids 430-454 of SEQ ID NO: 1; a di-chain loop region of BoNT/B comprising amino acids 437-446 of SEQ ID NO: 2; a di-chain loop region of BoNT/C1 comprising amino acids 437-453 of SEQ ID NO: 3; a di-chain loop region of BoNT/D comprising amino acids 437-450 of SEQ ID NO: 4; a di-chain loop region of BoNT/E comprising amino acids 412-426 of SEQ ID NO: 5; a di-chain loop region of BoNT/F comprising amino acids 429-445 of SEQ ID NO: 6; a di-chain loop region of BoNT/G comprising amino acids 436-450 of SEQ ID NO: 7; a di-chain loop region of TeNT comprising amino acids 439-467 of SEQ ID NO: 8; a di-chain loop region of BaNT comprising amino acids 421-435 of SEQ ID NO: 9; and a di-chain loop region of BuNT comprising amino acids 412-426 of SEQ ID NO: 10 (Table 2).

[0101] Table 2. Di-chain Loop Region of Clostridial Toxins

Toxin	Light Chain Region	Di-chain Loop Region Containing the Naturally-occurring Protease Cleavage Site	Heavy Chain Region
BoNT/A	NMNFTKLKNFTGLFEFYKLL	CVRGIITSKTKSLDKGYNK*----ALNDLC	IKVNNWDL
BoNT/B	KQAYEEISKEHLAVYKIQM	CKSVK*----------APGIC	IDVDNEDL
BoNT/C1	PALRKVNPENMLYLFTKF	CHKAIDGRSLYNK*----------TLDC	RELLVKNTDL
BoNT/D	PALQKLSSESVVDLFTKV	CLRLTKNSR*----------DDSTC	IKVKNNRL
BoNT/E	PRIITPITGRGLVKKIIRF	CKNIVSVKGIR*----------KSIC	IEINNGEL
BoNT/F	PKIIDSIPDKGLVEKIVKF	CKSVIPRKGTK*----------APPRLC	IRVNNSEL
BoNT/G	KEAYEEISLEHLVIYRIAM	CKPVMYKNTGK*----------SEQC	IIVNNEDL
TeNT	TNAFRNVDGSGLVSKLIGL	CKKIIPPTNIRENLYNRTA*SLTDLGGELC	IKIKNEDL
BaNT	SRIVGPIPDNGLVERFVGL	CKS-IVSKKGTK*----------NSLC	IKVNNRDL
BuNT	PRIITPITGRGLVKKIIRF	CKN-IVSVKGIR*----------KSIC	IEINNGEL

The amino acid sequence displayed are as follows: BoNT/A, residues 410-462 of SEQ ID No: 1; BoNT/B, residues 418-454 of SEQ ID No: 2; BoNT/C1, residues 419-463 of SEQ ID No: 3; BoNT/D, residues 419-458 of SEQ ID No: 4; BoNT/E, residues 393-434 of SEQ ID No: 5; BoNT/F, residues 410-453 of SEQ ID No: 6; BoNT/G, residues 419-458 of SEQ ID No: 7; TeNT, residues 422-475 of SEQ ID No: 8; BaNT, residues 402-443 of SEQ ID No: 9; and BuNT, residues 393-434 of SEQ ID No: 10. An asterisks (*) indicates the peptide bond that is cleaved by a Clostridial toxin protease.

[0102] As mentioned above, Clostridial toxins are translated as a single-chain polypeptide of approximately 150 kDa that is subsequently cleaved by proteolytic scission within a disulfide loop by a

naturally-occurring protease. This posttranslational processing yields a di-chain molecule comprising an approximately 50 kDa light chain (LC) and an approximately 100 kDa heavy chain (HC) held together by a single disulphide bond and noncovalent interactions. While the identity of the protease is currently unknown, the di-chain loop protease cleavage site for many Clostridial toxins has been determined. In BoNTs, cleavage at K448-A449 converts the single polypeptide form of BoNT/A into the di-chain form; cleavage at K441-A442 converts the single polypeptide form of BoNT/B into the di-chain form; cleavage at K449-T450 converts the single polypeptide form of BoNT/C1 into the di-chain form; cleavage at R445-D446 converts the single polypeptide form of BoNT/D into the di-chain form; cleavage at R422-K423 converts the single polypeptide form of BoNT/E into the di-chain form; cleavage at K439-A440 converts the single polypeptide form of BoNT/F into the di-chain form; and cleavage at K446-S447 converts the single polypeptide form of BoNT/G into the di-chain form. Proteolytic cleavage of the single polypeptide form of TeNT at A457-S458 results in the di-chain form. Proteolytic cleavage of the single polypeptide form of BaNT at K431-N432 results in the di-chain form. Proteolytic cleavage of the single polypeptide form of BuNT at R422-K423 results in the di-chain form. Such a di-chain loop protease cleavage site is operably-linked in-frame to a modified Clostridial toxin as a fusion protein. However, it should also be noted that additional cleavage sites within the di-chain loop also appear to be cleaved resulting in the generation of a small peptide fragment being lost. As a non-limiting example, cleavage of a BoNT/A single-chain polypeptide ultimately results in the loss of a ten amino acid fragment within the di-chain loop.

[0103] It is envisioned that any molecule that comprises a di-chain loop region can be modified to include an exogenous protease cleavage site useful for the disclosed methods. Examples of molecules that can have the di-chain loop modified to include an exogenous protease cleavage site useful for the disclosed methods include, e.g., Keith A. Foster et al., *Clostridial Toxin Derivatives Able To Modify Peripheral Sensory Afferent Functions,* U.S. Patent 5,989,545; Clifford C. Shone et al., *Recombinant Toxin Fragments,* U.S. Patent 6,461,617; Conrad P. Quinn et al., *Methods and Compounds for the Treatment of Mucus Hypersecretion,* U.S. Patent 6,632,440; Lance E. Steward et al., *Methods And Compositions For The Treatment Of Pancreatitis,* U.S. Patent 6,843,998; Stephan Donovan, *Clostridial Toxin Derivatives and Methods For Treating Pain,* U.S. Patent 7,244,437; Stephan Donovan, *Clostridial Toxin Derivatives and Methods For Treating Pain,* U.S. Patent 7,413,742; Stephan Donovan, *Clostridial Toxin Derivatives and Methods For Treating Pain,* U.S. Patent 7,425,338; each of which is hereby incorporated by reference in its entirety.

[0104] A di-chain loop region is modified by the addition of an exogenous protease cleavage site. As used herein, the term "exogenous protease cleavage site" is synonymous with a "non-naturally occurring protease cleavage site" or "non-native protease cleavage site" and refers to a protease cleavage site that is not normally present in a di-chain loop region from a naturally occurring Clostridial toxin. It is envisioned that any and all exogenous protease cleavage sites that can be used to convert the single-chain polypeptide form of a Clostridial toxin into the di-chain form are useful to practice aspects of the present invention. Non-limiting examples of exogenous protease cleavage sites include, e.g., an enterokinase protease cleavage site, a human rhinovirus 3C protease cleavage site, a human enterovirus 3C protease cleavage site, a tobacco etch virus (TEV) protease cleavage site, a Tobacco Vein Mottling

Virus (TVMV) protease cleavage site, a subtilisin protease cleavage site, or a Caspase 3 protease cleavage site.

[0105] It is envisioned that an exogenous protease cleavage site of any and all lengths can be useful in aspects of the present invention with the proviso that the exogenous protease cleavage site is capable of being cleaved by its respective protease. Thus, in aspects of this embodiment, an exogenous protease cleavage site can have a length of, e.g., at least 6, 7, 8, 9, 10, 15, 20, 25, 30, 40, 50, or 60 amino acids; or at most 6, 7, 8, 9, 10, 15, 20, 25, 30, 40, 50, or 60 amino acids.

[0106] In an embodiment, a di-chain loop region comprises an exogenous protease cleavage site. In aspects of this embodiment, a di-chain loop region is modified to comprise, e.g., an enterokinase protease cleavage site, a Tobacco Etch Virus protease cleavage site, a Tobacco Vein Mottling Virus protease cleavage site, a human rhinovirus 3C protease cleavage site, a human enterovirus 3C protease cleavage site, a subtilisin cleavage site, and a Caspase 3 cleavage site. In other aspects of this embodiment, an exogenous protease cleavage site is located within the di-chain loop of, e.g., a BoNT/A, a BoNT/B, a BoNT/C1 , a BoNT/D, a BoNT/E, a BoNT/F, a BoNT/G, a TeNT, a BaNT, or a BuNT. In other aspects of this embodiment, an exogenous protease cleavage site is located within the di-chain loop of a protein disclosed in, e.g., U.S. Patent 5,989,545; U.S. Patent 6,461 ,617; U.S. Patent 6,632,440; U.S. Patent 6,843,998; U.S. Patent 7,244,437; U.S. Patent 7,413,742; and U.S. Patent 7,425,338.

[0107] In an aspect of this embodiment, a di-chain loop region comprises a Tobacco Etch Virus protease cleavage site having the consensus sequence E-P5-P4-Y-P2-Q*-G (SEQ ID NO: 27) or E-P5-P4-Y-P2-Q*-S (SEQ ID NO: 28), where P2, P4 and P5 can be any amino acid. In other aspects of the embodiment, a di-chain loop region comprises a Tobacco Etch Virus protease cleavage site comprising SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37 or SEQ ID NO: 38. In still other aspects of this embodiment, a Tobacco Etch Virus protease cleavage site is located within the di-chain loop of, e.g., a BoNT/A, a BoNT/B, a BoNT/C1 , a BoNT/D, a BoNT/E, a BoNT/F, a BoNT/G, a TeNT, a BaNT, or a BuNT. In other aspects of this embodiment, a Tobacco Etch Virus protease cleavage site is located within the di-chain loop of a protein disclosed in, e.g., U.S. Patent 5,989,545; U.S. Patent 6,461 ,617; U.S. Patent 6,632,440; U.S. Patent 6,843,998; U.S. Patent 7,244,437; U.S. Patent 7,413,742; and U.S. Patent 7,425,338.

[0108] In another aspect of this embodiment, a di-chain loop region comprises a Tobacco Vein Mottling Virus protease cleavage site having the consensus sequence P6-P5-V-R-F-Q*-G (SEQ ID NO: 39) or P6-P5-V-R-F-Q*-S (SEQ ID NO: 40), where P5 and P6 can be any amino acid. In other aspects of the embodiment, a di-chain loop region comprises a Tobacco Vein Mottling Virus protease cleavage site comprising SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, or SEQ ID NO: 44. In still other aspects of this embodiment, a Tobacco Vein Mottling Virus protease cleavage site is located within the di-chain loop of, e.g., a BoNT/A, a BoNT/B, a BoNT/C 1, a BoNT/D, a BoNT/E, a BoNT/F, a BoNT/G, a TeNT, a BaNT, or a BuNT. In other aspects of this embodiment, a Tobacco Vein Mottling Virus protease cleavage site is located within the di-chain loop of a protein disclosed in, e.g., U.S. Patent 5,989,545; U.S. Patent 6,461 ,617; U.S. Patent 6,632,440; U.S. Patent 6,843,998; U.S. Patent 7,244,437; U.S. Patent 7,413,742; and U.S. Patent 7,425,338.

[0109] In yet another aspect of this embodiment, a di-chain loop region comprises a human rhinovirus 3C protease cleavage site having the consensus sequence P5-P4-L-F-Q*-G-P (SEQ ID NO: 45), where

P4 is G, A, V, L, I, M, S or T and P5 can any amino acid, with D or E preferred. In other aspects of the embodiment, a di-chain loop region comprises a human rhinovirus 3C protease cleavage site comprising SEQ ID NO: 46, SEQ ID NO: 47, SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50 or SEQ ID NO: 51. In still other aspects of this embodiment, a human rhinovirus 3C protease cleavage site is located within the di-chain loop of, e.g., a BoNT/A, a BoNT/B, a BoNT/C1, a BoNT/D, a BoNT/E, a BoNT/F, a BoNT/G, a TeNT, a BaNT, or a BuNT. In other aspects of this embodiment, a human rhinovirus 3C protease cleavage site is located within the di-chain loop of a protein disclosed in, e.g., U.S. Patent 5,989,545; U.S. Patent 6,461,617; U.S. Patent 6,632,440; U.S. Patent 6,843,998; U.S. Patent 7,244,437; U.S. Patent 7,413,742; and U.S. Patent 7,425,338.

[0110] In still another aspect of this embodiment, a di-chain loop region comprises a subtilisin protease cleavage site having the consensus sequence P6-P5-P4-P3-H*-Y (SEQ ID NO: 52) or P6-P5-P4-P3-Y-H* (SEQ ID NO: 53), where P3, P4 and P5 and P6 can be any amino acid. In other aspects of the embodiment, a di-chain loop region comprises a subtilisin protease cleavage site comprising SEQ ID NO: 54, SEQ ID NO: 55, or SEQ ID NO: 56. In still other aspects of this embodiment, a subtilisin protease cleavage site is located within the di-chain loop of, e.g., a BoNT/A, a BoNT/B, a BoNT/C 1, a BoNT/D, a BoNT/E, a BoNT/F, a BoNT/G, a TeNT, a BaNT, or a BuNT. In other aspects of this embodiment, a subtilisin protease cleavage site is located within the di-chain loop of a protein disclosed in, e.g., U.S. Patent 5,989,545; U.S. Patent 6,461,617; U.S. Patent 6,632,440; U.S. Patent 6,843,998; U.S. Patent 7,244,437; U.S. Patent 7,413,742; and U.S. Patent 7,425,338.

[0111] In a further aspect of this embodiment, a di-chain loop region comprises a Caspase 3 protease cleavage site having the consensus sequence D-P3-P2-D*P1' (SEQ ID NO: 57), where P3 can be any amino acid, with E preferred, P2 can be any amino acid and P1' can any amino acid, with G or S preferred. In other aspects of the embodiment, a di-chain loop region comprises a Caspase 3 protease cleavage site comprising SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, or SEQ ID NO: 63. In still other aspects of this embodiment, a Caspase 3 protease cleavage site is located within the di-chain loop of, e.g., a BoNT/A, a BoNT/B, a BoNT/C1, a BoNT/D, a BoNT/E, a BoNT/F, a BoNT/G, a TeNT, a BaNT, or a BuNT. In other aspects of this embodiment, a Caspase 3 protease cleavage site is located within the di-chain loop of a protein disclosed in, e.g., U.S. Patent 5,989,545; U.S. Patent 6,461,617; U.S. Patent 6,632,440; U.S. Patent 6,843,998; U.S. Patent 7,244,437; U.S. Patent 7,413,742; and U.S. Patent 7,425,338.

[0112] In yet another aspect of this embodiment, a di-chain loop region comprises an enterokinase protease cleavage site having the consensus sequence DDDDK (SEQ ID NO: 64). In other aspects of this embodiment, an enterokinase protease cleavage site is located within the di-chain loop of, e.g., a BoNT/A, a BoNT/B, a BoNT/C 1, a BoNT/D, a BoNT/E, a BoNT/F, a BoNT/G, a TeNT, a BaNT, or a BuNT. In yet other aspects of this embodiment, an enterokinase protease cleavage site is located within the di-chain loop of a protein disclosed in, e.g., U.S. Patent 5,989,545; U.S. Patent 6,461,617; U.S. Patent 6,632,440; U.S. Patent 6,843,998; U.S. Patent 7,244,437; U.S. Patent 7,413,742; and U.S. Patent 7,425,338.

[0113] A di-chain loop region is modified to replace a naturally-occurring di-chain loop protease cleavage site for an exogenous protease cleavage site. In this modification, the naturally-occurring di-chain loop protease cleavage site is made inoperable and thus can not be cleaved by its protease. Only

the exogenous protease cleavage site can be cleaved by its corresponding exogenous protease. In this type of modification, the exogenous protease site is operably-linked in-frame to a modified Clostridial toxin as a fusion protein and the site can be cleaved by its respective exogenous protease. Replacement of an endogenous di-chain loop protease cleavage site with an exogenous protease cleavage site can be a substitution of the sites where the exogenous site is engineered at the position approximating the cleavage site location of the endogenous site. Replacement of an endogenous di-chain loop protease cleavage site with an exogenous protease cleavage site can be the addition of an exogenous site where the exogenous site is engineered at a position different from the cleavage site location of the endogenous site, the endogenous site being engineered to be inoperable.

[01 14] A naturally-occurring protease cleavage site contained within the di-chain loop region can be made inoperable by altering at least the two amino acids flanking the peptide bond cleaved by the naturally-occurring di-chain loop protease. More extensive alterations can be made, with the proviso that the two cysteine residues of the di-chain loop region remain intact and the region can still form a disulfide bridge. Non-limiting examples of an amino acid alteration include deletion of an amino acid or replacement of the original amino acid with a different amino acid. Thus, in one embodiment, a naturally-occurring protease cleavage site contained within the di-chain loop region is made inoperable by altering the two amino acids flanking the peptide bond cleaved by a naturally-occurring protease. In other aspects of this embodiment, a naturally-occurring protease cleavage site contained within the di-chain loop region is made inoperable by altering, e.g., at least three amino acids including the two amino acids flanking the peptide bond cleaved by a naturally-occurring protease; at least four amino acids including the two amino acids flanking the peptide bond cleaved by a naturally-occurring protease; at least five amino acids including the two amino acids flanking the peptide bond cleaved by a naturally-occurring protease; at least six amino acids including the two amino acids flanking the peptide bond cleaved by a naturally-occurring protease; at least seven amino acids including the two amino acids flanking the peptide bond cleaved by a naturally-occurring protease; at least eight amino acids including the two amino acids flanking the peptide bond cleaved by a naturally-occurring protease; at least nine amino acids including the two amino acids flanking the peptide bond cleaved by a naturally-occurring protease; at least ten amino acids including the two amino acids flanking the peptide bond cleaved by a naturally-occurring protease; at least 15 amino acids including the two amino acids flanking the peptide bond cleaved by a naturally-occurring protease; or at least 20 amino acids including the two amino acids flanking the peptide bond cleaved by a naturally-occurring protease.

[01 15] In still other aspects of this embodiment, a naturally-occurring di-chain protease cleavage site contained within the di-chain loop region is made inoperable by altering, e.g., at most three amino acids including the two amino acids flanking the peptide bond cleaved by a naturally-occurring protease; at most four amino acids including the two amino acids flanking the peptide bond cleaved by a naturally-occurring protease; at most five amino acids including the two amino acids flanking the peptide bond cleaved by a naturally-occurring protease; at most six amino acids including the two amino acids flanking the peptide bond cleaved by a naturally-occurring protease; at most seven amino acids including the two amino acids flanking the peptide bond cleaved by a naturally-occurring protease; at most eight amino acids including the two amino acids flanking the peptide bond cleaved by a naturally-occurring protease; at most nine amino acids including the two amino acids flanking the peptide bond cleaved by a naturally-

occurring protease; at most ten amino acids including the two amino acids flanking the peptide bond cleaved by a naturally-occurring protease; at most 15 amino acids including the two amino acids flanking the peptide bond cleaved by a naturally-occurring protease; or at most 20 amino acids including the two amino acids flanking the peptide bond cleaved by a naturally-occurring protease.

[01 16] The methods disclosed in the present specification include, in part, a cell. It is envisioned that any and all cells can be used. Thus, aspects of this embodiment include, without limitation, prokaryotic cells including, without limitation, strains of aerobic, microaerophilic, capnophilic, facultative, anaerobic, gram-negative and gram-positive bacterial cells such as those derived from, e.g., *Escherichia coli, Bacillus subtilis, Bacillus licheniformis, Bacteroides fragilis, Clostridia perfringens, Clostridia* difficile, *Caulobacter crescentus, Lactococcus lactis, Methylobacterium extorquens, Neisseria meningirulls, Neisseria meningitidis, Pseudomonas fluorescens* and *Salmonella typhimurium;* and eukaryotic cells including, without limitation, yeast strains, such as, e.g., those derived from *Pichia pastoris, Pichia methanolica, Pichia angusta, Schizosaccharomyces pombe, Saccharomyces cerevisiae* and *Yarrowia lipolytica;* insect cells and cell lines derived from insects, such as, e.g., those derived from *Spodoptera frugiperda, Trichoplusia ni, Drosophila melanogaster* and *Manduca sexta;* and mammalian cells and cell lines derived from mammalian cells, such as, e.g., those derived from mouse, rat, hamster, porcine, bovine, equine, primate and human. Cell lines may be obtained from the American Type Culture Collection, European Collection of Cell Cultures and the German Collection of Microorganisms and Cell Cultures. Non-limiting examples of specific protocols for selecting, making and using an appropriate cell line are described in e.g., INSECT CELL CULTURE ENGINEERING (Mattheus F. A. Goosen et al. eds., Marcel Dekker, 1993); INSECT CELL CULTURES: FUNDAMENTAL AND APPLIED ASPECTS (J. M. Vlak et al. eds., Kluwer Academic Publishers, 1996); Maureen A. Harrison & Ian F. Rae, GENERAL TECHNIQUES OF CELL CULTURE (Cambridge University Press, 1997); CELL AND TISSUE CULTURE: LABORATORY PROCEDURES (Alan Doyle et al eds., John Wiley and Sons, 1998); R. Ian Freshney, CULTURE OF ANIMAL CELLS: A MANUAL OF BASIC TECHNIQUE (Wiley-Liss, 4TH ed. 2000); ANIMAL CELL CULTURE: A PRACTICAL APPROACH (John R. W. Masters ed., Oxford University Press, 3RD ed. 2000); MOLECULAR CLONING A LABORATORY MANUAL, *supra,* (2001); BASIC CELL CULTURE: A PRACTICAL APPROACH (John M. Davis, Oxford Press, 2ND ed. 2002); and CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, *supra,* (2004). These protocols are routine procedures within the scope of one skilled in the art and from the teaching herein.

[01 17] The methods disclosed in the present specification include, in part, introducing into a cell an expression construct or dual expression construct as disclosed in the present specification. An expression construct or dual expression construct introduced into a cell can be transiently or stably maintained by that cell. Stably-maintained expression constructs or dual expression constructs may be extra-chromosomal and replicate autonomously, or they may be integrated into the chromosomal material of the cell and replicate non-autonomously. It is envisioned that any and all methods for introducing an expression construct or a dual expression construct disclosed in the present specification into a cell can be used. Methods useful for introducing an expression construct or a dual expression construct into a cell include, without limitation, chemical-mediated transfection such as, e.g., calcium phosphate-mediated, diethyl-aminoethyl (DEAE) dextran-mediated, lipid-mediated, polyethyleneimine (PEI)-mediated, polylysine-mediated and polybrene-mediated ; physical-mediated tranfection, such as, e.g., biolistic particle delivery, microinjection, protoplast fusion and electroporation; and viral-mediated

transfection, such as, e.g., retroviral-mediated transfection, see, e.g., Introducing Cloned Genes into Cultured Mammalian Cells, pp. 16.1-16.62 (Sambrook & Russell, eds., Molecular Cloning A Laboratory Manual, Vol. 3, 3rd ed. 2001). One skilled in the art understands that selection of a specific method to introduce an expression construct or a dual expression construct into a cell will depend, in part, on whether the cell will transiently contain the expression construct or dual expression construct, or whether the cell will stably contain the expression construct or dual expression construct. These protocols are routine procedures within the scope of one skilled in the art and from the teaching herein.

[0118] In an aspect of this embodiment, a chemical-mediated method, termed transfection, is used to introduce an expression construct or a dual expression construct disclosed in the present specification into a cell. In chemical-mediated methods of transfection the chemical reagent forms a complex with the expression construct or dual expression construct that facilitates its uptake into the cells. Such chemical reagents include, without limitation, calcium phosphate-mediated, see, e.g., Martin Jordan & Florian Worm, *Transfection of adherent and suspended cells by calcium phosphate,* 33(2) Methods 136-143 (2004); diethyl-aminoethyl (DEAE) dextran-mediated, lipid-mediated, cationic polymer-mediated like polyethyleneimine (PEI)-mediated and polylysine-mediated and polybrene-mediated, see, e.g., Chun Zhang et al., *Polyethylenimine strategies for plasmid delivery to brain-derived cells,* 33(2) Methods 144-150 (2004). Such chemical-mediated delivery systems can be prepared by standard methods and are commercially available, see, e.g., CellPhect Transfection Kit (Amersham Biosciences, Piscataway, NJ); Mammalian Transfection Kit, Calcium phosphate and DEAE Dextran, (Stratagene, Inc., La Jolla, CA); Lipofectamine ™ Transfection Reagent (Invitrogen, Inc., Carlsbad, CA); ExGen 500 Transfection kit (Fermentas, Inc., Hanover, MD), and SuperFect and Effectene Transfection Kits (Qiagen, Inc., Valencia, CA).

[0119] In another aspect of this embodiment, a physically-mediated method is used to introduce an expression construct or a dual expression construct disclosed in the present specification into a cell. Physical techniques include, without limitation, electroporation, biolistic and microinjection. Biolistics and microinjection techniques perforate the cell wall in order to introduce the expression construct or dual expression construct into the cell, see, e.g., Jeike E. Biewenga et al., *Plasmid-mediated gene transfer in neurons using the biolistics technique,* 71(1) J. Neurosci. Methods. 67-75 (1997); and John O'Brien & Sarah C. R. Lummis, *Biolistic and diolistic transfection: using the gene gun to deliver DNA and lipophilic dyes into mammalian cells,* 33(2) Methods 121-125 (2004). Electroporation, also termed electropermeabilization, uses brief, high-voltage, electrical pulses to create transient pores in the membrane through which the polynucleotide molecules enter and can be used effectively for stable and transient transfections of all cell types, see, e.g., M. Golzio et al., *In vitro and in vivo electric field-mediated permeabilization, gene transfer, and expression,* 33(2) Methods 126-135 (2004); and Oliver Greschet al., *New non-viral method for gene transfer into primary cells,* 33(2) Methods 151-163 (2004).

[0120] In another aspect of this embodiment, a viral-mediated method, termed transduction, is used to introduce an expression construct or a dual expression construct disclosed in the present specification into a cell. In viral-mediated methods of transient transduction, the process by which viral particles infect and replicate in a host cell has been manipulated in order to use this mechanism to introduce the expression construct or dual expression construct into the cell. Viral-mediated methods have been developed from a wide variety of viruses including, without limitation, retroviruses, adenoviruses, adeno-

associated viruses, herpes simplex viruses, picornaviruses, alphaviruses and baculoviruses, see, e.g., Armin Blesch, *Lentiviral and MLV based retroviral vectors for ex vivo and in vivo gene transfer,* 33(2) Methods 164-172 (2004); and Maurizio Federico, *From lentiviruses to lentivirus vectors,* 229 Methods Mol. Biol. 3-15 (2003); E. M. Poeschla, *Non-primate lentiviral vectors,* 5(5) Curr. Opin. Mol. Ther. 529-540 (2003); Karim Benihoud et al, Adenovirus vectors for gene delivery, 10(5) Curr. Opin. Biotechnol. 440-447 (1999); H. Bueler, *Adeno-associated viral vectors for gene transfer and gene therapy,* 380(6) Biol. Chem. 613-622 (1999); Chooi M. Lai et al., *Adenovirus and adeno-associated virus vectors,* 21(12) DNA Cell Biol. 895-913 (2002); Edward A. Burton et al., *Gene delivery using herpes simplex virus vectors,* 21(12) DNA Cell Biol. 915-936 (2002); Paola Grandi et al., *Targeting HSV amplicon vectors,* 33(2) Methods 179-186 (2004); llya Frolov et al., *Alphavirus-based expression vectors: strategies and applications,* 93(21) Proc. Natl. Acad. Sci. U. S. A. 11371-11377 (1996); Markus U. Ehrengruber, *Alphaviral gene transfer in neurobiology,* 59(1) Brain Res. Bull. 13-22 (2002); Thomas A. Kost & J. Patrick Condreay, *Recombinant baculoviruses as mammalian cell gene-delivery vectors,* 20(4) Trends Biotechnol. 173-180 (2002); and A. Huser & C. Hofmann, *Baculovirus vectors: novel mammalian cell gene-delivery vehicles and their applications,* 3(1) Am. J. Pharmacogenomics 53-63 (2003).

[0121] Adenoviruses, which are non-enveloped, double-stranded DNA viruses, are often selected for mammalian cell transduction because adenoviruses handle relatively large polynucleotide molecules of about 36 kb, are produced at high titer, and can efficiently infect a wide variety of both dividing and non-dividing cells, see, *e.g.,* Wim T. J. M. C. Hermens et al., Transient gene transfer to neurons and glia: analysis of adenoviral vector performance in the CNS and PNS, 71(1) J. Neurosci. Methods 85-98 (1997); and Hiroyuki Mizuguchi et al., Approaches for generating recombinant adenovirus vectors, 52(3) Adv. Drug Deliv. Rev. 165-176 (2001). Transduction using adenoviral-based system do not support prolonged protein expression because the nucleic acid molecule is carried by an episome in the cell nucleus, rather than being integrated into the host cell chromosome. Adenoviral vector systems and specific protocols for how to use such vectors are disclosed in, *e.g.,* VIRAPOWER™ Adenoviral Expression System (Invitrogen, Inc., Carlsbad, CA) and VIRAPOWER™ Adenoviral Expression System Instruction Manual 25-0543 version A, Invitrogen, Inc., (Jul. 15, 2002); and ADEASY™ Adenoviral Vector System (Stratagene, Inc., La Jolla, CA) and ADEASY™ Adenoviral Vector System Instruction Manual 064004f, Stratagene, Inc..

[0122] Introduction of an expression construct or dual expression construct disclosed in the present specification into a cell can also be achieved using single-stranded RNA retroviruses, such as, *e.g.,* oncoretroviruses and lentiviruses. Retroviral-mediated transduction often produce transduction efficiencies close to 100%, can easily control the proviral copy number by varying the multiplicity of infection (MOI), and can be used to either transiently or stably transduce cells, see, *e.g.,* Tiziana Tonini et al., *Transient production of retroviral- and lentiviral-based vectors for the transduction of Mammalian cells,* 285 Methods Mol. Biol. 141-148 (2004); Armin Blesch, *Lentiviral and MLV based retroviral vectors for ex vivo and in vivo gene transfer,* 33(2) Methods 164-172 (2004); Felix Recillas-Targa, *Gene transfer and expression in mammalian cell lines and transgenic animals,* 267 Methods Mol. Biol. 417-433 (2004); and Roland Wolkowicz et al., *Lentiviral vectors for the delivery of DNA into mammalian cells,* 246 Methods Mol. Biol. 391-411 (2004). Retroviral particles consist of an RNA genome packaged in a protein capsid, surrounded by a lipid envelope. The retrovirus infects a host cell by injecting its RNA into the

cytoplasm along with the reverse transcriptase enzyme. The RNA template is then reverse transcribed into a linear, double stranded cDNA that replicates itself by integrating into the host cell genome. Viral particles are spread both vertically (from parent cell to daughter cells via the provirus) as well as horizontally (from cell to cell via virions). This replication strategy enables long-term persistent expression since the nucleic acid molecules of interest are stably integrated into a chromosome of the host cell, thereby enabling long-term expression of the protein. For instance, animal studies have shown that lentiviral vectors injected into a variety of tissues produced sustained protein expression for more than 1 year, see, e.g., Luigi Naldini et al., *In vivo gene delivery and stable transduction of non-dividing cells by a lentiviral vector,* 272(5259) Science 263-267 (1996). The Oncoretroviruses-derived vector systems, such as, *e.g.,* Moloney murine leukemia virus (MoMLV), are widely used and infect many different non-dividing cells. Lentiviruses can also infect many different cell types, including dividing and non-dividing cells and possess complex envelope proteins, which allows for highly specific cellular targeting.

[0123] Retroviral vectors and specific protocols for how to use such vectors are disclosed in, *e.g.,* Manfred Gossen & Hermann Bujard, *Tight control of gene expression in eukaryotic cells by tetracycline-responsive promoters,* U.S. Patent 5,464,758, Hermann Bujard & Manfred Gossen, *Methods for regulating gene expression,* U.S. Patent 5,814,618, David S. Hogness, *Polynucleotides encoding insect steroid hormone receptor polypeptides and cells transformed with same,* U.S. Patent 5,514,578, and David S. Hogness, Polynucleotide encoding insect ecdysone receptor, U.S. Patent 6,245,531; Elisabetta Vegeto et al., *Progesterone receptor having C. terminal hormone binding domain truncations,* U.S. Patent 5,364,791,, Elisabetta Vegeto et al., *Mutated steroid hormone receptors, methods for their use and molecular switch for gene therapy,* U.S. Patent 5,874,534, and Elisabetta Vegeto et al., *Mutated steroid hormone receptors, methods for their use and molecular switch for gene therapy,* U.S. Patent 5,935,934. Furthermore, such viral delivery systems can be prepared by standard methods and are commercially available, see, *e.g.,* BD™ Tet-Off and Tet-On Gene Expression Systems (BD Biosciences-Clonetech, Palo Alto, CA) and BD™ Tet-Off and Tet-On Gene Expression Systems User Manual, PT3001-1, BD Biosciences Clonetech, (Mar. 14, 2003), GENESWITCH ™ System (Invitrogen, Inc., Carlsbad, CA) and GENESWITCH ™ System A Mifepristone-Regulated Expression System for Mammalian Cells version D, 25-0313, Invitrogen, Inc., (Nov. 4, 2002); VIRAPOWER™ Lentiviral Expression System (Invitrogen, Inc., Carlsbad, CA) and VIRAPOWER™ Lentiviral Expression System Instruction Manual 25-0501 version E, Invitrogen, Inc., (Dec. 8, 2003); and COMPLETE CONTROL® Retroviral Inducible Mammalian Expression System (Stratagene, La Jolla, CA) and COMPLETE CONTROL® Retroviral Inducible Mammalian Expression System Instruction Manual, 064005e.

[0124] The methods disclosed in the present specification include, in part, expressing an expression construct or dual expression construct disclosed in the present specification. It is envisioned that any of a variety of expression systems may be useful for expressing an expression construct or a dual expression construct disclosed in the present specification, including, without limitation, cell-based systems, and cell-free expression systems. Cell-based systems include, without limitation, viral expression systems, prokaryotic expression systems, yeast expression systems, baculoviral expression systems, insect expression systems and mammalian expression systems. Cell-free systems include, without limitation, wheat germ extracts, rabbit reticulocyte extracts and *E. coli* extracts and generally are equivalent to the

method disclosed herein. Expression of an expression construct or dual expression construct using an expression system can include any of a variety of characteristics including, without limitation, inducible expression, non-inducible expression, constitutive expression, viral-mediated expression, stably-integrated expression, and transient expression. Expression systems that include well-characterized vectors, reagents, conditions and cells are well-established and are readily available from commercial vendors that include, without limitation, Ambion, Inc. Austin, TX; BD Biosciences-Clontech, Palo Alto, CA; BD Biosciences Pharmingen, San Diego, CA; Invitrogen, Inc, Carlsbad, CA; QIAGEN, Inc., Valencia, CA; Roche Applied Science, Indianapolis, IN; and Stratagene, La Jolla, CA. Non-limiting examples on the selection and use of appropriate heterologous expression systems are described in e.g., PROTEIN EXPRESSION. A PRACTICAL APPROACH (S. J. Higgins and B. David Hames eds., Oxford University Press, 1999); Joseph M. Fernandez & James P. Hoeffler, GENE EXPRESSION SYSTEMS. USING NATURE FOR THE ART OF EXPRESSION (Academic Press, 1999); and Meena Rai & Harish Padh, *Expression Systems for Production of Heterologous Proteins,* 80(9) CURRENT SCIENCE 1121-1128, (2001). These protocols are routine procedures well within the scope of one skilled in the art and from the teaching herein.

[0125] A variety of cell-based expression procedures are useful for expressing an expression construct or a dual expression construct disclosed in the present specification. Examples included, without limitation, viral expression systems, prokaryotic expression systems, yeast expression systems, baculoviral expression systems, insect expression systems and mammalian expression systems. Viral expression systems include, without limitation, the VIRAPOWER™ Lentiviral (Invitrogen, Inc., Carlsbad, CA), the Adenoviral Expression Systems (Invitrogen, Inc., Carlsbad, CA), the ADEASY™ XL Adenoviral Vector System (Stratagene, La Jolla, CA) and the VIRAPORT® Retroviral Gene Expression System (Stratagene, La Jolla, CA). Non-limiting examples of prokaryotic expression systems include the CHAMPION ™ pET Expression System (EMD Biosciences-Novagen, Madison, WI), the TRIEX™ Bacterial Expression System (EMD Biosciences-Novagen, Madison, WI), the QIAEXPRESS® Expression System (QIAGEN, Inc.), and the AFFINITY® Protein Expression and Purification System (Stratagene, La Jolla, CA). Yeast expression systems include, without limitation, the EASYSELECT™ *Pichia* Expression Kit (Invitrogen, Inc., Carlsbad, CA), the YES-ECHO™ Expression Vector Kits (Invitrogen, Inc., Carlsbad, CA) and the SPECTRA™ S. *pombe* Expression System (Invitrogen, Inc., Carlsbad, CA). Non-limiting examples of baculoviral expression systems include the BACULODIRECT™ (Invitrogen, Inc., Carlsbad, CA), the BAC-TO-BAC® (Invitrogen, Inc., Carlsbad, CA), and the BD BACULOGOLD™ (BD Biosciences-Pharmigen, San Diego, CA). Insect expression systems include, without limitation, the *Drosophila* Expression System (DES®) (Invitrogen, Inc., Carlsbad, CA), INSECTSELECT™ System (Invitrogen, Inc., Carlsbad, CA) and INSECTDIRECT™ System (EMD Biosciences-Novagen, Madison, WI). Non-limiting examples of mammalian expression systems include the T-REX™ (Tetracycline-Regulated Expression) System (Invitrogen, Inc., Carlsbad, CA), the FLP-IN ™ T-REX™ System (Invitrogen, Inc., Carlsbad, CA), the pcDNA™ system (Invitrogen, Inc., Carlsbad, CA), the pSecTag2 system (Invitrogen, Inc., Carlsbad, CA), the EXCHANGER® System, INTERPLAY™ Mammalian TAP System (Stratagene, La Jolla, CA), COMPLETE CONTROL® Inducible Mammalian Expression System (Stratagene, La Jolla, CA) and LACSWITCH® II Inducible Mammalian Expression System (Stratagene, La Jolla, CA).

[0126] Another procedure of expressing an expression construct or a dual expression construct disclosed in the present specification employs a cell-free expression system such as, without limitation,

prokaryotic extracts and eukaryotic extracts. Non-limiting examples of prokaryotic cell extracts include the RTS 100 *E. coli* HY Kit (Roche Applied Science, Indianapolis, IN), the ACTIVEPRO™ In Vitro Translation Kit (Ambion, Inc., Austin, TX), the ECOPRO™ System (EMD Biosciences-Novagen, Madison, WI) and the Expressway™ Plus Expression System (Invitrogen, Inc., Carlsbad, CA). Eukaryotic cell extract include, without limitation, the RTS 100 Wheat Germ CECF Kit (Roche Applied Science, Indianapolis, IN), the TNT® Coupled Wheat Germ Extract Systems (Promega Corp., Madison, WI), the Wheat Germ IVT™ Kit (Ambion, Inc., Austin, TX), the Retic Lysate IVT™ Kit (Ambion, Inc., Austin, TX), the PROTEINSCRIPT® II System (Ambion, Inc., Austin, TX) and the TNT® Coupled Reticulocyte Lysate Systems (Promega Corp., Madison, WI).

[0127] The methods disclosed in the present specification include, in part, growing a cell at a first temperature for a certain period of time and then growing the cell at a second temperature for a certain period of time. The first and second temperatures and the periods of time the cells are grown at the first and second temperatures are determined based on the desired amount of protein to be expressed by the cell, and the desired cleavage efficiency at the exogenous protease cleavage site located within the di-chain loop region to convert the single-chain protein into its di-chain form.

[0128] In one embodiment, a cell is grown at a first temperature for a certain period of time in order to achieve maximum cell density. In aspects of this embodiment, a cell is grown at about 37 °C for about 0.5 hours, about 1.0 hour, about 1.5 hours, about 2.0 hours, about 3.0 hours, about 3.5 hours, about 4.0 hours, about 5.0 hours, about 6.0 hours, about 7.0 hours, about 8.0 hours, about 9.0 hours or about 10 hours. In other aspects of this embodiment, a cell is grown at about 42 °C for about 0.5 hours, about 1.0 hour, about 1.5 hours, about 2.0 hours, about 3.0 hours, about 3.5 hours, about 4.0 hours, about 5.0 hours. In aspects of this embodiment, a cell is grown at about 30 °C for about 0.5 hours, about 1.0 hour, about 1.5 hours, about 2.0 hours, about 3.0 hours, about 3.5 hours, about 4.0 hours, or about 5.0 hours. In yet other aspects, of this embodiment, a cell is grown at about 12 °C for about 2 hours to about 8 hours, at about 16 °C for about 2 hours to about 8 hours, at about 20 °C for about 2 hours to about 8 hours, or at about 24 °C for about 2 hours to about 8 hours. In still other aspects, of this embodiment, a cell is grown at about 12 °C to about 16 °C for about 2 hours to about 8 hours, or at about 20 °C to about 24 °C for about 2 hours to about 8 hours.

[0129] In another embodiment, a cell is grown at a second temperature for a certain period of time in order to achieve maximum induction of protein expression. In aspects of this embodiment, a cell is grown at about 37 °C for about 1.5 hours, about 2.5 hours, about 3.5 hours, about 4.5 hours, about 5.5 hours, about 6.5 hours, about 7.5 hours, about 8.5 hours, about 9.5 hours, about 10.5 hours, about 11.5 hours, about 12.5 hours, about 13.5 hours, about 14.5 hours, about 15.5 hours, about 16.5 hours, or about 24.5 hours. In other aspects of this embodiment, a cell is grown at about 30 °C for about 1.5 hours, about 2.5 hours, about 3.5 hours, about 4.5 hours, about 5.5 hours, about 6.5 hours, about 7.5 hours, about 8.5 hours, about 9.5 hours, about 10.5 hours, about 11.5 hours, about 12.5 hours, about 13.5 hours, about 14.5 hours, about 15.5 hours, about 16.5 hours, or about 24.5 hours. In yet other aspects of this embodiment, a cell is grown at about 25 °C for about 1.5 hours, about 2.5 hours, about 3.5 hours, about 4.5 hours, about 5.5 hours, about 6.5 hours, about 7.5 hours, about 8.5 hours, about 9.5 hours, about 10.5 hours, about 11.5 hours, about 12.5 hours, about 13.5 hours, about 14.5 hours, about 15.5 hours, about 16.5 hours, or about 24.5 hours. In still other aspects of this embodiment, a cell is grown at about

22 °C for about 1.5 hours, about 2.5 hours, about 3.5 hours, about 4.5 hours, about 5.5 hours, about 6.5 hours, about 7.5 hours, about 8.5 hours, about 9.5 hours, about 10.5 hours, about 11.5 hours, about 12.5 hours, about 13.5 hours, about 14.5 hours, about 15.5 hours, about 16.5 hours, or about 24.5 hours. In further aspects of this embodiment, a cell is grown at about 16 °C for about 1.5 hours, about 2.5 hours, about 3.5 hours, about 4.5 hours, about 5.5 hours, about 6.5 hours, about 7.5 hours, about 8.5 hours, about 9.5 hours, about 10.5 hours, about 11.5 hours, about 12.5 hours, about 13.5 hours, about 14.5 hours, about 15.5 hours, about 16.5 hours, or about 24.5 hours. In yet further aspects of this embodiment, a cell is grown at about 12 °C for about 1.5 hours, about 2.5 hours, about 3.5 hours, about 4.5 hours, about 5.5 hours, about 6.5 hours, about 7.5 hours, about 8.5 hours, about 9.5 hours, about 10.5 hours, about 11.5 hours, about 12.5 hours, about 13.5 hours, about 14.5 hours, about 15.5 hours, about 16.5 hours, or about 24.5 hours.

[0130] Aspects of the present invention can also be described as follows:

1. An intracellular method of converting a single-chain protein into its di-chain form, the method comprising the steps of:
   a) growing a cell comprising a dual expression construct at a first temperature for a certain period of time in order to achieve maximal cell density, the dual expression construct comprising;
      i) an open reading frame encoding a single-chain protein comprising a di-chain loop region comprising an exogenous protease cleavage site; and
      ii) an open reading frame encoding a protease; wherein the protease can cleave the exogenous protease cleavage site located within the di-chain loop;
   b) growing the cell at a second temperature for a certain period of time in order to achieve maximal induction of protein expression from the open reading frame encoding the single-chain protein,
   wherein growth at step (b) induces expression of the single-chain protein and the protease from the dual expression construct; and
   wherein the produced protease cleaves the single-chain protein at the exogenous protease cleavage site located within the di-chain loop region, thereby converting the single-chain protein into its di-chain form.
2. An intracellular method of converting a single-chain Clostridial toxin into its di-chain form, the method comprising the steps of:
   a) growing a cell comprising a dual expression construct at 37 °C for about 3.5 hours, the dual expression construct comprising;
      i) an open reading frame encoding a single-chain Clostridial toxin, the single-chain Clostridial toxin comprising an enzymatic domain, a translocation domain, a binding domain, and a di-chain loop region comprising an exogenous protease cleavage site; and
      ii) an open reading frame encoding a protease; wherein the protease can cleave the exogenous protease cleavage site located within the di-chain loop;
   b) growing the cell at 22 °C for about 16 to about 18 hours,
   wherein growth at step (b) induces expression of the single-chain Clostridial toxin and the protease from the dual expression construct; and

wherein the produced protease cleaves the single-chain Clostridial toxin at the exogenous protease cleavage site located within the di-chain loop region, thereby converting the single-chain Clostridial toxin into its di-chain form.

3. The intracellular method according to 2, wherein the single-chain Clostridial toxin comprises a linear amino-to-carboxyl single polypeptide order of 1) the Clostridial toxin enzymatic domain, the di-chain loop region comprising an exogenous protease cleavage site, the Clostridial toxin translocation domain and the Clostridial toxin binding domain; 2) the Clostridial toxin enzymatic domain, the di-chain loop region comprising an exogenous protease cleavage site, the Clostridial toxin binding domain and the Clostridial toxin translocation domain; 3) the Clostridial toxin binding domain, the Clostridial toxin translocation domain, the di-chain loop region comprising an exogenous protease cleavage site and the Clostridial toxin enzymatic domain; 4) the Clostridial toxin binding domain, the Clostridial toxin enzymatic domain, the di-chain loop region comprising an exogenous protease cleavage site and the Clostridial toxin translocation domain; 5) the Clostridial toxin translocation domain, the di-chain loop region comprising an exogenous protease cleavage site, the Clostridial toxin enzymatic domain and the Clostridial toxin binding domain; or 6) the Clostridial toxin translocation domain, the di-chain loop region comprising an exogenous protease cleavage site, the Clostridial binding domain and the Clostridial toxin enzymatic domain.

4. The intracellular method according to 2, wherein the Clostridial toxin enzymatic domain is a BoNT/A enzymatic domain, a BoNT/B enzymatic domain, a BoNT/C1 enzymatic domain, a BoNT/D enzymatic domain, a BoNT/E enzymatic domain, a BoNT/F enzymatic domain, a BoNT/G enzymatic domain, a TeNT enzymatic domain, a BaNT enzymatic domain, or a BuNT enzymatic domain.

5. The intracellular method according to 2, wherein the Clostridial toxin translocation domain is a BoNT/A translocation domain, a BoNT/B translocation domain, a BoNT/C1 translocation domain, a BoNT/D translocation domain, a BoNT/E translocation domain, a BoNT/F translocation domain, a BoNT/G translocation domain, a TeNT translocation domain, a BaNT translocation domain, or a BuNT translocation domain.

6. The intracellular method according to 2, wherein the Clostridial toxin binding domain is a BoNT/A binding domain, a BoNT/B binding domain, a BoNT/C1 binding domain, a BoNT/D binding domain, a BoNT/E binding domain, a BoNT/F binding domain, a BoNT/G binding domain, a TeNT binding domain, a BaNT binding domain, or a BuNT binding domain.

7. The intracellular method according to 2, wherein the exogenous protease cleavage site is an enterokinase protease cleavage site, a human rhinovirus 3C protease cleavage site, a human enterovirus 3C protease cleavage site, a tobacco etch virus (TEV) protease cleavage site, a Tobacco Vein Mottling Virus (TVMV) protease cleavage site, a subtilisin protease cleavage site, or a Caspase 3 protease cleavage site.

8. The intracellular method according to 2, wherein the protease is an enterokinase protease, a human rhinovirus 3C protease, a human enterovirus 3C protease, a tobacco etch virus (TEV) protease, a Tobacco Vein Mottling Virus (TVMV) protease, a subtilisin protease, or a Caspase 3 protease.

9. An intracellular method of converting a single-chain protein into its di-chain form, the method comprising the steps of

a) growing a cell comprising a dual expression construct at 37 °C for about 8 hours, the dual expression construct comprising;

i) an open reading frame encoding a single-chain protein, the single-chain protein comprising an enzymatic domain, a translocation domain, an integrated TEV protease cleavage site-opioid binding domain; and

ii) an open reading frame encoding a TEV protease;

b) growing the cell at about 12 to about 16 °C for about 16 to about 18 hours,

wherein growth at step (b) induces expression of the single-chain protein and the TEV protease from the dual expression construct; and

wherein the produced TEV protease cleaves the single-chain protein at the TEV protease cleavage site located within the integrated TEV cleavage site opioid binding domain, thereby converting the single-chain protein into its di-chain form.

10. The intracellular method according to 9, wherein the protein comprises a linear amino-to-carboxyl single polypeptide order of 1) the Clostridial toxin enzymatic domain, the Clostridial toxin translocation domain, and the integrated TEV protease cleavage site-opioid binding domain, 2) the Clostridial toxin enzymatic domain, the integrated TEV protease cleavage site-opioid binding domain, and the Clostridial toxin translocation domain, 3) the integrated TEV protease cleavage site-opioid binding domain, the Clostridial toxin translocation domain, and the Clostridial toxin enzymatic domain, 4) the integrated TEV protease cleavage site-opioid binding domain, the Clostridial toxin enzymatic domain, and the Clostridial toxin translocation domain, 5) the Clostridial toxin translocation domain, the integrated TEV protease cleavage site-opioid binding domain, and the Clostridial toxin enzymatic domain, or 6) the Clostridial toxin translocation domain, the Clostridial toxin enzymatic domain, and the integrated TEV protease cleavage site-opioid binding domain.

11. The intracellular method according to 9, wherein the Clostridial toxin enzymatic domain is a BoNT/A enzymatic domain, a BoNT/B enzymatic domain, a BoNT/C1 enzymatic domain, a BoNT/D enzymatic domain, a BoNT/E enzymatic domain, a BoNT/F enzymatic domain, a BoNT/G enzymatic domain, a TeNT enzymatic domain, a BaNT enzymatic domain, or a BuNT enzymatic domain.

12. The intracellular method according to 9, wherein the Clostridial toxin translocation domain is a BoNT/A translocation domain, a BoNT/B translocation domain, a BoNT/C1 translocation domain, a BoNT/D translocation domain, a BoNT/E translocation domain, a BoNT/F translocation domain, a BoNT/G translocation domain, a TeNT translocation domain, a BaNT translocation domain, or a BuNT translocation domain.

13. The intracellular method according to 9, wherein the integrated TEV protease cleavage site-opiod binding domain is an integrated TEV protease cleavage site-nociceptin binding domain, an integrated TEV protease cleavage site-dynorphin binding domain, an integrated TEV protease cleavage site-enkephalin binding domain, an integrated TEV protease cleavage site-BAM22 binding domain, an integrated TEV protease cleavage site-endomorphin binding domain, an integrated TEV protease cleavage site-endorphin binding domain, an integrated TEV protease cleavage site-hemorphin binding domain, or an integrated TEV protease cleavage site-rimorphin binding domain.

14. An intracellular method of converting a single-chain protein into its di-chain form, the method comprising the steps of

a) growing a cell comprising a dual expression construct at 37 °C for about 8 hours, the dual expression construct comprising;

i) an open reading frame encoding a single-chain protein, the single-chain protein comprising an enzymatic domain, a translocation domain, a non-Clostridial toxin binding domain and a di-chain loop region comprising a TEV protease cleavage site; and

ii) an open reading frame encoding a TEV protease;

b) growing the cell at about 12 to about 16 °C for about 16 to about 18 hours,

wherein growth at step (b) induces expression of the single-chain protein and the TEV protease from the dual expression construct; and

wherein the produced TEV protease cleaves the single-chain protein at the TEV protease cleavage site located within the di-chain loop region, thereby converting the single-chain protein into its di-chain form.

15. The intracellular method according to 14, wherein the single-chain Clostridial toxin comprises a linear amino-to-carboxyl single polypeptide order of 1) the Clostridial toxin enzymatic domain, the di-chain loop region comprising a TEV protease cleavage site, the Clostridial toxin translocation domain and the non-Clostridial toxin binding domain; 2) the Clostridial toxin enzymatic domain, the di-chain loop region comprising a TEV protease cleavage site, the non-Clostridial toxin binding domain and the Clostridial toxin translocation domain; 3) the non-Clostridial toxin binding domain, the Clostridial toxin translocation domain, the di-chain loop region comprising a TEV protease cleavage site and the Clostridial toxin enzymatic domain; 4) the non-Clostridial toxin binding domain, the Clostridial toxin enzymatic domain, the di-chain loop region comprising a TEV protease cleavage site and the Clostridial toxin translocation domain; 5) the Clostridial toxin translocation domain, the di-chain loop region comprising a TEV protease cleavage site, the Clostridial toxin enzymatic domain and the non-Clostridial toxin binding domain; or 6) the Clostridial toxin translocation domain, the di-chain loop region comprising an exogenous protease cleavage site, the non-Clostridial binding domain and the Clostridial toxin enzymatic domain.

16. The intracellular method according to 14, wherein the Clostridial toxin enzymatic domain is a BoNT/A enzymatic domain, a BoNT/B enzymatic domain, a BoNT/C1 enzymatic domain, a BoNT/D enzymatic domain, a BoNT/E enzymatic domain, a BoNT/F enzymatic domain, a BoNT/G enzymatic domain, a TeNT enzymatic domain, a BaNT enzymatic domain, or a BuNT enzymatic domain.

17. The intracellular method according to 14, wherein the Clostridial toxin translocation domain is a BoNT/A translocation domain, a BoNT/B translocation domain, a BoNT/C1 translocation domain, a BoNT/D translocation domain, a BoNT/E translocation domain, a BoNT/F translocation domain, a BoNT/G translocation domain, a TeNT translocation domain, a BaNT translocation domain, or a BuNT translocation domain.

18. The intracellular method according to 14, wherein the non-Clostridial toxin binding domain is an opioid peptide binding domain, a melanocortin peptide binding domain, a galanin peptide binding domain, a granin peptide binding domain, a tachykinin peptide binding domain, a neuropeptide Y related peptide binding domain, a neurohormone peptide binding domain, a cytokine peptide binding domain, a kinin peptide binding domain, a fibroblast growth factor peptide binding domain, a neurotrophin peptide binding domain, a tumor necrosis factor peptide binding domain, a glial derived

neurotrophic factor peptide binding domain, a transformation growth factor β peptide binding domain, a bone morphogenetic protein peptide binding domain, a growth and differentiation factor peptide binding domain, an activin peptide binding domain, a vascular endothelial growth factor peptide binding domain, an insulin growth factor peptide binding domain, an epidermial growth factor peptide binding domain, a glucagon like hormone peptide binding domain, a pituitary adenylate cyclase activating peptide binding domain, a growth hormone-releasing hormone peptide binding domain, a vasoactive intestinal peptide binding domain, a gastric inhibitory polypeptide peptide binding domain, a calcitonin-related peptidesvisceral gut peptide binding domain, or a protease activated receptor peptide binding domain.

EXAMPLES

Example 1

TEV Protease Variants

[0131] The following example illustrates how to make and use TEV protease variants that have increased stability and/or solubility.

A. Construction of pET29/TEV expression constructs.

[0132] In order to produce a TEV protease recombinantly, an open reading frame encoding the desired TEV protease was synthesized using standard procedures (BlueHeron Biotechnology, Bothell, WA). Complementary oligonucleotides of 20 to 50 bases in length, spanning the entire open reading frame, were synthesized using standard phosphoramidite synthesis. These oligonucleotides were hybridized into double stranded duplexes that were sequentially ligated together to assemble the full-length polynucleotide molecule. This polynucleotide molecule was cloned using standard molecular biology methods into a pUCBHBI carrier vector at the *Sma*I site to generate pUCBHB1/TEV plasmids. The synthesized polynucleotide molecule was verified by sequencing using BIG DYE TERMINATOR™ Chemistry 3.1 (Applied Biosystems, Foster City, CA) and an ABI 3100 sequencer (Applied Biosystems, Foster City, CA).

[0133] The open reading frame encoding the TEV variants were codon-optimized for *E. coli* expression and all encode an approximately 250 amino acid proteolytic fragment of approximately 27.5 kDa, corresponding to residues 2038-2279 of the full-length TEV polyprotein fused to either an N- or C-terminal poly-histidine affinity purification tag. Recombinant expression of wild-type TEV protease results in a protein that has a propensity to cleave itself at Serine 219 to generate a truncated protease with greatly diminished proteolytic activity. Thus, to largely eliminate autoproteolysis and subsequent generation of this truncated product, TEV variants were synthesized where Serine 219 was changed to either Asparagine (S219N) or Valine (S219V). In addition, it is well documented that although recombinant wild-type TEV protease is expressed at very high levels in *E. coli,* it is almost entirely insoluble (Kapust et al., 2001). Thus, to improve solubility of the expressed TEV, several amino acid variants were made and tested to determine whether the changes resulted in increased protein solubility. The TEV variants synthesized are shown in Table 3. Variant 1 represented a codon-optimized TEV construct engineered with a C-terminal His-tag and the S219N mutation. Variant 11 was a construct with native DNA sequence of TEV protease engineered with an N-terminal tag and the S219N mutation.

Table 3. TEV Protease Variants

Variant	Autoproteolysis Elimination Change	Solubility Enhancing Changes	Affinity Tag	DNA SEQ ID NO:	Protein SEQ ID NO:
1	S219N	—	C-term	65	66
2	S219N	L56V, S135G	N-term	67	68
3	S219N	T17S, N68D, I77V	N-term	69	70
4	S219N	N44V, L56V, S135G	N-term	71	72
5	S219N	L56V, N68D, S135G	N-term	73	74
6	S219N	T17S, L56V, N68D, I77V	N-term	75	76
7	S219N	T17S, N68D, I77V, S135G	N-term	77	78
8	S219N	T17S, N44V, L56V, N68D, I77V, S135G	C-term	79	80
9	S219V	T17S, N44V, L56V, N68D, I77V, S135G	N-term	81	82
10	S219N	T17S, N44V, L56V, N68D, I77V, S135G	N-term	83	84
11	S219N	—	N-term	85	86

[0134] To construct pET29/TEV variant expression constructs, a pUCBHB1/TEV construct was digested with restriction endonucleases that 1) excise the insert comprising the open reading frame encoding the TEV; and 2) enable this insert to be operably-linked to a pET29 vector (EMD Biosciences-Novagen, Madison, WI). Using a T4 DNA ligase procedure this insert was directionally ligated into a pET29 vector digested with the same restriction endonucleases in the multiple cloning site. The ligation mixture was transformed into electro-competent *E. coli* BL21(DE3) Acella cells (Edge BioSystems, Gaithersburg, MD) by electroporation, plated on 1.5% Luria-Bertani agar plates (pH 7.0) containing 50 μg/mL of kanamycin, and placed in a 37 °C incubator for overnight growth. Bacteria containing expression constructs were identified as kanamycin resistant colonies. Candidate constructs were isolated using an alkaline lysis plasmid mini-preparation procedure and analyzed by restriction endonuclease digest mapping and sequencing both DNA strands to confirm the presence and integrity of the TEV gene insert. This cloning strategy yielded a pET29 expression construct comprising the polynucleotide molecule encoding TEV variants operably-linked to either a carboxyl terminal or amino-terminal polyhistidine affinity purification peptide.

B. Analysis of TEV expression under different induction conditions.

[0135] To determine the best growth and protein induction conditions to use, pET29/TEV variants 9 and 10 (Table 3) were grown and induced in an IPTG induced media and an auto-inducing media. In addition, the length of induction was examined.

[0136] To induce expression with IPTG, cells harboring the TEV expression construct were first grown overnight to produce a starter culture. Fresh LB media was inoculated at 1:1000 with the overnight culture and allowed to grow, with shaking, at 37°C until OD_{600} reached 0.7, at which time IPTG was added to a final concentration of 0.6 mM. Cells were harvested 4 hrs. following induction and total cell lysates evaluated to detect target expression.

[0137] To express constructs under auto-induction conditions, 3.0 mL of PA-0.5G media containing 50 μg/mL kanamycin was inoculated with a single colony of BL21(DE3) cells harboring the appropriate expression construct and grown at 37 °C with shaking overnight. 1.0 μL of this starter culture was used

to inoculate 1.0 mL of ZYP-5052 auto-induction media containing 50 μg/mL kanamycin. Cells were grown at 37 °C with shaking and aliquots removed at 5, 8, 12, 20, and 28 hours.

[0138] To determine total TEV protease expression, 40 *[it* of the induced cell culture from each time-point was mixed with an equal volume of 2x Laemmi Sample Buffer and incubated at 95 °C for 10 minutes. 2 *[it* of 1 unit/μL Benzonase in 1 M $MgSO_4$ was added to this mixture and incubated at 95 °C for 5 minutes. A 15 *[it* aliquot was loaded and separated by MOPS polyacrylamide gel electrophoresis using NuPAGE® Novex 4-12% Bis-Tris precast polyacrylamide gels (Invitrogen, Inc, Carlsbad, CA) under denaturing, reducing conditions. The gel was washed and fixed in Fix Solution comprising 10% methanol, 7% acetic acid for 30 minutes. After fixing, the Fix Solution was removed and the gel incubated with SYPRO Ruby Protein Gel Stain at room temperature for 3 hours. The gel was then destained in Destain Solution comprising 10% methanol, 7% acetic acid at room temperature for 3 hours. The image was visualized with a Typhoon 9410 Variable Mode Imager and Imager Analysis software (GE Healthcare, Amersham Biosciences, Piscataway, NJ).

[0139] To determine soluble TEV protease expression, 1.0 mL of the induced cell culture was lysed by adding 100 *[it* of a Cell Lysis Solution comprising 1x FASTBREAK™ Cell Lysis reagent (Promega Corp., Madison, WI), 500 mM NaCl, 250 units/mL benzonase nuclease (EMD Biosciences-Novagen, Madison, WI), and 1x Protease Inhibitor Cocktail III (EMD Biosciences-Calbiochem, Gibbstown, NJ) and incubated at room temperature for 25 minutes with constant vortexing. The lysate was centrifuged at 4300 rpm for 15 minutes to pellet debris. 800 *[it* of the supernatant was transferred to a clean tube, to which 30 *[it* of MagneHis magnetic beads were added and the mixture incubated for 5 minutes with constant rotation. After incubation, the magnetic beads were sequestered on a magnetic stand, the solution was removed, and the beads washed three times with 150 *[it* wash buffer comprising 500 mM NaCl. The protein was eluted with 80 *[it* of elution buffer, an equal volume of 2x Laemmli Sample Buffer was added, and the mixture incubated at 95 °C for 10 minutes. A 15 *[it* aliquot was loaded and separated by MOPS polyacrylamide gel electrophoresis using NuPAGE® Novex 4-12% Bis-Tris precast polyacrylamide gels (Invitrogen, Inc, Carlsbad, CA) under denaturing, reducing conditions.

[0140] Results of the induction experiments indicated that auto-induction conditions resulted in 5-10-fold more expressed TEV protease relative to IPTG-induction. Comparison of total and soluble TEV protease expression in the auto-induction media revealed that although longer induction times resulted in more total protein, the amount of recoverable soluble TEV protease decreased. In fact, about 8 hours of expression at 37 °C yielded the largest amount of soluble protein. Lastly, although both the TEV S219N and TEV S219V variants exhibited significantly less autoproteolysis, the TEV S219V variant showed more truncated product at prolonged induction times suggesting that the TEV S219V variant was more prone to autoproteolysis.

[0141] Once the growth and induction conditions were optimized using pET29/TEV variants 9 and 10, expression of all eleven pET29/TEV variants was examined in parallel under these conditions. The results indicated that the order of increasing yield of soluble TEV protease, from greatest to least of the five highest expressers, was from pET29/TEV variants 5, 10, 7, 3, and 6. In comparison, the TEV variant 11 was expressed at the lowest level of all.

C. Large-scale expression and purification.

[0142] To rigorously compare TEV protease expression levels from the top five pET29/TEV variants, along with variant 11 as a control, under large-scale conditions, 3.0 mL of PA-0.5G media containing 50 μg/mL Kanamycin was inoculated with a single colony of BL21(DE3) cells harboring the appropriate expression construct and grown at 37 °C with shaking overnight. 250 ***[it*** of this starter culture was used to inoculate 250 mL of ZYP-5052 containing 50 μg/mL kanamycin and grown at 37 °C with shaking for 8 hours. The cells were pelleted by centrifugation.

[0143] To lyse cells, the cell pellet was resuspended in a 5.0 mL/gram cell pellet of Lysis Solution comprising BUGBUSTER™ Protein Extraction Reagent (EMD Biosciences-Novagen, Madison, WI), 1 x protease Inhibitor Cocktail Set III (EMD Biosciences-Calbiochem, Gibbstown, NJ), 25 units/mL Benzonase nuclease, and 1 Kunit/mL rLysozyme (EMD Biosciences-Novagen, Madison, WI). The cell suspension was incubated at room temperature on a platform rocker for 20 minutes, followed by incubation on ice for 15 minutes. The suspension was centrifuged at 4 °C for 30 minutes at 30,350 rcf to pellet debris and the supernatant was transferred to a clean tube. To prepare the insoluble cell extract pellet for SDS-PAGE analysis, the pellet was resuspended to the original volume with 1x BUGBUSTER™ Protein Extraction Reagent.

[0144] To purify a TEV protease variant by IMAC purification, the clarified lysate was mixed with TALON ™ SuperFlow Metal Affinity Cobalt Resin equilibrated with IMAC Wash Solution comprising 25 mM Sodium phosphate, pH 7.0, 500 mM NaCl, 10% glycerol and 35 mM imidazole. The lysate-resin mixture was incubated on a platform rocker at 4 °C for 1 hour and then transferred to a 20 mL disposable column support attached to a vacuum manifold. The column was washed twice with five column volumes of IMAC Wash Solution. The TEV protease was eluted from the resin with two column volumes of IMAC Elution Solution, comprising 25 mM sodium phosphate, pH 7.8, 500 mM NaCl, 10% glycerol and 500 mM imidazole, and collected in 1.0 mL fractions. Each fraction containing protein was identified by mixing 10 ***[it*** aliquot with 200 ***[it*** of QUICKSTART™ Bradford Dye reagent. Peak elution fractions were pooled and dialyzed for secondary ion exchange chromatography purification.

[0145] To dialyze an IMAC-purified TEV protease variant, the pooled sample comprising the peak elution fraction was dialyzed in a FASTDIALYZER® fitted with 25 kD MWCO membrane at 4 °C in 1 L of a Desalting Buffer with constant stirring overnight. For cation exchange chromatography, the desalting buffer (Buffer A) comprised 50 mM Tris-HCl, pH 8.0.

[0146] To purify a TEV protease variant by cation exchange chromatography, the desalted protein solution was loaded onto a 1 mL UNO-S1 cation exchange column, pre-equilibrated with Buffer A, at a flow rate of 0.5 mL/min. Bound protein was eluted by NaCl gradient with Buffer B comprising 25 mM sodium phosphate, pH 7.0, 1 M NaCl at a flow rate of 1.0 mL/min as follows: 5% Buffer B for 3 mL, 20% Buffer B for 10 mL, 20% to 100% Buffer B over 10 mL. Elution of proteins from the column was detected with a UV-Visible detector at 214 nm, 260 nm, and 280 nm, and all peak fractions were pooled and protein concentration determined. Aliquots were flash frozen in liquid nitrogen and stored at -80 °C. TEV variant 7 had the highest yield of soluble protease (ca. 35 mg/L) followed by variant 3 (ca. 24 mg/L) and variant 10 (ca. 23 mg/L). The remaining two variants, 5 and 6, had yields of 18 and 8 mg/L, respectively. Yield of the TEV variant 11 was ca. 0.6 mg/L. As such, all of the top five TEV variants containing a solubility enhancing amino acid change resulted in at least a 10-fold increase in soluble TEV protease purified relative to the TEV variant 11 that only comprised the autoproteolysis eliminating amino acid

change (S219N). When comparing the rank order of yield of TEV protease from small- and large-scale expression studies, variant 5 exhibited the highest yield in small-scale expressions (Example 1C). However, it was variant 7 that had the highest yield in large-scale expressions. Repeat comparison of yields from large-scale batches consistently revealed variant 7 to be the highest expressing variant. As a result, variant 7 represented the lead TEV protease construct and was used for all subsequent studies described here.

[0147] To determine the proteolytic activity of TEV protease variants, a TEV protease variant, or AcTEV protease as a positive control, was added to 30 μl of a Reaction Solution comprising 50 mM Tris-HCl, pH 8.0, 1 mM DTT, and 2.5 μg of a TEV substrate and incubated at 30 °C for 30 minutes, 60 minutes, and 120 minutes. The reactions were quenched by adding 2 x Laemmi Sample Buffer and incubating the sample at 95 °C for 10 minutes. A 15 μl aliquot was loaded and separated by MOPS polyacrylamide gel electrophoresis using NuPAGE® Novex 4-12% Bis-Tris precast polyacrylamide gels (Invitrogen, Inc, Carlsbad, CA) under denaturing, reducing conditions. The gel was washed and fixed in Fix Solution comprising 10% methanol, 7% acetic acid for 30 minutes. After fixing, the Fix Solution was removed and the gel incubated with SYPRO Ruby Protein Gel Stain at room temperature for 3 hours. The gel was then destained in Destain Solution comprising 10% methanol, 7% acetic acid at room temperature for 3 hours. The image was visualized with a Typhoon 9410 Variable Mode Imager and analyzed with ImageQuantTL Image Analysis software (GE Healthcare, Amersham Biosciences, Piscataway, NJ). The ratio of intensities of uncleaved substrate and cleaved product was used to calculate percentage of cleaved TEV substrate. The results of the TEV protease activity assay are given in Table 4.

Table 4. TEV Protease Activity Assay

TEV Protease	TEV Substrate Cleavage (%)		
	30 minute	60 minute	120 minute
AcTEV	73.9	91.6	97.2
TEV variant 3	96.5	97.7	98.1
TEV variant 5	95.6	97.8	95.6
TEV variant 6	90.8	96.8	97.2
TEV variant 7	96.6	97.8	97.7
TEV variant 10	74.2	93.3	96.1

Example 2

Intracellular activation of a Clostridial toxin with a TEV protease cleavage site using two different expression constructs

[0148] The following example illustrates a procedure useful for expressing in a cell a Clostridial toxin comprising a di-chain loop region comprising an exogenous protease cleavage site as disclosed in the present specification.

A. Construction of pET29/BoNT/A-TEV expression construct.

[0149] In order to produce a BoNT/A comprising a TEV protease cleavage site located within the di-chain loop region, an open reading frame (SEQ ID NO: 87) encoding the desired BoNT/A-TEV (SEQ ID NO: 88) was synthesized using standard procedures (BlueHeron Biotechnology, Bothell, WA).

Complementary oligonucleotides of 20 to 50 bases in length, spanning the entire open reading frame of BoNT/A-TEV were synthesized using standard phosphoramidite synthesis. These oligonucleotides were hybridized into double stranded duplexes that were sequentially ligated together to assemble the full-length polynucleotide molecule. This polynucleotide molecule was cloned using standard molecular biology methods into a pUCBHBI carrier vector at the *Sma*I site to generate the pUCBHB1/BoNT/A-TEV constructs. The synthesized polynucleotide molecule was verified by sequencing using BIG DYE TERMINATOR™ Chemistry 3.1 (Applied Biosystems, Foster City, CA) and an ABI 3100 sequencer (Applied Biosystems, Foster City, CA).

[0150] To generate the pET29/BoNT/A-TEV expression construct, pUCBHB1/BoNT/A-TEV was digested with restriction endonucleases that 1) excise the insert comprising the open reading frame encoding BoNT/A-TEV; and 2) enable this insert to be operably-linked to a pET29 vector (EMD Biosciences-Novagen, Madison, WI). This insert was subcloned using a T4 DNA ligase procedure into a pET29 vector digested with the analogous restriction endonucleases to yield the appropriate pET29/BoNT/A-TEV expression construct. The ligation mixture was transformed into electro-competent *E. coli* BL21 (DE3) Acella cells (Edge BioSystems, Gaithersburg, MD) by electroporation, plated on 1.5% Luria-Bertani agar plates (pH 7.0) containing 50 μg/mL of kanamycin, and placed in a 37 °C incubator for overnight growth. Bacteria containing expression constructs were identified as kanamycin resistant colonies. Candidate constructs were isolated using an alkaline lysis plasmid mini-preparation procedure and analyzed by restriction endonuclease digest mapping and sequencing both DNA strands to confirm the presence and integrity of the insert. This cloning strategy yielded a pET29 expression construct comprising the polynucleotide molecule encoding BoNT/A-TEV operably-linked to a carboxyl-terminal polyhistidine affinity purification peptide.

B. Construction of pET22/TEV expression constructs.

[0151] To generate a pET22/TEV variant expression construct, a pET29/TEV variant 7 expression construct was digested with restriction endonucleases that 1) excise the insert comprising the open reading frame (SEQ ID NO: 77) encoding the TEV protease (SEQ ID NO: 78); and 2) enable this insert to be operably-linked to a pET22 vector (EMD Biosciences-Novagen, Madison, WI). This insert was subcloned using a T4 DNA ligase procedure into a pET22 vector digested with the analogous restriction endonucleases to yield the appropriate pET22/TEV expression construct. The ligation mixture was transformed into electro-competent *E. coli* BL21(DE3) Acella cells (Edge BioSystems, Gaithersburg, MD) by electroporation, plated on 1.5% Luria-Bertani agar plates (pH 7.0) containing 50 μg/mL of ampicillin, and placed in a 37 °C incubator for overnight growth. Bacteria containing expression constructs were identified as ampicillin resistant colonies. Candidate constructs were isolated using an alkaline lysis plasmid mini-preparation procedure and analyzed by restriction endonuclease digest mapping and sequencing both DNA strands to confirm the presence and integrity of the insert. This cloning strategy yielded a pET22 expression construct comprising the polynucleotide molecule encoding TEV variant 7 operably-linked to an amino-terminal polyhistidine affinity purification peptide.

C. Construction of cells comprising pET29/BoNT/A-TEV and pET22/TEV expression constructs.

[0152] To make a cell comprising pET29/BoNT/A-TEV and pET22/TEV expression constructs, a pET29/BoNT/A-TEV expression construct was transformed into electro-competent *E. coli* BL21 (DE3) cells harboring pET22/TEV variant 7 expression construct using electroporation, plated on 1.5% Luria-

Bertani agar plates (pH 7.0) containing 50 μg/mL of ampicillin and 50 μg/mL of kanamycin, and placed in a 37 °C incubator for overnight growth. Bacteria containing both expression constructs were identified as ampicillian-kanamycin resistant colonies. Candidate constructs were isolated using an alkaline lysis plasmid mini-preparation procedure and analyzed by restriction endonuclease digest mapping to determine the presence of both constructs. This cloning strategy yielded cells comprising pET29/BoNT/A-TEV and pET22/TEV expression constructs.

D. In situ activation of BoNT/A-TEV.

[0153] To produce di-chain forms of BoNT/A-TEV under auto-induction conditions, 3.0 mL of PA-0.5G media containing 50 μg/mL kanamycin and 50 μg/mL ampicillin was inoculated with a single colony of BL21 (DE3) cells harboring pET29/BoNT/A-TEV and pET22/TEV expression constructs and grown at 37 °C with shaking overnight. About 1.0 ***[it*** of this starter culture was used to inoculate a 1.0 mL of ZYP-5052 containing 50 μg/mL kanamycin and 50 μg/mL ampicillin and grown at 37 °C with shaking for 3.5 hours and then at 22 °C with shaking for 18.5 hours. As a control, BL21 (DE3) cells harboring pET29/BoNT/A-TEV alone were grown and induced as described above, except only 50 μg/mL kanamycin was used as a selective agent.

[0154] Following growth and induction, the cells were lysed and IMAC purified essentially as described in Example 1B. The IMAC purified samples were analyzed by SDS-PAGE and the gels stained essentially as described in Example 1B.

[0155] The results indicate that when pET29/BoNT/A-TEV is expressed alone, an approximately 150 kDa band corresponding to the single-chain for of BoNT/A-TEV was detected under both reducing and non-reducing conditions. In contrast, when BoNT/A-TEV was co-expressed with TEV protease, two bands were observed under reducing conditions, one of approximately 50 kDa and the other of approximately 100 kDa. Moreover, when the same samples were run under non-reducing conditions, the approximately 50 kDa and approximately 100 kDa bands disappeared and a new band of approximately 150 kDa was observed. Taken together, these observations indicate that the approximately 50 kDa and approximately 100 kDa bands seen under reducing conditions correspond to the light and heavy chains of the BoNT/A-TEV, and that the presence of these two bands was indicative of di-chain formation of BoNT/A-TEV. Thus, co-expression of BoNT/A-TEV and TEV protease in these cells results in cleavage of BoNT/A-TEV at the TEV protease cleavage site located within the di-chain loop and the subsequent formation of the di-chain form of BoNT/A-TEV.

[0156] To confirm these results, a large scale expression of BL21 (DE3) cells harboring pET29/BoNT/A-TEV and pET22/TEV expression constructs was done. 3.0 mL of PA-0.5G media containing 50 μg/mL kanamycin and 50 μg/mL ampicillin was inoculated with a single colony of BL21 (DE3) cells comprising pET29/BoNT/A-TEV and pET22/TEV expression constructs and grown at 37 °C with shaking overnight. About 250 ***[it*** of this starter culture was used to inoculate 250 mL of ZYP-5052 containing 50 μg/mL kanamycin and 50 μg/mL ampicillin and grown at 37 °C with shaking for 3.5 hours and then at 22 °C with shaking for 18.5 hours. The cells were pelleted by centrifugation. The cells were lysed and IMAC purified as described in Example 1C.

[0157] To dialyze the IMAC-purified BoNT/A-TEV for secondary ion exchange chromatography, the pooled sample comprising the peak elution fractions were dialyzed in a FASTDIALYZER ® fitted with 25

kD MWCO membrane at 4 °C in 1 L of a Desalting Buffer with constant stirring overnight. For anion exchange chromatography, the desalting buffer (Buffer A) comprised 50 mM Tris-HCl, pH 8.0.

[0158] To purify BoNT/A-TEV by anion exchange chromatography, the desalted protein solution was loaded onto a 1 mL UNO-Q1 anion exchange column, pre-equilibrated with Buffer A, at a flow rate of 0.5 mL/min. Bound protein was eluted by NaCl gradient with Buffer B comprising 50 mM Tris-HCl, pH 8.0, 1 M NaCl at a flow rate of 0.5 mL/min as follows: 3% Buffer B for 3 mL, 7% Buffer B for 10 mL, 7% to 100% Buffer B over 10 mL. Elution of proteins from the column was detected with a UV-Visible detector at 214 nm, 260 nm, and 280 nm, and all peak fractions were pooled and protein concentration determined. Aliquots were flash frozen in liquid nitrogen and stored at -80 °C. Purified BoNT/A-TEV protein was analyzed by SDS-PAGE, and the gels stained essentially as described in Example 1B. The results confirm the initial small scale experiments and indicate that the single-chain BoNT/A-TEV is converted to its di-chain form with near 100% efficiency.

[0159] To assess the activity of the BoNT/A-TEV di-chains, these toxins were evaulated in a cell-based assay and animal-based assay.

[0160] To test the activity of BoNT/A-TEV di-chains using a cell-based assay, an immuno-based BoNT/A activity assay using multiplex ECL sandwich ELISA was performed essentially as described in patent application Fernandez-Salas, et al., *Immuno-Based BoNT/A Activity Assays,* Attorney Docket No. 18383 (BOT), which is hereby incorporated by reference in its entirety.

[0161] To obtain a BoNT/A-TEV treated cell lysate for analysis, approximately 50,000 cells from a stock culture of a SiMa cell line were seeded into a poly-D-lysine 96-well plate containing a serum-free medium containing Minimum Essential Medium, 2 mM GlutaMAX™ I with Earle's salts, 1 x B27 supplement, 1 x N2 supplement, 0.1 mM Non-Essential Amino Acids, 10 mM HEPES and 25 μg/mL of GTb1. These cells were incubated in a 37 °C incubator under 5% carbon dioxide until the cells differentiated, as assessed by standard and routine morphological criteria, such as growth arrest and neurite extension (approximately 3 days). The media was aspirated from each well and replaced with fresh media containing either 0 (untreated sample), 0.01 nM, 0.04 nM, 0.12 nM, 0.37 nM, 1.11 nM, 3.33 nM and 10.0 nM of a BoNT/A-TEV. After a 24 hr treatment, the cells were washed, incubated for an additional two days without toxin. To harvest the cells, the medium was aspirated, washed with 1 x PBS, and lysed by adding 30 μl of Lysis Buffer comprising 50 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100 to each well, and the plate incubated on a shaker rotating at 500 rpm for 30 minutes at 4 °C. The plate was centrifuged at 4000 rpm for 20 minutes at 4 °C to pellet cell debris and the supernatant was transferred to a capture antibody coated 96-well plate to perform the detection step.

[0162] To prepare the oSNAP-25 capture antibody solution, the oSNAP-25 monoclonal antibody contained in the ascites from hybridoma cell line 2E2A6 was purified using a standard Protein A purification protocol To prepare the a-SNAP-25 detection antibody solution, a-SNAP-25 rabbit polyclonal antibody S9684 (Sigma, St. Louis, MO) was conjugated to Ruthenium(II)-tris-bipyridine-(4-methysulfonate) NHS ester labeling reagent (Meso Scale Discovery, Gaithersburg, MD) according to the manufacturer's instructions (Meso Scale Discovery, Gaithersburg, MD). To prepare the solid phase support comprising the capture antibody that was specific for a SNAP-25 cleaved product, approximately 5 μL of a-SNAP-25 monoclonal antibody 2E2A6 solution (20 μg/mL in 1 x PBS) was added to each well of a 96-well MSD High Bind plate and the solution was allowed to air dry in a biological safety cabinet for

2-3 hours in order to liquid evaporate the solution. The capture antibody-bound wells were then blocked and used directly to detect BoNT/A activity.

[0163] To detect the presence of a cleaved SNAP-25 product by ECL sandwich ELISA analysis, the Blocking Buffer from stored plates was aspirated, 25 µl of a lysate from cells treated with BoNT/A was added to each well and the plates were incubated at 4 °C for 2 hrs. Plate wells were washed three times by aspirating the cell lysate and rinsing each well three times with 200 µl 1 x PBS, 0.1% TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate). After washing, 25 µl of 5 µg/mL oSNAP-25 detection antibody solution comprising 2% Amersham Blocking Reagent in 1 x PBS, 0.1% TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate) was added to each well, the plate was sealed, and the sealed plate was incubated at room temperature for 1 hour with shaking. After a-SNAP-25 detection antibody incubation, the wells were washed three times with 200 µL 1 x PBS, 0.1% TWEEN-20® (polyoxyethylene (20) sorbitan monolaureate). The raw data obtained from the ECL imager was then transferred to SigmaPlot v. 9.0 and a 4-parameter logistics fit was used to define the dose-response curves. There were no constraints used for the 4-parameter logistic function when plotting the data. Graphical reports were generated using the following analysis: R2 (correlation coefficient), a (Max for data set), b (hillslope), and X0 ± SE (EC_{50} value ± standard error). The results from two independent runs indicate that the activity of both di-chains was nearly identical and within 2-fold of the native di-chain.

[0164] To test the activity of BoNT/A-TEV di-chains using an animal-based assay, an *in vivo* Digit Abduction Score (DAS) assay was performed. CD-1 Fe mice were weighed and placed into subsets of 10 animals for each discrete DAS assay. Mice were included into a particular subset based on the following criteria: 1) good health; 2) robust baseline DAS response of 0; 3) inclusion in a median weight range of X ± 2 g established for the selected subset and 4) weight greater than 17.0 g.

[0165] Each mouse was injected with 5 µl of one of seven different doses of BoNT/A-TEV (0.01 nM, 0.04 nM, 0.12 nM, 0.37 nM, 1.11 nM, 3.33 nM and 10.0 nM) with a 30-gauge needle in the gastrocnemius muscle of the right hind limb. As a control, the gastrocnemius muscle of the left hind limb was injected with 5 µl of a solution not containing any BoNT/A-TEV. Mice were observed for the DAS response consecutively for the first 4 days. The DAS was read by lifting each mouse by the tail and precisely observing the injected hind limbs. The abduction or no abduction of the hind digits reveals the effect of paralysis due to the test toxin injected in the muscle. The digit abduction of the injected hind limb was compared with that of the non-injected hind limb and scored accordingly. DAS data was analyzed by calculating the ED_{50} dose based on peak mean DAS score and AUC (area under the curve) in terms of u/Kg and/or ng/Kg. This was accomplished as follows: 1) the mean peak DAS score for each dose was calculated in each study; 2) any dose that elicited more than five deaths in any study was eliminated from consideration; 3) the highest dose used in a given individual study was the lowest dose which elicited an average peak of 4.0; 4) the lowest dose used in a given individual study was the highest dose which elicited an average peak of 0; 5) curves were constructed for each individual study of average peak DAS vs. log (dose); 6) an AUC value was calculated for each group of 10 mice of the multiple groups in some studies; 7) curves were constructed for each individual study of average AUC vs. log (dose); 8) an x, y replicate response curve was constructed for each set of multiple identical studies; for each test toxin; 9) dose-response data were analyzed by non-linear regression (non-weighted) using a three-parameter logistic equation (Sigma Plot v 8.0; SPSS Science, Chicago, Illinois) using the following equation:

$$y = a/(1 + (x/x0)^b)$$

where y is the response, a is the asymptotic y_{max}, b is the slope, x is the dose, and 0 is the ED_{50} dose, For peak ED_{50} determinations, Ymax was set to 4 (maximum DAS reading on scale). Mean (peak and/or AUC) ED_{50} values were computed for each eight-dose study performed.

[01 66] The results from two independent runs indicate that the level of activity of both di-chains was nearly identical and within 2-fold of the native di-chain. Taken together, the cell-based assay and DAS assay data indicate that the process of intracellular activation yields di-chain rBoNT/A which was not only structurally comparable to the *in-vitro* nicked material but also functionally indistinguishable.

Example 3

Intracellular activation of a Clostridial toxin with a TEV protease cleavage site using two different expression constructs under control of independent promoters

[01 67] The following example illustrates a procedure useful for expressing in a cell a Clostridial toxin comprising a di-chain loop region comprising an exogenous protease cleavage site as disclosed in the present specification. In this case, the formation of the di-chain form of the toxin is regulated by TEV protease under control of an independent promoter.

A. Construction of pBAD/TEV expression construct.

[01 68] In order to produce a TEV protease recombinantly, the expression of which was under control of an arabinose promoter ($_{PBAD}$), the open reading frame encoding the TEV protease variant 7 (Table 3 [130]), minus an N-terminal His tag, was cloned into the expression vector pBAD/Myc-HisA to construct pBAD/TEV. To construct pBAD/TEV, an open reading frame encoding the TEV protease variant 7 (SEQ ID NO: 106), minus an N-terminal poly-histidine tag, was synthesized using standard procedures (BlueHeron Biotechnology, Carlsbad, CA). The synthetic fragment was also flanked by restriction sites to enable this insert to be operably-linked to a pBAD/Myc-HisA vector (Life Technologies, Madison, WI). Using a T4 DNA ligase procedure this insert was directionally ligated into a pBAD/Myc-HisA vector digested with the same restriction endonucleases in the multiple cloning site. The ligation mixture was transformed into electro-competent *E. coli* BL21(DE3) Acella cells (Edge BioSystems, Gaithersburg, MD) by electroporation, plated on 1.5% Luria-Bertani agar plates (pH 7.0) containing 50 μg/mL of ampicillin, and placed in a 37 °C incubator for overnight growth. Bacteria containing expression constructs were identified as ampicillin resistant colonies. Candidate constructs were isolated using an alkaline lysis plasmid mini-preparation procedure and analyzed by restriction endonuclease digest mapping and sequencing both DNA strands to confirm the presence and integrity of the TEV gene insert. This cloning strategy yielded a pBAD/TEV expression construct comprising the polynucleotide molecule encoding TEV variant 7 free of a polyhistidine affinity purification peptide.

B. Construction of cells comprising pET29/BoNT/A-TEV and pBAD/TEV expression constructs.

[01 69] To make a cell comprising pET29/BoNT/A-TEV and pBAD/TEV expression constructs, a pET29/BoNT/A-TEV expression construct (described in Example 2A) was transformed into electro-competent *E. coli* BL21(DE3) cells harboring pBAD/TEV variant 7 expression construct using electroporation, plated on 1.5% Luria-Bertani agar plates (pH 7.0) containing 50 μg/mL of ampicillin and 50 μg/mL of kanamycin, and placed in a 37 °C incubator for overnight growth. Bacteria containing both expression constructs were identified as ampicillian-kanamycin resistant colonies. Candidate constructs were isolated using an alkaline lysis plasmid mini-preparation procedure and analyzed by restriction

endonuclease digest mapping to determine the presence of both constructs. This cloning strategy yielded cells comprising pET29/BoNT/A-TEV and pBAD/TEV expression constructs.

C. In situ activation of BoNT/A-TEV.

[01 70] To produce di-chain forms of BoNT/A-TEV under auto-induction conditions, 3.0 mL of PA-0.5G media containing 50 μg/mL kanamycin and 50 μg/mL ampicillin was inoculated with a single colony of BL21 (DE3) cells harboring pET29/BoNT/A-TEV and pBAD/TEV expression constructs and grown at 37 °C with shaking overnight. 250 ***[it*** of this starter culture was used to inoculate 250 mL of ZYP-5052 containing 50 μg/mL kanamycin and 100 μg/mL ampicillin and grown at 37 °C with shaking for 8 hours and then at 22 °C with shaking for 14 hours. At this point, TEV expression was induced with 0.2% L-arabinose and the culture was grown for an additional 4 hours at 22°C. As a control, BL21 (DE3) cells harboring pET29/BoNT/A-TEV alone were grown and induced as described above, except only 50 μg/mL kanamycin was used as a selective agent.

[01 71] Following growth and induction, the cells were lysed and IMAC purified essentially as described in Example 1C. To dialyze the IMAC-purified BoNT/A-TEV for secondary ion exchange chromatography, the pooled sample comprising the peak elution fractions were dialyzed in a FASTDIALYZER ® fitted with 25 kD MWCO membrane at 4 °C in 1 L of a Desalting Buffer with constant stirring overnight. For anion exchange chromatography, the desalting buffer (Buffer A) comprised 50 mM Tris-HCl, pH 8.0.

[01 72] To purify BoNT/A-TEV by anion exchange chromatography, the desalted protein solution was loaded onto a 1 mL UNO-Q1 anion exchange column, pre-equilibrated with Buffer A, at a flow rate of 0.5 mL/min. Bound protein was eluted by NaCl gradient with Buffer B comprising 50 mM Tris-HCl, pH 8.0, 1 M NaCl at a flow rate of 0.5 mL/min as follows: 3% Buffer B for 3 mL, 7% Buffer B for 10 mL, 7% to 100% Buffer B over 10 mL. Elution of proteins from the column was detected with a UV-Visible detector at 214 nm, 260 nm, and 280 nm, and all peak fractions were pooled and protein concentration determined .

[01 73] Purified BoNT/A-TEV protein was analyzed by SDS-PAGE, and the gels stained essentially as described in Example 1B. The results indicate that when pET29/BoNT/A-TEV is expressed alone, an approximately 150 kDa band corresponding to the single-chain for of BoNT/A-TEV was detected under both reducing and non-reducing conditions. In contrast, when BoNT/A-TEV was co-expressed with TEV protease under control of the P_{BAD} promoter and induced with arabinose, two bands were observed under reducing conditions, one of approximately 50 kDa and the other of approximately 100 kDa. Moreover, when the same samples were run under non-reducing conditions, the approximately 50 kDa and approximately 100 kDa bands disappeared and a new band of approximately 150 kDa was observed . Taken together, these observations indicate that the approximately 50 kDa and approximately 100 kDa bands seen under reducing conditions correspond to the light and heavy chains of the BoNT/A-TEV, and that the presence of these two bands was indicative of di-chain formation of BoNT/A-TEV. Thus, co-expression of BoNT/A-TEV and TEV protease in these cells results in cleavage of BoNT/A-TEV at the TEV protease cleavage site located within the di-chain loop and the subsequent formation of the di-chain form of BoNT/A-TEV. The results indicate that between 90-95% of the single-chain BoNT/A-TEV is converted to its di-chain form.

Example 4

Intracellular activation of a Clostridial toxin with a TEV protease cleavage site

using a dual expression construct

[0174] The following example illustrates methods useful for purifying and quantifying a Clostridial toxin comprising an exogenous protease cleavage site as disclosed in the present specification.

A. Construction of pET29/BoNT/A-TEV/2xTEV dual expression construct.

[0175] To construct pET29/BoNT/A-TEV/2xTEV dual expression construct, a synthetic fragment (SEQ ID NO: 89) encoding the last 37 amino acids of BoNT/A-TEV as well as transcription (T7 promoter, lac operator site) and translation (RBS) elements necessary for *E. coli* expression and the entire coding region of TEV variant 7 was synthesized using standard procedures (BlueHeron Biotechnology, Bothell, WA). Complementary oligonucleotides of 20 to 50 bases in length, were synthesized using standard phosphoramidite synthesis. These oligonucleotides were hybridized into double stranded duplexes that were sequentially ligated together to assemble the full-length polynucleotide molecule. This polynucleotide molecule was cloned using standard molecular biology methods into a pUCBHBI carrier vector at the *Sma*I site to generate the pUCBHB1/BoNT/A-TEV_C-term/T7Prom/TEV plasmid. The synthesized polynucleotide molecule was verified by sequencing using BIG DYE TERMINATOR™ Chemistry 3.1 (Applied Biosystems, Foster City, CA) and an ABI 3100 sequencer (Applied Biosystems, Foster City, CA.

[0176] To generate the pET29/BoNT/A-TEV/2xTEV expression construct, pUCBHB1/BoNT/A-TEV_C-term/T7Prom/TEV was digested with restriction endonucleases that 1) excise the insert comprising the C-terminus of BoNT/A-TEV, transcription and translation motifs necessary for *E. coli* expression of a second open reading frame, and the entire coding region of TEV variant 7; and 2) enable this insert to be operably-linked behind the BoNT/A gene in pET29/BoNT/A-TEV vector from Example 1A. This insert was subcloned using a T4 DNA ligase procedure into the pET29/BoNT/A-TEV vector digested with the analogous restriction endonucleases to yield the appropriate pET29/BoNT/A-TEV/2xTEV dual expression construct comprising the BoNT/A-TEV and TEV protease variant 7 open reading frames with the intervening transcription and translation elements of SEQ ID NO: 89. The ligation mixture was transformed into electro-competent *E. coli* BL21(DE3) Acella cells (Edge BioSystems, Gaithersburg, MD) by electroporation, plated on 1.5% Luria-Bertani agar plates (pH 7.0) containing 50 μg/mL of kanamycin, and placed in a 37 °C incubator for overnight growth. Bacteria containing expression constructs were identified as kanamycin resistant colonies. Candidate constructs were isolated using an alkaline lysis plasmid mini-preparation procedure and analyzed by restriction endonuclease digest mapping and sequencing both DNA strands to confirm the presence and integrity of the insert. This cloning strategy yielded a pET29 dual expression construct comprising the polynucleotide molecule encoding a BoNT/A-TEV variant operably-linked to a carboxyl terminal polyhistidine affinity purification tag and a TEV protease. The open reading frame organization was such that transcription initiation from the first T7 promoter yields an mRNA with the open reading frame encoding BoNT/A-TEV and the open reading frame encoding TEV protease. In addition, transcription initiation from the second T7 promoter yields mRNA with the open reading frame encoding only TEV protease. Thus, there would be twice as many transcripts encoding TEV protease compared to BoNT/A-TEV.

B. In situ activation of BoNT/A-TEV from pET29/BoNT/A-TEV/2xTEV.

[0177] To produce di-chain forms of BoNT/A-TEV under auto-induction conditions, 3.0 mL of PA-0.5G media containing 50 μg/mL Kanamycin was inoculated with a single colony of BL21(DE3) cells

comprising pET29/BoNT/A-TEV/TEV dual expression construct and grown at 37 °C with shaking overnight. About 250 µL of this starter culture was used to inoculate 250 mL of ZYP-5052 containing 50 µg/mL kanamycin and grown at 37 °C with shaking for 3.5 hours and then at 22 °C with shaking for 18.5 hours. The cells were pelleted by centrifugation. The cells were lysed, IMAC purified, desalted , purified by anion exchange chromatography, analyzed by SDS-PAGE, and the gels stained essentially as described in Example 2D. As a control, BL21 (DE3) cells harboring pET29/BoNT/A-TEV alone were grown and induced as described above, except only 50 µg/mL kanamycin was used as a selective agent.

[0178] The results indicate that when expressed alone, an approximately 150 kDa band corresponding to the single-chain for of BoNT/A-TEV was detected under both reducing and non-reducing conditions. In contrast, when BoNT/A-TEV was co-expressed with TEV protease, two bands were observed under reducing conditions, one of approximately 50 kDa and the other of approximately 100 kDa. Moreover, when the same samples were run under non-reducing conditions, the approximately 50 kDa and approximately 100 kDa bands disappeared and a new band of approximately 150 kDa was observed . Taken together, these observations indicate that the approximately 50 kDa and approximately 100 kDa bands seen under reducing conditions correspond to the light and heavy chains of the BoNT/A-TEV, and that the presence of these two bands was indicative of di-chain formation of BoNT/A-TEV. The results also indicated that the single-chain BoNT/A-TEV was converted to its di-chain form with greater than 95% efficiency. Thus, co-expression of BoNT/A-TEV and TEV protease from a dual expression construct in these cells results in cleavage of BoNT/A-TEV at the TEV protease cleavage site located within the di-chain loop and the subsequent formation of the di-chain form of BoNT/A-TEV.

C. Construction of pRSFduet/TEV/2xBoNT/A-TEV dual expression constructs.

[0179] To determine if reversing the organization of the open reading frames encoding BoNT/A-TEV and the TEV protease would affect yield and cleavage efficiency of BoNT/A-TEV, a dual expression construct was made where transcription initiation from the first T7 promoter yields an mRNA with the open reading frames encoding TEV and BoNT/A-TEV and transcription initiation from the second T7 promoter yields mRNA with the open reading frame encoding only BoNT/A-TEV. Thus, there would be twice as many mRNA's encoding BoNT/A-TEV compared to TEV protease.

[0180] To construct pRSFduet/TEV/2xBoNT/A-TEV dual expression construct, two sequential cloning reactions were performed. First, the open reading frame (SEQ ID NO: 91) encoding TEV variant 7 (SEQ ID NO: 22) was amplified by PCR from the pET29/TEV variant 7 expression construct. The 5'-end of the open reading frame encoding the poly-histidine affinity tag was excluded from the amplification to encode a tag-less protease. Following amplification, the PCR product was digested at the unique restriction sites, incorporated at the ends of the PCR product by means of the PCR primers, and cloned into the corresponding sites in MCSI (multiple cloning site) of the dual expression plasmid pRSFduet-1 (EMD Biosciences-Novagen, Madison, WI) using a T4 DNA ligase procedure. This intermediate construct was designated pRSduet/TEV. Next, a pET29/BoNT-A/TEV expression construct was digested with restriction endonucleases that 1) excise the insert comprising the open reading frame (SEQ ID NO: 87) encoding the BoNT/A-TEV (SEQ ID NO: 88); and 2) enable this insert to be operably-linked to the MCS2 in pRSFduet/TEV. The BoNT/A-TEV insert was subcloned into the MCS2 of the pRSFduet vector using a T4 DNA ligase procedure to yield the appropriate pRSFduet/TEV/2xBoNT/A-TEV dual expression construct. This cloning strategy yielded a pRSFduet dual expression construct where transcription from

the first T7 promoter would produce mRNA's encoding TEV and BoNT/A-TEV and transcription from the second T7 promoter would produce mRNA's encoding only BoNT/A-TEV.

[0181] This cloning strategy will yield a pRSFduet dual expression construct where the first T7 promoter will transcribe the open reading frame encoding BoNT/A-TEV and the second T7 promoter will transcribe the open reading encoding TEV protease.

D. Construction of pET29/BoNT/A-TEV/TEV dual expression construct.

[0182] To determine BoNT/A-TEV yields and efficiency of conversion to di-chain from a transcription unit configuration where BoNT/A-TEV and TEV could only be produced from their own independent mRNA's, pET29/BoNT/A-TEV/TEV was constructed. To generate the pET29/BoNT/A-TEV/TEV dual expression construct, a short synthetic DNA fragment was used to incorporate a T7 terminator site (SEQ ID NO: 92) in the intervening sequence between the open reading frames of BoNT/A-TEV and TEV in the dual expression construct pET29/BoNT/A-TEV/2xTEV (Example 3A above). Using a T4 DNA ligase procedure, this was essentially accomplished by swapping the intervening region in pET29/BoNT/A-TEV/2xTEV which lacked a T7 terminator site with a synthetic DNA fragment harboring the intervening transcription and translation elements along with a T7 termination site of SEQ ID NO: 93. The resulting dual expression construct, designated pET29/BoNT/A-TEV/TEV, comprises the polynucleotide molecule encoding a BoNT/A-TEV variant operably-linked to a carboxyl terminal polyhistidine affinity tag and TEV protease, transcribed from the first and second T7 promoters, respectively.

E. In situ activation of BoNT/A-TEV.

[0183] The growth and induction of di-chain forms of BoNT/A-TEV under auto-induction conditions was done essentially as described in Example 2D, except the BL21 (DE3) cells comprising a pET29/BoNT/A-TEV/2xTEV dual expression construct, a pRSF/TEV/2xBoNT/A-TEV dual expression construct, or a pET29/BoNT/A-TEV/TEV dual expression construct were used and single colonies from each of these cell lines were used to inoculate four 1.0 mL cultures in parallel. After growth and induction, the four 1.0 mL replicates were pooled together for processing. The cells were lysed and IMAC purified, and analyzed by SDS-PAGE, and the gels stained essentially as described in Example 1B. As a control, BL21 (DE3) cells harboring pET29/BoNT/A-TEV alone were grown and induced as described above, except only 50 μg/mL kanamycin was used as a selective agent. The results indicate that BoNT/A-TEV was expressed at very comparable levels from cells containing any one of the three dual expression constructs; however, the extent of conversion to di-chain varied. Single-chain BoNT/A-TEV was converted to its di-chain form with ca. 96% efficiency when the proteins were expressed from pET29/BoNT/A-TEV/2xTEV, with ca. 81% efficiency when the proteins were expressed from pET29/BoNT/A-TEV/TEV, and with greater than 99% efficiency when the proteins were expressed from pRSFduet/TEV/2xBoNT/A-TEV.

Example 5

Intracellular activation of a protein comprising an integrated TEV protease cleavage site-opioid binding domain using a dual expression construct

[0184] The following example illustrates methods useful for purifying and quantifying any of the proteins comprising a di-chain loop comprising an exogenous protease cleavage site disclosed in the present specification.

A. Construction of pRSFduet/TEV/2xNociLHN/A-TEV dual expression construct.

[0185] To construct pRSFduet/TEV/2xNocil_HN/A-TEV dual expression construct, a pET29/Nocil_HN/A-TEV expression construct was digested with restriction endonucleases that 1) excise the insert comprising the open reading frame (SEQ ID NO: 94) encoding the NociLHN/A-TEV (SEQ ID NO: 95); and 2) enable this insert to be operably-linked to the MCS2 of pRSFduet/TEV, a pRSFduet-1 vector harboring TEV variant 7 in MCSI (Described in Example 3C). The NociLHN/A-TEV insert was subcloned into the MCS2 of the pRSFduet/TEV construct using a T4 DNA ligase procedure to yield the appropriate pRSFduet/TEV/2xNociLHN/A-TEV dual expression construct. The ligation mixture was transformed into electro-competent *E. coli* BL21(DE3) Acella cells (Edge BioSystems, Gaithersburg, MD) by electroporation, plated on 1.5% Luria-Bertani agar plates (pH 7.0) containing 50 μg/mL of kanamycin, and placed in a 37 °C incubator for overnight growth. Bacteria containing expression constructs were identified as kanamycin resistant colonies. Candidate constructs were isolated using an alkaline lysis plasmid mini-preparation procedure and analyzed by restriction endonuclease digest mapping and sequencing both DNA strands to confirm the presence and integrity of the insert. This cloning strategy yielded a pRSFduet dual expression construct where transcription from the first T7 promoter would produce mRNA's encoding TEV and NociLHN/A-TEV and transcription from the second T7 promoter would produce mRNA's encoding only NociLHN/A-TEV.

B. In situ activation of NociLHN/A-TEV.

[0186] To produce di-chain forms of NociLHN/A-TEV under auto-induction conditions, 3.0 mL of PA-0.5G media containing 50 μg/mL kanamycin was inoculated with a single colony of BL21(DE3) cells comprising pRSFduet/TEV/2xNociLHN/A-TEV dual expression construct and grown at 37 °C with shaking overnight. 250 ***[it*** of this starter culture was used to inoculate 250 mL of ZYP-5052 containing 50 μg/mL kanamycin and grown at 37 °C with shaking for 8 hours and then at 16 °C with shaking for 18 hours. The cells were pelleted by centrifugation. The cells were lysed, IMAC purified, desalted, purified by anion exchange chromatography, analyzed by SDS-PAGE, and the gels stained essentially as described in Example 2D. As a control, BL21(DE3) cells harboring NociLHN/A-TEV alone were grown and induced as described above.

[0187] The results indicate that when expressed alone, an approximately 102 kDa band corresponding to the single-chain of NociLHN/A-TEV was detected under both reducing and non-reducing conditions. In contrast, when NociLHN/A-TEV was co-expressed with TEV protease, two bands were observed under reducing conditions, one of approximately 50.8 kDa and the other of approximately 51.3 kDa. Moreover, when the same samples were run under non-reducing conditions, the approximately 50.8 kDa and approximately 51.3 kDa bands disappeared and a new band of approximately 102 kDa was observed. Taken together, these observations indicate that the approximately 50.8 kDa and approximately 51.3 kDa bands seen under reducing conditions respectively correspond to the Clostridial toxin enzymatic domain and the Clostridial toxin translocation domain with the nociceptin targeting moiety attached to its amino terminus. The presence of these two bands was indicative of di-chain formation of NociLHN/A-TEVand that the single-chain NociLHN/A-TEV was converted to its di-chain form with greater than 95% efficiency. Thus, co-expression of NociLHN/A-TEV and TEV protease from a dual expression construct in these cells results in cleavage of NociLHN/A-TEV at the TEV protease cleavage site located within the integrated TEV protease cleavage site-opioid binding domain and the subsequent formation of the di-chain form of NociLHN/A-TEV.

C. Construction of pRSFduet/TEV/2xDynLHN/A-TEV dual expression construct.

[0188] pRSFduet/TEV/2xDynl_HN/A-TEV dual expression construct was generated almost exactly as pRSFduet/TEV/2xNocil_HN/A-TEV. A pET29/Dynl_HN/A-TEV expression construct was digested with restriction endonucleases that 1) excise the insert comprising the open reading frame (SEQ ID NO: 96) encoding the DynLHN/A-TEV (SEQ ID NO: 97); and 2) enable this insert to be operably-linked to the MCS2 of of pRSFduet/TEV (Described in Example 3C). The DynLHN/A-TEV insert was subcloned into the MCS2 of the pRSFduet/TEV construct using a T4 DNA ligase procedure to yield the appropriate pRSFduet/TEV/2xDynLHN/A-TEV dual expression construct. The ligation mixture was transformed into electro-competent *E. coli* BL21(DE3) Acella cells (Edge BioSystems, Gaithersburg, MD) by electroporation, plated on 1.5% Luria-Bertani agar plates (pH 7.0) containing 50 μg/mL of kanamycin, and placed in a 37 °C incubator for overnight growth. Bacteria containing expression constructs were identified as kanamycin resistant colonies. Candidate constructs were isolated using an alkaline lysis plasmid mini-preparation procedure and analyzed by restriction endonuclease digest mapping and sequencing both DNA strands to confirm the presence and integrity of the insert. This cloning strategy yielded a pRSFduet dual expression construct where transcription from the first T7 promoter would produce mRNAs encoding TEV and DynLHN/A-TEV and transcription from the second T7 promoter would produce mRNA's encoding only DynLHN/A-TEV.

D. In situ activation of DynLHN/A-TEV.

[0189] To produce di-chain forms of NociLHN/A-TEV under auto-induction conditions, 3.0 mL of PA-0.5G media containing 50 μg/mL kanamycin was inoculated with a single colony of BL21(DE3) cells comprising pRSFduet/TEV/2xDynLHN/A-TEV dual expression construct and grown at 37 °C with shaking overnight. 250 ***[it*** of this starter culture was used to inoculate 250 mL of ZYP-5052 containing 50 μg/mL kanamycin and grown at 37 °C with shaking for 8 hours and then at 16 °C with shaking for 18 hours. The cells were pelleted by centrifugation. The cells were lysed, IMAC purified, desalted, purified by anion exchange chromatography, analyzed by SDS-PAGE, and the gels stained essentially as described in Example 2D. As a control, BL21 (DE3) cells harboring DynLHN/A-TEV alone were grown and induced as described above.

[0190] The results indicate that when expressed alone, an approximately 102 kDa band corresponding to the single-chain for of DynLHN/A-TEV was detected under both reducing and non-reducing conditions. In contrast, when DynLHN/A-TEV was co-expressed with TEV protease, two bands were observed under reducing conditions, one of approximately 50.8 kDa and the other of approximately 52 kDa. Moreover, when the same samples were run under non-reducing conditions, the approximately 50.8 kDa and approximately 52 kDa bands disappeared and a new band of approximately 102 kDa was observed. Taken together, these observations indicate that the approximately 50.8 kDa band corresponds to the Clostridial toxin enzymatic domain and an approximately 52 kDa band corresponds to the Clostridial toxin translocation domain with the dynorphin targeting moiety attached to its amino terminus. The presence of these two bands was indicative of di-chain formation of DynLHN/A-TEV and also that the single-chain DynLHN/A-TEV was converted to its di-chain form with greater than 95% efficiency. Thus, co-expression of DynLHN/A-TEV and TEV protease from a dual expression construct in these cells results in cleavage of DynLHN/A-TEV at the TEV protease cleavage site located within the integrated TEV protease

cleavage site-opioid binding domain and the subsequent formation of the di-chain form of DynLHN/A-TEV.

Example 6

Intracellular activation of a protein comprising an integrated TEV protease cleavage site-Galanin binding domain using two different expression constructs

[0191] The following example illustrates methods useful for purifying and quantifying any of the proteins comprising a di-chain loop comprising an integrated TEV protease cleavage site-opioid binding domain disclosed in the present specification where the target protein and the protease are expressed from separate plasmids and under control of different promoters.

A. Construction of pET29/GalLHN/A-TEV expression construct.

[0192] To construct the pET29/GalLHN/A-TEV expression construct, a pET29/Dynl_HN/A-TEV expression construct was first digested with restriction endonucleases to excise a DNA segment encoding the di-chain loop comprising an integrated TEV protease cleavage site- dynorphin binding domain. The resulting pET29/LHn/A framework fragment was ligated with a synthetic DNA fragment bracketed with the compatible restriction sites (SEQ ID NO: 98), comprising the di-chain loop comprising an integrated TEV protease cleavage site-galanin binding domain (SEQ ID NO: 99). The ligation mixture was transformed into electro-competent *E. coli* BL21 (DE3) Acella cells (Edge BioSystems, Gaithersburg, MD) by electroporation, plated on 1.5% Luria-Bertani agar plates (pH 7.0) containing 50 μg/mL of kanamycin, and placed in a 37 °C incubator for overnight growth. Bacteria containing expression constructs were identified as kanamycin resistant colonies. Candidate constructs were isolated using an alkaline lysis plasmid mini-preparation procedure and analyzed by restriction endonuclease digest mapping and sequencing both DNA strands to confirm the presence and integrity of the insert. This cloning strategy yielded the pET29/GalLHN/A-TEV expression construct comprising the open reading frame (SEQ ID NO: 100) encoding the GalLHN/A-TEV (SEQ ID NO: 101) in which expression of GalLHN/A-TEV is under control of the T7 promoter.

B. Construction of pColdIV/TEV expression construct.

[0193] To generate an expression construct in which TEV is under control of the cold-shock promoter (csp), the open reading frame (SEQ ID NO: 91) encoding TEV variant 7 (SEQ ID NO: 22) was amplified by PCR from the pET29/TEV variant 7 expression construct. The 5'-end of the open reading frame encoding the poly-histidine affinity tag was excluded from the amplification to encode a tag-less protease. Following amplification, the PCR product was digested at the unique restriction sites, incorporated at the ends of the PCR product by means of the PCR primers, and cloned into the corresponding sites in the multiple cloning site of the expression plasmid pColdIV (Clontech Laboratories, Inc. Madison, WI) using a T4 DNA ligase procedure. The ligation mixture was transformed into electro-competent *E. coli* BL21 (DE3) Acella cells (Edge BioSystems, Gaithersburg , MD) by electroporation, plated on 1.5% Luria-Bertani agar plates (pH 7.0) containing 50 μg/mL of ampicillin, and placed in a 37 °C incubator for overnight growth. Bacteria containing expression constructs were identified as ampicillin resistant colonies. Candidate constructs were isolated using an alkaline lysis plasmid mini-preparation procedure and analyzed by restriction endonuclease digest mapping and sequencing both DNA strands to confirm the presence and integrity of the insert. This cloning strategy yielded the pColdIV/TEV expression

construct comprising the polynucleotide molecule encoding TEV variant 7 under control of the cold-shock promoter.

C. Construction of cells comprising pET29/GalLHN/A-TEV and pColdIV/TEV expression constructs.

[0194] To make a cell comprising pET29/GalLHN/A-TEV and pColdIV/TEV expression constructs, the pET29/GalLHN/A-TEV expression construct was transformed into electro-competent *E. coli* BL21 (DE3) cells harboring pColdIV/TEV using electroporation, plated on 1.5% Luria-Bertani agar plates (pH 7.0) containing 100 μg/mL of ampicillin and 50 μg/mL of kanamycin, and placed in a 37 °C incubator for overnight growth. Bacteria containing both expression constructs were identified as ampicillian-kanamycin resistant colonies. Candidate constructs were isolated using an alkaline lysis plasmid mini-preparation procedure and analyzed by restriction endonuclease digest mapping to determine the presence of both constructs. This cloning strategy yielded cells comprising pET29/GalLHN/A-TEV and pColdIV/TEV expression constructs.

D. In situ activation of pET29/GalLHN/A.

[0195] To produce di-chain forms of GalLHN/A-TEV under auto-induction conditions, 3.0 mL of PA-0.5G media containing 50 μg/mL kanamycin and 100 μg/mL ampicillin was inoculated with a single colony of BL21 (DE3) cells harboring pET29/GalLHN/A-TEV and pColdIV/TEV expression constructs and grown at 37 °C with shaking overnight. About 250 ***[it*** of this starter culture was used to inoculate 250 mL of ZYP-5052 containing 50 μg/mL kanamycin and 100 μg/mL ampicillin and grown at 37 °C with shaking for 8 hours and then at 15 °C with shaking for 18 hours. The cells were lysed and IMAC purified using Magne-His resin.

[0196] To purify di-chain GalLHN/A-TEV by Magne-His purification, induced cells from 250 mL expression cultures were resuspended in 16 mL of cold (4-6 °C) IMAC Wash Buffer consisting of 100 mM HEPES, pH 7.5, 10 % v/v glycerol, 10 mM imidazole, 1 M NaCl . The cell suspension was transferred to a sealed-atmosphere treatment chamber (#101-021-006, Branson Ultrasonics Corporation) and sonicated by 15 pulses (10 sec, 30% amplitude, 0.5-inch disruptor horn) with 1 minute in between pulses (Sonifier® Digital 450, Branson Ultrasonics Corporation). During sonication the sealed-atmosphere treatment chamber was cooled by passing chilled water from a circulating water bath (3.5 °C) through the outer jacket of the chamber. Sonicated material was transferred from the treatment chamber to a clean Oakridge tube and centrifuged at 30,500 RCF for 30 min (SL-50T Rotor, Sorvall; FIBERLite® F21 S-8X50 Rotor, Piramoon Technologies Inc.) at 4 °C to remove insoluble cellular debris. The clarified lysate was aspirated by syringe and passed first through a 0.8 μm and then a 0.45 μm syringe filter (Sartorius) in series into a clean 50 mL conical tube. Magne-His™ Protein Purification Resin (Promega Corp., Madison, WI) was vortexed to a uniform suspension and 4 mL of the suspension transferred to the clarified lysate. The tube was sealed and inverted several times to mix the particles well. The mixture was incubated for 30 min with gentle rocking to bind the target protein at 16 °C. The tube was transferred to a MagneSil Magnetic Separation Unit (Promega Corp., Madison, WI) and ~2 min were allowed for capture of the resin particles. The supernatant solution was removed and the tube removed from the separation unit. The resin was then resuspended in 10 mL IMAC Wash Buffer,captured on the magnetic separation unit, and the wash buffer removed. The wash step was repeated two more times. To elute the target

protein, the resin was resuspended in 5 mL of the Magne-His™ Elution Buffer (100 mM HEPES, pH 7.5, 500mM Imidazole) incubated at room temperature for 2 min, the resin captured on the magnetic separation unit and the supernatant solution transferred to a new tube. The elution step was repeated once.

[0197] To dialyze the IMAC-purified GalLHN/A-TEV for secondary ion exchange chromatography, the pooled elution fractions were dialyzed in a FASTDIALYZER ® fitted with 25 kD MWCO membrane at 4 °C in 1 L of a Desalting Buffer (Buffer A: 50 mM Tris-HCl, pH 8.0) with constant stirring overnight.

[0198] To purify di-chain GalLHN/A-TEV by anion exchange chromatography, the desalted protein solution was loaded onto a 1 mL UNO-Q1 anion exchange column, pre-equilibrated with Buffer A, at a flow rate of 1 mL/min. Bound protein was eluted by NaCl gradient with Buffer B comprising 50 mM Tris-HCl, pH 8.0, 1 M NaCl at a flow rate of 1 mL/min as follows: 7% Buffer B for 3 mL, 15% Buffer B for 7 mL, 10% to 50% Buffer B over 10 mL. Elution of proteins from the column was detected with a UV-Visible detector at 214 nm, 260 nm, and 280 nm, and all peak fractions were pooled and protein concentration determined. Aliquots were flash frozen in liquid nitrogen and stored at -80 °C. Purified BoNT/A-TEV protein was analyzed by SDS-PAGE, and the gels stained essentially as described in Example 1B.

[0199] The results indicate that when GalLHN/A-TEV was co-expressed with TEV protease, two nearly superimposing bands were observed under reducing conditions, one of approximately 51.1 kDa and another of approximately 52.1 kDa. Moreover, when the same samples were run under non-reducing conditions, the two approximately 51.1 kDa and 52.1 kDa bands disappeared and a new band of approximately 103 kDa was observed. Taken together, these observations indicate that the approximately 51.1 kDa band corresponds to the Clostridial toxin enzymatic domain and the approximately 52.1 kDa band corresponds to the Clostridial toxin translocation domain with the galanin targeting moiety attached to its amino terminus. The presence of these two bands was indicative of di-chain formation of GalLHN/A-TEV and also that the single-chain GalLHN/A-TEV was converted to its di-chain form with approximately 90% efficiency. Thus, co-expression of GalLHN/A-TEV and TEV protease in these cells from independent plasmids results in cleavage of GalLHN/A-TEV at the TEV protease cleavage site located within the integrated TEV protease cleavage site-galanin binding domain and the subsequent formation of the di-chain form of GalLHN/A-TEV.

Example 7: Prophetic

Intracellular activation of a protein comprising an integrated TEV protease cleavage site-Galanin binding domain using a dual expression construct

[0200] The following example illustrates methods useful for purifying and quantifying any of the proteins comprising a di-chain loop comprising an integrated TEV protease cleavage site-opioid bidning domain disclosed in the present specification where the target protein and the protease are expressed from a dual expression plasmid.

A. Construction of pRSFduet/TEV/2xGalLHN/A-TEV dual expression construct.

[0201] To construct pRSFduet/TEV/2xGalLHN/A-TEV dual expression construct similar to *pRSFduet/TEV/2xNociLHN/A-TEV and pRSFduet/TEV/2xDynLHN/A-TEV* constructed before (See Example 4), a pET29/GalLHN/A-TEV expression construct will be digested with restriction

endonucleases to 1) excise the insert comprising the open reading frame (SEQ ID NO: 100) encoding the GalLHN/A-TEV (SEQ ID NO: 101); and 2) enable this insert to be operably-linked to the MCS2 of pRSFduet/TEV, a pRSFduet-1 vector harboring TEV variant 7 in MCSI (Described in Example 3C). The GalLHN/A-TEV insert will be subcloned into the MCS2 of the pRSFduet/TEV construct using a T4 DNA ligase procedure to yield the pRSFduet/TEV/2xGalLHN/A-TEV dual expression construct. The ligation mixture will be transformed into electro-competent *E. coli* BL21(DE3) Acella cells (Edge BioSystems, Gaithersburg, MD) by electroporation, plated on 1.5% Luria-Bertani agar plates (pH 7.0) containing 50 μg/mL of kanamycin, and placed in a 37 °C incubator for overnight growth. Bacteria containing expression constructs will be identified as kanamycin resistant colonies and candidate constructs confirmed by restriction endonuclease digest mapping and sequencing both DNA strands to confirm the presence and integrity of the insert. This cloning strategy will yield a pRSFduet dual expression construct where transcription from the first T7 promoter will produce mRNAs encoding TEV and GalLHN/A-TEV and transcription from the second T7 promoter will produce mRNAs encoding only GalLHN/A-TEV.

B. In situ activation of GalLHN/A-TEV.

[0202] To produce di-chain forms of GalLHN/A-TEV under auto-induction conditions, 3.0 mL of PA-0.5G media containing 50 μg/mL kanamycin will be inoculated with a single colony of BL21(DE3) cells comprising pRSFduet/TEV/2xGalLHN/A-TEV dual expression construct and grown at 37 °C with shaking overnight. 250 μL of this starter culture will be used to inoculate 250 mL of ZYP-5052 containing 50 μg/mL kanamycin and grown at 37 °C with shaking for 8 hours and then at 16 °C with shaking for 18 hours. The cells will be pelleted by centrifugation, lysed, IMAC purified, desalted, and purified by anion exchange chromatography as described in Example 5D. Purified target protein will be analyzed by SDS-PAGE under both reducing and non-reducing conditons, and the gels stained essentially as described in Example 1B to assess expression levels and the extent to which GalLHN/A-TEV produced from the pRSFduet/TEV/2xGalLHN/A-TEV dual expression construct is converted to its di-chain form.

Example 8

Intracellular activation of a protein comprising an integrated TEV protease cleavage site-Dynorphin binding domain using a dual expression construct in BEVS

[0203] The following example illustrates methods useful for purifying and quantifying any of the proteins comprising a di-chain loop comprising an integrated TEV protease cleavage site-opioid binding domain disclosed in the present specification where the target protein and the protease are co-expressed in a dual expression construct and under control of two independent promoters in the baculovirus expression vector system (BEVS).

A. Construction of pBAC-6/TEV/DynLHN/A-TEV dual expression construct.

[0204] To construct the pBAC-6/TEV/DynLHN/A-TEV dual expression construct, a synthetic fragment (SEQ ID NO: 107) encoding recombinant TEV variant 7 downstream of the p10 promoter sequence and DynLHn/A-TEV downstream of the polH promoter sequence in the opposite orientation was synthesized using standard procedures (BlueHeron Biotechnology, Bothell, WA). Complementary oligonucleotides of 20 to 50 bases in length were synthesized using standard phosphoramidite synthesis. These oligonucleotides were hybridized into double stranded duplexes that were sequentially ligated together to assemble the full-length polynucleotide molecule. This polynucleotide molecule was cloned using

standard molecular biology methods into a pUCBHBI carrier vector at the *Sma*\ site to generate the pUCBHB1/p10-TEV/polH-DynI_HN/A-TEV plasmid. The synthesized polynucleotide molecule was verified by sequencing using BIG DYE TERMINATOR™ Chemistry 3.1 (Applied Biosystems, Foster City, CA) and an ABI 3100 sequencer (Applied Biosystems, Foster City, CA.

[0205] To generate the pBAC-6/TEV/DynLHN/A-TEV dual expression construct, pUCBHB1/p10-TEV/polH-DynLHN/A-TEV was digested with restriction endonucleases that 1) excise the insert comprising the entire coding region of TEV variant 7 under control of the p10 promoter and DynLHN/A-TEV in the opposite direction under control of the polH promoter; and 2) enable this insert to be operably-linked to a pBAC-6 transfer vector (EMD Biosciences-Novagen, Madison, WI). This insert was subcloned using a T4 DNA ligase procedure into the pBAC-6 transfer vector digested with the analogous restriction endonucleases to yield the engineered pBAC-6 dual expression construct comprising TEV protease variant 7 open reading frame downstream of the p10 promoter and a second open reading frame of DynLHN/A-TEV downstream of the polH promoter. The ligation mixture was transformed into electro-competent *E. coli* BL21(DE3) Acella cells (Edge BioSystems, Gaithersburg, MD) by electroporation, plated on 1.5% Luria-Bertani agar plates (pH 7.0) containing 100 μg/mL of ampicillin, and placed in a 37 °C incubator for overnight growth. Bacteria containing expression construct were identified as ampicillin resistant colonies. Candidate constructs were isolated using an alkaline lysis plasmid mini-preparation procedure and analyzed by restriction endonuclease digest mapping and sequencing both DNA strands to confirm the presence and integrity of the insert. This cloning strategy yielded a pBAC-6 dual expression construct comprising the polynucleotide molecule encoding a DynLH/A-TEV operably-linked to a carboxyl terminal polyhistidine affinity purification tag and TEV protease.

B. Generation of high titer TEV/DynLHN/A-TEV recombinant baculovirus stock.

[0206] Before di-chain forms of DynLHN/A-TEV could be produced, high titre recombinant baculovirus stock comprising TEV/DynLHN/A-TEV were generated. Approximately $2x10^6$ Sf9 insect cells were seeded in 35 mm dishes in a 2 mL volume of insect cell culture medium ESF921. A transfection solution was prepared by mixing Solution A (comprising 2 μg of pBAC-6/TEV/DynLHN/A-TEV, 0.5 μg of linearized *flashBAC* baculovirus DNA (Oxford Expression Technologies, Oxford, UK), and 100 μL of Transfection Medium) with solution B (comprising 6 μL of TRANSLT®-2020 transfection reagent and 100 μL of Transfection Medium) and incubating at room temperature for 30 minutes. An additional 800 μL of Transfection Medium was next added to the Solution A/B mixture, mixed gently, and added dropwise onto the cells. Cells were incubated at 28°C for 5 hours, at the end which 3 mL of ESF 921 was added to bring the final volume up to 4 mL in each well. The incubation was continued at 28°C for 4-5 days for the production of P0 recombinant virus. To generate higher titer P1 recombinant baculovirus seed stocks, virus isolated from P0 supernatant was titered using bacu/oQUANT (Oxford Expression Technologies, Oxford, UK) and further amplified in shake flasks. About 100-200 mL of Sf9 cells at a density of $2x10^6$ cells/mL were infected with P0 virus at an MOI (multiplicity of infection) < 1 pfu/cell and incubated with shaking for 4 - 5 days. Following quantification, the high titer P1 stock was used to infect Tni cells for high-level protein expression.

C. In situ activation of DynLHN/A-TEV.

[0207] To produce di-chain forms of DynHN/A-TEV, 50 mL Tni cells at a concentration of 1 x 10^6/ml_ were infected at an MOI of 5 with recombinant P1 virus stock comprising TEV/DynLHN/A-TEV and harvested 3 days post-infection (pi). The cells were lysed and IMAC purified using Magne-His resin.

[0208] To purify di-chain DynLHN/A-TEV by Magne-His purification, the cell pellet was resuspended in 20 mL of PBS w/o Ca^{2+} or Mg^{2+} in the presence of 100 *[it* Insect PopCulture Reagent and 20 *[i*L (10U) Benzonase Nuclease, mixed gently and incubated for 15 minutes at room temperature. After clarifying the cell lysate by centrifugation at 16,000 rpm for 15 minutes at 4 °C, the supernatant was mixed with 4 mL of uniformly suspended Magne-His™ Protein Purification Resin (Promega Corp., Madison, Wl). The mixture was incubated for 20 min at room temperature with gentle rocking to bind the target protein. The tube was transferred to a MagneSil magnetic separation unit for about 2 min to allow capture of the resin particles. After removing the supernatant, the tube was removed from the separation unit and the resin resuspended in 10 mL of IMAC wash buffer. Again, the resin was captured on the magnetic separation unit and the wash buffer removed. The wash step was repeated two more times. To elute the target protein, the resin was resuspended in 2.5 mL of the Magne-His™ Elution Buffer (100 mM HEPES, pH 7.5, 500mM Imidazole), incubated at room temperature for 2 min, the resin captured on the magnetic separation unit, and the supernatant solution transferred to a new tube. The elution step was repeated again to maximize target recovery from the magnetic resin.

[0209] To dialyze the IMAC-purified DynLHN/A-TEV for secondary ion exchange chromatography, the pooled elution fractions were dialyzed in a FASTDIALYZER® fitted with 25 kD MWCO membrane at 4 °C in 1 L of a Desalting Buffer (Buffer A: 50 mM Tris-HCI, pH 8.0) with constant stirring overnight.

[0210] To purify di-chain DynLHN/A-TEV by anion exchange chromatography, the desalted protein solution was loaded onto a 1 mL UNO-Q1 anion exchange column, pre-equilibrated with Buffer A, at a flow rate of 1 mL/min. Bound protein was eluted by NaCI gradient with Buffer B comprising 50 mM Tris-HCI, pH 8.0, 1 M NaCI at a flow rate of 1 mL/min as follows: 7% Buffer B for 3 mL, 15% Buffer B for 7 mL, 10% to 50% Buffer B over 10 mL. Elution of proteins from the column was detected with a UV-Visible detector at 214 nm, 260 nm, and 280 nm, and all peak fractions were pooled and protein concentration determined. Aliquots were stored at -20 °C. Purified DynLHN/A-TEV protein was analyzed by SDS-PAGE, and the gels stained essentially as described in Example 1B.

[0211] The results indicate that when DynLHN/A-TEV was co-expressed with TEV protease in insect cells and purified to near homogeneity, two nearly superimposing bands were observed under reducing conditions, one of approximately 51 kDa and another of approximately 52 kDa. Moreover, when the same samples were run under non-reducing conditions, the two approximately 50 kDa and 52 kDa bands disappeared and a new band of approximately 102 kDa was observed. Taken together, these observations indicate that the approximately 51 kDa band corresponds to the Clostridial toxin enzymatic domain and the approximately 52 kDa band corresponds to the Clostridial toxin translocation domain with the dynorphin targeting moiety attached to its amino terminus. The presence of these two bands was indicative of di-chain formation of DynLHN/A-TEV and also that the single-chain DynLHN/A-TEV was converted to its di-chain form with 80-90% efficiency. Thus, co-expression of DynLHN/A-TEV and TEV protease in insect cells infected with TEV/DynLHN/A-TEV recombinant baculovirus generated from pBAC-6/TEV/DynLHN/A-TEV dual expression construct results in cleavage of DynLHN/A-TEV at the TEV

protease cleavage site located within the integrated TEV protease cleavage site-dynorphin binding domain and the subsequent formation of the di-chain form of DynLHN/A-TEV.

[0212] Although aspects of the present invention have been described with reference to the disclosed embodiments, one skilled in the art will readily appreciate that the specific examples disclosed are only illustrative of these aspects and in no way limit the present invention. Various modifications can be made without departing from the spirit of the present invention.

CLAIMS

What is claimed:

1. An intracellular method of converting a single-chain Clostridial toxin comprising a di-chain loop region into its di-chain form, the method comprising the steps of:
   a) growing a cell comprising a dual expression construct at 37 °C for about 3.5 hours, the dual expression construct comprising;
      i) an open reading frame encoding a single-chain Clostridial toxin, the single-chain Clostridial toxin comprising an enzymatic domain, a translocation domain, a binding domain, and a di-chain loop region comprising a TEV protease cleavage site; and
      ii) an open reading frame encoding a TEV protease;
   b) growing the cell at 22 °C for about 16 to about 18 hours,
   wherein growth at step (b) induces expression of the single-chain Clostridial toxin and the TEV protease from the dual expression construct; and
   wherein the produced TEV protease cleaves the single-chain Clostridial toxin at the TEV protease cleavage site located within the di-chain loop region, thereby converting the single-chain Clostridial toxin into its di-chain form.
2. The intracellular method according to Claim 1, wherein the single-chain Clostridial toxin comprises a linear amino-to-carboxyl single polypeptide order of 1) the Clostridial toxin enzymatic domain, the di-chain loop region comprising a TEV protease cleavage site, the Clostridial toxin translocation domain and the Clostridial toxin binding domain; 2) the Clostridial toxin enzymatic domain, the di-chain loop region comprising a TEV protease cleavage site, the Clostridial toxin binding domain and the Clostridial toxin translocation domain; 3) the Clostridial toxin binding domain, the Clostridial toxin translocation domain, the di-chain loop region comprising a TEV protease cleavage site and the Clostridial toxin enzymatic domain; 4) the Clostridial toxin binding domain, the Clostridial toxin enzymatic domain, the di-chain loop region comprising a TEV protease cleavage site and the Clostridial toxin translocation domain; 5) the Clostridial toxin translocation domain, the di-chain loop region comprising a TEV protease cleavage site, the Clostridial toxin enzymatic domain and the Clostridial toxin binding domain; or 6) the Clostridial toxin translocation domain, the di-chain loop region comprising a TEV protease cleavage site, the Clostridial binding domain and the Clostridial toxin enzymatic domain.
3. The intracellular method according to Claim 1, wherein the Clostridial toxin enzymatic domain is a BoNT/A enzymatic domain, a BoNT/B enzymatic domain, a BoNT/C1 enzymatic domain, a BoNT/D enzymatic domain, a BoNT/E enzymatic domain, a BoNT/F enzymatic domain, a BoNT/G enzymatic domain, a TeNT enzymatic domain, a BaNT enzymatic domain, or a BuNT enzymatic domain.
4. The intracellular method according to Claim 1, wherein the Clostridial toxin translocation domain is a BoNT/A translocation domain, a BoNT/B translocation domain, a BoNT/C1 translocation domain, a BoNT/D translocation domain, a BoNT/E translocation domain, a BoNT/F translocation domain, a BoNT/G translocation domain, a TeNT translocation domain, a BaNT translocation domain, or a BuNT translocation domain.
5. The intracellular method according to Claim 1, wherein the Clostridial toxin binding domain is a BoNT/A binding domain, a BoNT/B binding domain, a BoNT/C1 binding domain, a BoNT/D binding

domain, a BoNT/E binding domain, a BoNT/F binding domain, a BoNT/G binding domain, a TeNT binding domain, a BaNT binding domain, or a BuNT binding domain.

6. An intracellular method of converting a single-chain protein into its di-chain form, the method comprising the steps of

a) growing a cell comprising a dual expression construct at 37 °C for about 8 hours, the dual expression construct comprising;

i) an open reading frame encoding a single-chain protein, the single-chain protein comprising an enzymatic domain, a translocation domain, an integrated TEV protease cleavage site-opioid binding domain; and

ii) an open reading frame encoding a TEV protease;

b) growing the cell at about 12 to about 16 °C for about 16 to about 18 hours,

wherein growth at step (b) induces expression of the single-chain protein and the TEV protease from the dual expression construct; and

wherein the produced TEV protease cleaves the single-chain protein at the TEV protease cleavage site located within the integrated TEV cleavage site opioid binding domain, thereby converting the single-chain protein into its di-chain form.

7. The intracellular method according to Claim 6, wherein the protein comprises a linear amino-to-carboxyl single polypeptide order of 1) the Clostridial toxin enzymatic domain, the Clostridial toxin translocation domain, and the integrated TEV protease cleavage site-opioid binding domain, 2) the Clostridial toxin enzymatic domain, the integrated TEV protease cleavage site-opioid binding domain, and the Clostridial toxin translocation domain, 3) the integrated TEV protease cleavage site-opioid binding domain, the Clostridial toxin translocation domain, and the Clostridial toxin enzymatic domain, 4) the integrated TEV protease cleavage site-opioid binding domain, the Clostridial toxin enzymatic domain, and the Clostridial toxin translocation domain, 5) the Clostridial toxin translocation domain, the integrated TEV protease cleavage site-opioid binding domain, and the Clostridial toxin enzymatic domain; or 6) the Clostridial toxin translocation domain, the Clostridial toxin enzymatic domain, and the integrated TEV protease cleavage site-opioid binding domain

8. The intracellular method according to Claim 1, wherein the Clostridial toxin enzymatic domain is a BoNT/A enzymatic domain, a BoNT/B enzymatic domain, a BoNT/C1 enzymatic domain, a BoNT/D enzymatic domain, a BoNT/E enzymatic domain, a BoNT/F enzymatic domain, a BoNT/G enzymatic domain, a TeNT enzymatic domain, a BaNT enzymatic domain, or a BuNT enzymatic domain.

9. The intracellular method according to Claim 1, wherein the Clostridial toxin translocation domain is a BoNT/A translocation domain, a BoNT/B translocation domain, a BoNT/C1 translocation domain, a BoNT/D translocation domain, a BoNT/E translocation domain, a BoNT/F translocation domain, a BoNT/G translocation domain, a TeNT translocation domain, a BaNT translocation domain, or a BuNT translocation domain.

10. The intracellular method according to Claim 1, wherein the integrated TEV protease cleavage site-opioid binding domain is an integrated TEV protease cleavage site-nociceptin binding domain, an integrated TEV protease cleavage site-dynorphin binding domain, an integrated TEV protease cleavage site-enkephalin binding domain, an integrated TEV protease cleavage site-BAM22 binding domain, an integrated TEV protease cleavage site-endomorphin binding domain, an integrated TEV

protease cleavage site-endorphin binding domain region, an integrated TEV protease cleavage site-hemorphin binding domain, or an integrated TEV protease cleavage site-rimorphin binding domain.

11. An intracellular method of converting a single-chain protein into its di-chain form, the method comprising the steps of

a) growing a cell comprising a dual expression construct at 37 °C for about 8 hours, the dual expression construct comprising;

i) an open reading frame encoding a single-chain protein, the single-chain protein comprising an enzymatic domain, a translocation domain, a non-Clostridial toxin binding domain and a di-chain loop region comprising a TEV protease cleavage site; and

ii) an open reading frame encoding a TEV protease;

b) growing the cell at about 12 to about 16 °C for about 16 to about 18 hours,

wherein growth at step (b) induces expression of the single-chain protein and the TEV protease from the dual expression construct; and

wherein the produced TEV protease cleaves the single-chain protein at the TEV protease cleavage site located within the di-chain loop region, thereby converting the single-chain protein into its di-chain form.

12. The intracellular method according to Claim 11, wherein the single-chain Clostridial toxin comprises a linear amino-to-carboxyl single polypeptide order of 1) the Clostridial toxin enzymatic domain, the di-chain loop region comprising a TEV protease cleavage site, the Clostridial toxin translocation domain and the non-Clostridial toxin binding domain; 2) the Clostridial toxin enzymatic domain, the di-chain loop region comprising a TEV protease cleavage site, the non-Clostridial toxin binding domain and the Clostridial toxin translocation domain; 3) the non-Clostridial toxin binding domain, the Clostridial toxin translocation domain, the di-chain loop region comprising a TEV protease cleavage site and the Clostridial toxin enzymatic domain; 4) the non-Clostridial toxin binding domain, the Clostridial toxin enzymatic domain, the di-chain loop region comprising a TEV protease cleavage site and the Clostridial toxin translocation domain; 5) the Clostridial toxin translocation domain, the di-chain loop region comprising a TEV protease cleavage site, the Clostridial toxin enzymatic domain and the non-Clostridial toxin binding domain; or 6) the Clostridial toxin translocation domain, the di-chain loop region comprising a TEV protease cleavage site, the non-Clostridial binding domain and the Clostridial toxin enzymatic domain.

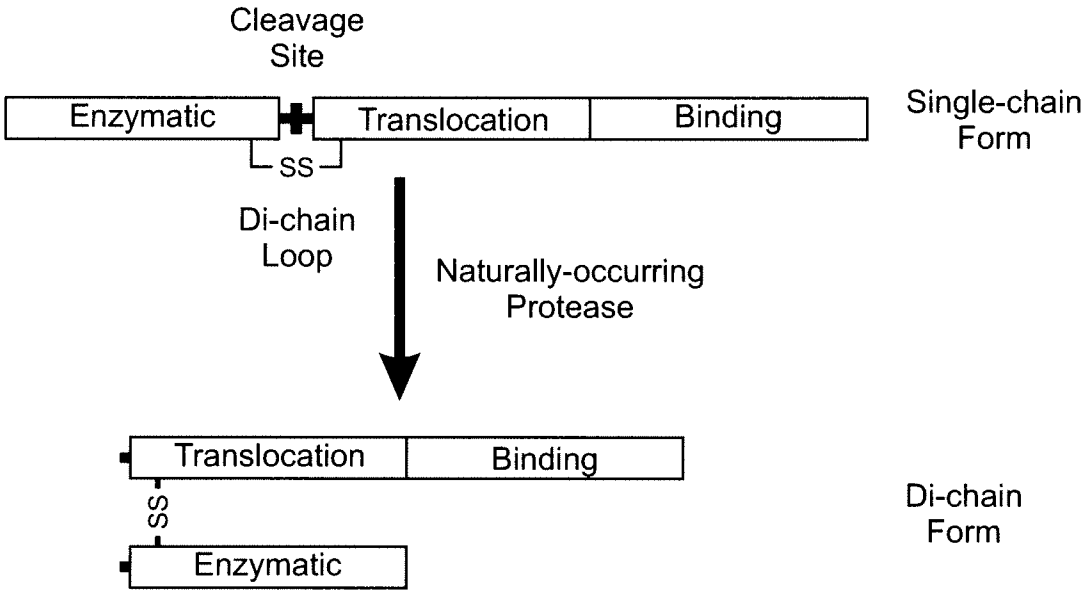
13. The intracellular method according to Claim 11, wherein the Clostridial toxin enzymatic domain is a BoNT/A enzymatic domain, a BoNT/B enzymatic domain, a BoNT/C1 enzymatic domain, a BoNT/D enzymatic domain, a BoNT/E enzymatic domain, a BoNT/F enzymatic domain, a BoNT/G enzymatic domain, a TeNT enzymatic domain, a BaNT enzymatic domain, or a BuNT enzymatic domain.

14. The intracellular method according to Claim 11, wherein the Clostridial toxin translocation domain is a BoNT/A translocation domain, a BoNT/B translocation domain, a BoNT/C1 translocation domain, a BoNT/D translocation domain, a BoNT/E translocation domain, a BoNT/F translocation domain, a BoNT/G translocation domain, a TeNT translocation domain, a BaNT translocation domain, or a BuNT translocation domain.

15. The intracellular method according to Claim 11, wherein the non-Clostridial toxin binding domain is an opioid peptide binding domain, a melanocortin peptide binding domain, a galanin peptide binding

domain, a granin peptide binding domain, a tachykinin peptide binding domain, a neuropeptide Y related peptide binding domain, a neurohormone peptide binding domain, a cytokine peptide binding domain, a kinin peptide binding domain, a fibroblast growth factor peptide binding domain, a neurotrophin peptide binding domain, a tumor necrosis factor peptide binding domain, a glial derived neurotrophic factor peptide binding domain, a transformation growth factor β peptide binding domain, a bone morphogenetic protein peptide binding domain, a growth and differentiation factor peptide binding domain, an activin peptide binding domain, a vascular endothelial growth factor peptide binding domain, an insulin growth factor peptide binding domain, an epidermial growth factor peptide binding domain, a glucagon like hormone peptide binding domain, a pituitary adenylate cyclase activating peptide binding domain, a growth hormone-releasing hormone peptide binding domain, a vasoactive intestinal peptide binding domain, a gastric inhibitory polypeptide peptide binding domain, a calcitonin-related peptidesvisceral gut peptide binding domain, or a protease activated receptor peptide binding domain.

FIG. 1.


Cleavage Site
Enzymatic
Translocation
Binding
SS
Single-chain Form
Di-chain Loop
Naturally-occurring Protease
Translocation
Binding
SS
Enzymatic
Di-chain Form

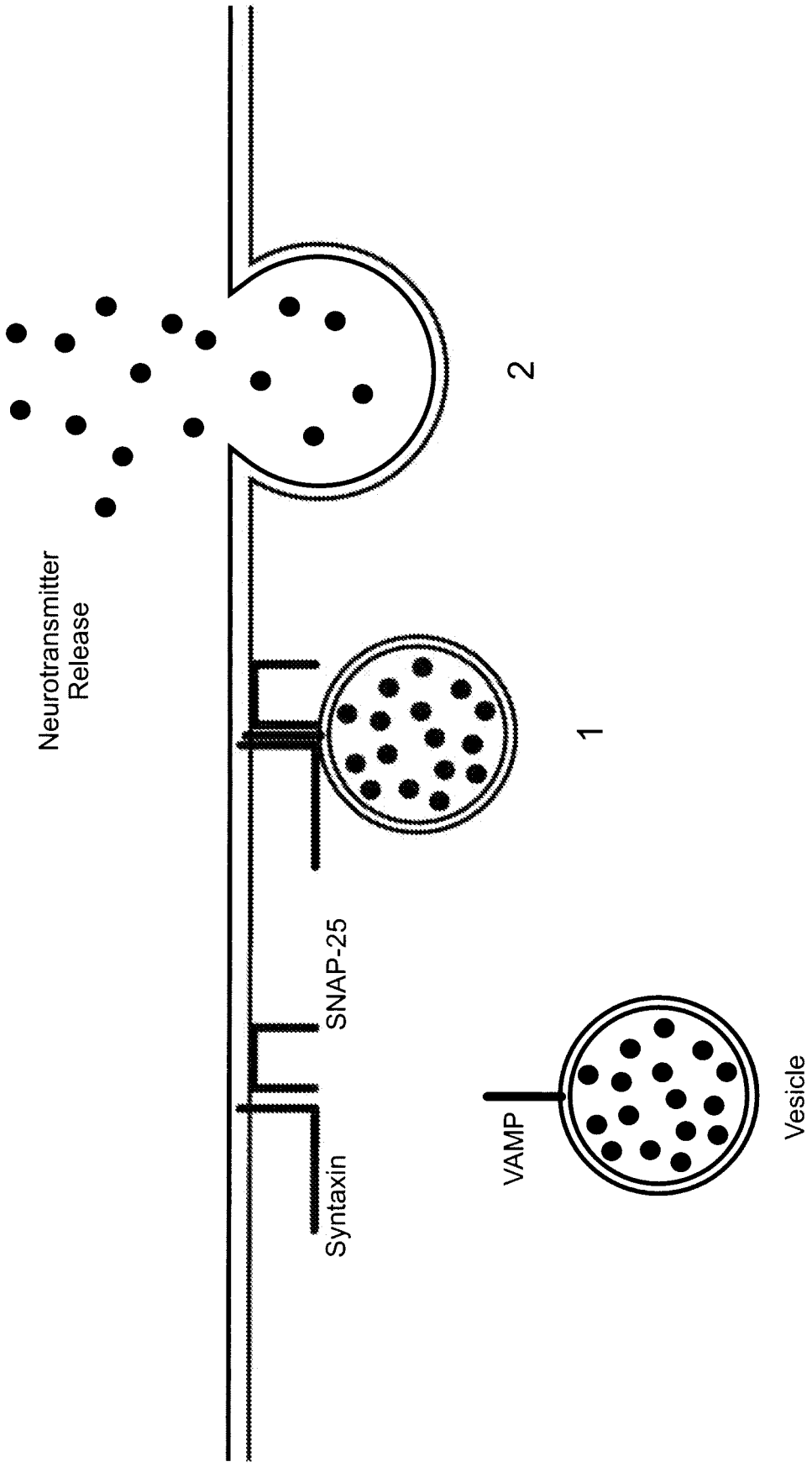
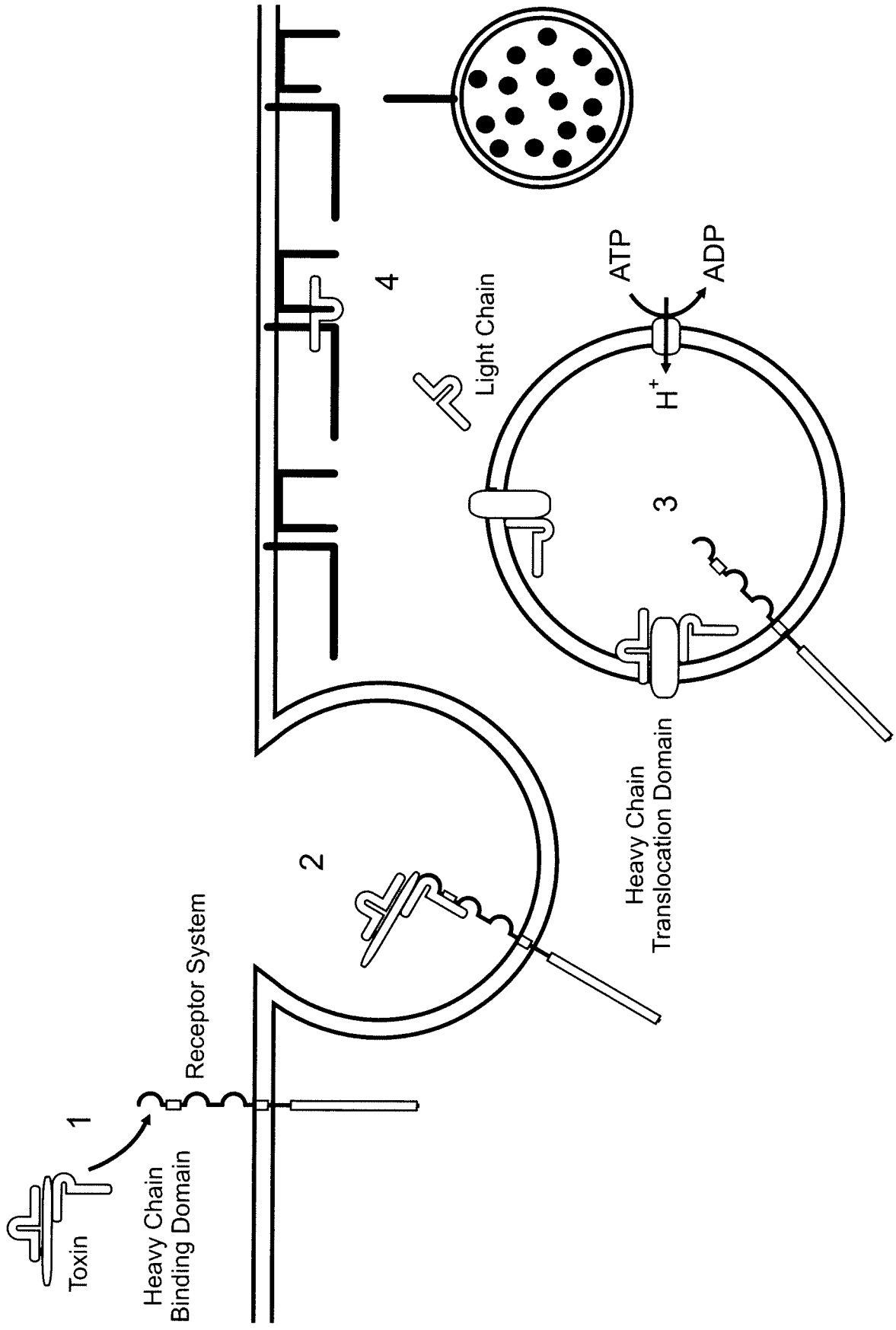

Neurotransmitter Release
2
1
SNAP-25
Syntaxin
VAMP
Vesicle

FIG. 2a.

FIG. 2b.


Toxin
1
Heavy Chain
Binding Domain
Receptor System
2
Heavy Chain
Translocation Domain
3
ATP
ADP
H+
Light Chain
4

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2011/022272

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/33 C12P21/02 C12N15/62 C12N9/50 C12N15/70
C12N15/866 C12P21/06
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K C12P C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal , BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2004/018589 Al (ZHONG JUN [US]) 29 January 2004 (2004-01-29) abstract paragraphs [0017] , [0020]	1-15
A	KAPUST R B ET AL: "Control led Intracel lular Processi ng of Fusi on Protei ns by TEV Protease" , PROTEIN EXPRESSION AND PURI FICATION, ACADEMIC PRESS, SAN DI EGO, CA, vol . 19, no. 2, 1 July 2000 (2000-07-01) , pages 312-318, XP027414856, ISSN: 1046-5928, D0I : D01: 10. 1006/PREP.2000. 1251 [retri eved on 2000-07-01] abstract; f i gure 2	1,6, 11

-/- ·

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" documentwhich may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such docu¬ments, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 June 2011	06/07/2011

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Wi ame, U se

1

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2011/022272

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 7 419 676 B2 (DOLLY J 0 [GB] ET AL DOLLY J OLIVER [GB] ET AL) 2 September 2008 (2008-09-02) cited in the application column 5, line 20 - line 41	1-5
A	US 2009/048431 Al (STEWARD LANCE E [US] ET AL) 19 February 2009 (2009-02-19) cited in the application abstract; claims	6-15
A	LISA D. CABRITA ET AL: "Enhancing the stability and solubility of TEV protease using in silico design", PROTEIN SCIENCE, vol. 16, no. 11, 1 November 2007 (2007-11-01), pages 2360-2367, XP55000980, ISSN: 0961-8368, D0I: 10.1110/ps.072822507 page 2364, column 1, line 3 - column 2, line 6	1,6, 11
A	VAN DEN BERG S ET AL: "Improved solubility of TEV protease by directed evolution", JOURNAL OF BIOTECHNOLOGY, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, vol. 121, no. 3, 10 February 2006 (2006-02-10), pages 291-298, XP024956889, ISSN: 0168-1656, D0I: DOI:10.1016/J.JBI0TEC.2005.08.006 [retrieved on 2006-02-10] abstract; table 1	1,6, 11

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2011/022272

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2004018589 A1	29-01-2004	NONE	
US 7419676 B2	02-09-2008	AT 348178 T	15-01-2007
		AU 777556 B2	21-10-2004
		AU 7573100 A	19-03-2001
		BR 0012759 A	02-04-2002
		CA 2380457 A1	01-03-2001
		CN 1371424 A	25-09-2002
		DE 60032367 T2	27-09-2007
		EP 1206554 A1	22-05-2002
		EP 1700918 A2	13-09-2006
		EP 2267008 A2	29-12-2010
		EP 2267009 A2	29-12-2010
		EP 2267010 A2	29-12-2010
		EP 2264052 A2	22-12-2010
		ES 2277854 T3	01-08-2007
		HK 1046020 A1	11-05-2007
		JP 2003507073 T	25-02-2003
		WO 0114570 A1	01-03-2001
		US 2009087458 A1	02-04-2009
		US 2008311622 A1	18-12-2008
		US 2009005313 A1	01-01-2009
		US 2009004224 A1	01-01-2009
		US 2009069238 A1	12-03-2009
		US 2009081730 A1	26-03-2009
		US 2009030188 A1	29-01-2009
		US 2009042270 A1	12-02-2009
		US 2009030182 A1	29-01-2009
		US 2006099672 A1	11-05-2006
		us 2007259401 A1	08-11-2007
		us 7132259 B1	07-11-2006
		us 2008081355 A1	03-04-2008
		us 2008161226 A1	03-07-2008
		us 2008221012 A1	11-09-2008
		us 2008182294 A1	31-07-2008
US 2009048431 A1	19-02-2009	US 2011070621 A1	24-03-2011