

2011

# Purification of a recombinant heavy chain fragment C vaccine candidate against botulinum serotype C neurotoxin [rBoNTC(H<sub>C</sub>)] expressed in *Pichia pastoris*

Michael P. Dux

*Novartis Animal Health US Inc.*

Jicai Huang

*Nektar Therapeutics*

Rick Barent

*Intervet*

Mehmet Inan

*Alder Pharmaceuticals*

S. Todd Swanson

*University of Nebraska-Lincoln*

*See next page for additional authors*

Follow this and additional works at: <http://digitalcommons.unl.edu/usarmyresearch>



Part of the [Operations Research, Systems Engineering and Industrial Engineering Commons](#)

---

Dux, Michael P.; Huang, Jicai; Barent, Rick; Inan, Mehmet; Swanson, S. Todd; Sinha, Jayanta; Ross, John T.; Smith, Leonard A.; Smith, Theresa J.; Henderson, Ian; and Meagher, Michael M., "Purification of a recombinant heavy chain fragment C vaccine candidate against botulinum serotype C neurotoxin [rBoNTC(H<sub>C</sub>)] expressed in *Pichia pastoris*" (2011). *US Army Research*. 120.

<http://digitalcommons.unl.edu/usarmyresearch/120>

This Article is brought to you for free and open access by the U.S. Department of Defense at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in US Army Research by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.

---

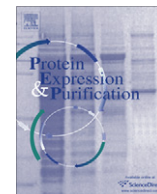
**Authors**

Michael P. Dux, Jicai Huang, Rick Barent, Mehmet Inan, S. Todd Swanson, Jayanta Sinha, John T. Ross, Leonard A. Smith, Theresa J. Smith, Ian Henderson, and Michael M. Meagher



Contents lists available at ScienceDirect

## Protein Expression and Purification

journal homepage: [www.elsevier.com/locate/yprep](http://www.elsevier.com/locate/yprep)Purification of a recombinant heavy chain fragment C vaccine candidate against botulinum serotype C neurotoxin [rBoNTC(H<sub>c</sub>)] expressed in *Pichia pastoris*Michael P. Dux<sup>a,e</sup>, Jicai Huang<sup>b,e</sup>, Rick Barent<sup>c,e</sup>, Mehmet Inan<sup>d,e</sup>, S. Todd Swanson<sup>e</sup>, Jayanta Sinha<sup>e</sup>, John T. Ross<sup>f</sup>, Leonard A. Smith<sup>g</sup>, Theresa J. Smith<sup>h</sup>, Ian Henderson<sup>f,i</sup>, Michael M. Meagher<sup>e,\*</sup><sup>a</sup> Novartis Animal Health US Inc., 1447 140[th] Street, Larchwood, IA 51241, United States<sup>b</sup> Nektar Therapeutics, 490 Discovery Drive, Huntsville, AL 35806, United States<sup>c</sup> Intervet, 21401 West Center Road, Elkhorn, NE 68022, United States<sup>d</sup> Alder Pharmaceuticals, 11804 North Creek Parkway South, Bothell, WA 98011, United States<sup>e</sup> Biological Process Development Facility (BPDF), 304B Othmer Hall, College of Engineering, University of Nebraska-Lincoln, Lincoln, NE 68588-0668, United States<sup>f</sup> DynPort Vaccine Company (DVC) LLC, A CSC Company, 64 Thomas Johnson Drive, Frederick, MD 21702, United States<sup>g</sup> United States Army Medical Research and Materiel Command, Fort Detrick, MD 21702-5011, United States<sup>h</sup> Integrated Toxicology Division, United States Army Medical Research Institute of Infectious Diseases (USAMRIID), Fort Detrick, Frederick, MD 21702-5011, United States<sup>i</sup> Emergent Product Development Gaithersburg Inc., Suite 100, 300 Professional Drive, Gaithersburg, MD 20879, United States

## ARTICLE INFO

## Article history:

Received 15 July 2010

and in revised form 23 September 2010

Available online 7 October 2010

## Keywords:

*Pichia pastoris*

Botulinum toxin vaccine

Protein purification

Recombinant

## ABSTRACT

A purification process for the manufacture of a recombinant C-terminus heavy chain fragment from botulinum neurotoxin serotype C [rBoNTC(H<sub>c</sub>)], a potential vaccine candidate, has been defined and successfully scaled-up. The rBoNTC(H<sub>c</sub>) was produced intracellularly in *Pichia pastoris* X-33 using a three step fermentation process, i.e., glycerol batch phase, a glycerol fed-batch phase to achieve high cell densities, followed by a methanol induction phase. The rBoNTC(H<sub>c</sub>) was captured from the soluble protein fraction of cell lysate using hydrophobic charge induction chromatography (HCIC; MEP HyperCel™), and then further purified using a CM 650M ion exchange chromatography step followed by a polishing step using HCIC once again. Method development at the bench scale was achieved using 5–100 mL columns and the process was performed at the pilot scale using 0.6–1.6 L columns in preparation for technology transfer to cGMP manufacturing. The process yielded approximately 2.5 g of rBoNTC(H<sub>c</sub>)/kg wet cell-weight (WCW) at the bench scale and 1.6 g rBoNTC(H<sub>c</sub>)/kg WCW at the pilot scale. The purified rBoNTC(H<sub>c</sub>) was stable for at least 3 months at 5 and –80 °C as determined by reverse phase-HPLC and SDS-PAGE and was stable for 24 months at –80 °C based on mouse potency bioassay. N-Terminal amino acid sequencing confirmed that the N-terminus of the purified rBoNTC(H<sub>c</sub>) was intact.

© 2010 Elsevier Inc. All rights reserved.

## Introduction

*Clostridium botulinum* produces seven antigenically distinct neurotoxins differentiated serologically by specific neutralization [1]. These botulinum neurotoxins (BoNT),<sup>1</sup> designated as serotypes

A–G, are the most toxic natural substances known [2]. The BoNT have been weaponized and are classified as a Category A select agent by the National Institute of Allergy and Infectious Disease [3]. There is a need for a safe, well characterized, and efficacious recombinant vaccine that will protect against all seven known serotypes of the toxin as well as their subtypes [4,5]. The BoNT have been extensively researched and are well characterized [6–11]. The heavy chain (H<sub>c</sub>) fragments of BoNT (A–F) were shown to be non-toxic, antigenic [11] and capable of eliciting a protective immunity in vaccinated animals challenged with homologous BoNT [12]. These results prompted an effort to develop a recombinant botulinum (rBoNT) vaccine against all seven serotypes using the H<sub>c</sub> fragments as vaccine antigens [10,13–17]. The H<sub>c</sub> fragments from each of the seven serotypes [rBoNT(H<sub>c</sub>)] are distinctly different proteins with varying degrees of homology. Due to the differences in the characteristics of each protein and the level of expression of each in *Pichia pastoris*, a standardized fermentation

\* Corresponding author. Fax: +1 402 472 4985.

E-mail address: [mmeagher1@unl.edu](mailto:mmeagher1@unl.edu) (M.M. Meagher).

<sup>1</sup> Abbreviations used: BCA, bicinechonic acid; BoNT, botulinum neurotoxin; rBoNT, recombinant botulinum neurotoxin; rBoNTC(H<sub>c</sub>), recombinant botulinum neurotoxin serotype C, heavy chain C-terminal fragment; BSA, bovine serum albumin; CV, column volume; H<sub>c</sub>, heavy chain C-terminal fragment; kDa, kilo-Dalton; MW, molecular weight; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HCIC, hydrophobic charge induction chromatography; HIC, hydrophobic interaction chromatography; WCW, wet cell weight; TBS, tris base-buffered saline; PMSF, phenylmethylsulfonyl fluoride; cGMP, current Good Manufacturing Practices; PDS, purified drug substance; i.p., intraperitoneal; A280, protein absorbance at 280 nm; pI, isoelectric point; RP, reverse phase; CM, carboxy methyl.

and purification process was not feasible, resulting in the development of separate processes for rBoNTA(H<sub>c</sub>), rBoNTB(H<sub>c</sub>), rBoNTE(H<sub>c</sub>) and rBoNTF(H<sub>c</sub>) [14,16–18] and now rBoNTC(H<sub>c</sub>). Serotypes D and G are still under development.

During process development it was noted that rBoNTC(H<sub>c</sub>) is sensitive to proteolytic degradation during the purification process. This was alleviated by the addition of protease inhibitors; however, the use of protease inhibitors is not desirable in a current Good Manufacturing Practice (cGMP) process. Proteolytic degradation of recombinant proteins expressed in *P. pastoris* can be problematic [19]. Alternative methods such as limiting growth rate [20,21], manipulation of growth temperature [22,23] and addition of protease inhibitors to the fermentation medium have been used to reduce protease activity, but in the case of rBoNTC(H<sub>c</sub>) these manipulations proved to be unsuccessful, requiring a different approach. Analysis of in-process purification samples by Western blot and N-terminal sequencing indicated several truncated forms of rBoNTC(H<sub>c</sub>). The prominent forms were missing the first 8 and 22 amino acids from the N-terminus based on the full length sequence listed in Table 1. Preliminary stability studies at 4 °C determined that the N-22 form was stable enough to move into process development and scale-up. The potency of the N-22 rBoNTC(H<sub>c</sub>) was compared to the intact rBoNTC(H<sub>c</sub>) produced with the aid of protease inhibitors and was demonstrated to have similar potency as compared to the intact rBoNTC(H<sub>c</sub>) in a mouse potency assay. Based on the potency results a new high copy rBoNTC(H<sub>c</sub>) clone was constructed using the truncated amino acid sequence and forwarded into process development.

This paper describes the research, development and scale-up of a process to produce a N-22 rBoNTC(H<sub>c</sub>) candidate vaccine suitable for transfer to a cGMP facility for production of clinical material. Two controlled non-cGMP pilot-scale runs were performed to demonstrate scalability of the process.

## Materials and methods

### Molecular biology

A synthetic gene, codon optimized for *P. pastoris*, encoding the rBoNTC(H<sub>c</sub>) N-22 fragment was produced based on the native sequence of the C-terminus from the heavy chain domain of the BoNT/C [5]. The full length sequence of the gene was synthesized without the first 22 amino acids from the N-terminus because of degradation seen during early purification development. The truncated gene was inserted into a pPICZA expression vector at the *EcoRI* site. The pPICZA-truncated rBoNTC(H<sub>c</sub>) was linearized with *SacI*, transformed into *P. pastoris* X-33 generating Mut<sup>+</sup> phenotypes, as indicated by their growth on methanol plates, and clones were selected on increasing amounts of Zeocin. Clones that contained

1–5 copies of the rBoNTC(H<sub>c</sub>) were confirmed by Southern blot, grown under standard shake flask conditions, induced with methanol for 10 h and evaluated by Western blot. Results from Western blot showed that there was a direct correlation between increased gene copy number and increased intracellular expression of rBoNTC(H<sub>c</sub>).

### Bench and pilot-scale fermentation

Fermentations were performed in 5 L fermentors (Bioflo 3000, New Brunswick Scientific Edison, NJ) or in 19 L fermentors (NLF, Bioengineering AG, Sagenrainstrasse 7, CH-8636 Wald, Switzerland) as described by Zhang et al. [21]. After a batch phase at pH 5.0 and 30 °C the cells were grown to the desired cell density by feeding glycerol at a feed rate of 20 g/L/h for 6 h and transitioned to methanol. Instead of using a standard exponential methanol feed rate, the induction phase was performed using a methanol sensor and feed-back control system with a set point of 2 mL/L residual methanol for 9 h because of the limited methanol consumption of the rBoNTC(H<sub>c</sub>) expressing clone. The methanol feed solution contained 12 mL/L of PTM1 salts [24]. Samples of the fermentation culture were periodically withdrawn for analyses in order to determine the WCW and rBoNTC(H<sub>c</sub>) production.

### Bench-scale cell harvest and disruption

Cells were harvested by centrifugation at 11,300 × g at 4 °C for 30 min using a Sorvall Evolution RC Centrifuge (Kendro Laboratory Products, Asheville, NC). Cell paste was frozen at –80 °C until processed. Eighty grams of WCW were suspended in 400 mL of 50 mM sodium phosphate, 2 M sodium chloride (NaCl), pH 6.5 to remove residual fermentation medium. The cell suspension was centrifuged at 11,300 × g at 4 °C for 5 min. Cell paste was resuspended into the same amount of 50 mM sodium phosphate, 2 M NaCl, pH 6.5 and homogenized using a Microfluidizer M-110EH (Microfluidics Corp., Newton, CA) set at 22,000 psi with the lysate chilled to <10 °C before processing. Two passes were required for >90% cell disruption based on hemocytometer and soluble protein released. The homogenate was clarified at 11,300 × g for 30 min at 4 °C followed by filtration through a Pall AcroPak 200 with a 0.8/0.2 μm Supor membrane (Pall Corp., East Hills, NY) and loaded immediately onto the first column.

### Pilot-scale cell harvest and disruption

Cells harvested from a 19 L fermentation (10 L working volume) were centrifuged at 11,300 × g at 4 °C for 30 min using a Sorvall Evolution RC Centrifuge (Kendro Laboratory Products, Asheville, NC) and then stored at –80 °C until processed. One kilogram of

**Table 1**  
N-Terminal sequence of purified rBoNTC(H<sub>c</sub>) full length.

Sample	N-Terminal sequence
Intact rBoNTC(H <sub>c</sub> )	pl of protein: 5.50 Protein MW: 52,370 Amino acid composition: A9 R17 N 61 D28 C1 Q12 E20 G27 H3 I51 L25 K26 M12 F27 P9 S35 T28 W8 Y30 V21 1 MTIPFNIFSY TNNLLKDII NEYFNININDS KILSLQNRKN TLVDTSGYNA EVSEEGDVQL 61 NPFPDFDKL GSSGEDRGKV IVTQENIVY NSMYESFSIS FWIRINKWVS NLPGYTIIDS 121 VKNNSGWSIG IISNLFVFL QKNEDESESI NFSYDISNNA PGYNKWFVFT VTNNMMGNMK 181 IYINGKLIDT IKVKELGIN FSKTITFEIN KIPDTGLITS DSDNINMWIR DFYIFAKELD 241 GKDINILFNS LQYTNVVKDY WGNLDRYNKE YMVNIDYLN RYMYANSRQI VFNTRRRNND 301 FNEGYSKIIK RIRGNTNDR VRGGDILYFD MTINNKAYNL FMKNETMYAD NHSTEDIYAI 361 GLREQTKDIN DNIIFQIQPM NNTYYASQI FKSFNNGENI SGICSIGTYR FRLGGDWYRH 421 NYLVPTVKQG NYASLLESTS THWGFVPVSE
N-Terminus of purified intact rBoNTC(H <sub>c</sub> )	T I P F N I F S Y T N N S L L
N-Terminus of purified N-22 rBoNTC(H <sub>c</sub> )	Y F N N I

WCW was suspended in 5 L of 50 mM sodium phosphate, 2 M NaCl, pH 6.5 and centrifuged at  $11,300 \times g$  for 5 min and resuspended into the same amount of 50 mM sodium phosphate, 2 M NaCl, pH 6.5 as a wash step. Cells were homogenized by two passes through a Microfluidizer M-110EH (Microfluidics Corp., Newton, CA) set at 22,000 psi. The homogenate was centrifuged at  $11,300 \times g$  for 60 min at 4 °C and filtered through a Pall AcroPak 1500, with a 0.8/0.2  $\mu\text{m}$  Supor membrane (Pall Corp., East Hills, NY) and loaded immediately onto the first column.

#### Bench-scale purification

All bench-scale chromatographic separations were performed on a BioCad Workstation (Applied Biosystems, Foster City, CA) at room temperature, with the load material on ice. A 2.7 cm  $\times$  17.3 cm column (GE Healthcare Piscataway, NJ) packed with 99 mL of MEP HyperCel™ resin (Pall Corp., East Hills, NY) was equilibrated with five column volumes (CV) of 50 mM sodium phosphate, 2 M NaCl, pH 6.5. Clarified cell lysate was loaded at a linear velocity of 300 cm/h followed by a 5 CV wash with the equilibration buffer. This was followed by a 5 CV 50 mM succinate, pH 4.5 wash and a 3 CV wash of 80% 50 mM succinate, pH 4.5, 20% 25 mM succinate, pH 3.0. Elution of the rBoNTC(H<sub>c</sub>) was performed by a 5 CV gradient from 70% 50 mM succinate, pH 4.5, 30% 25 mM succinate, pH 3.0 to 20% 50 mM succinate, pH 4.5, 80% 25 mM succinate, pH 3.0 followed by a 3 CV hold step of 20% 50 mM succinate, pH 4.5, 80% 25 mM succinate, pH 3.0.

The product from the capture step was loaded directly onto a 2.7 cm  $\times$  20.0 cm column (Applied Biosystems, Foster City, CA) packed with 115 mL weak cation exchange CM 650M resin (Toso Haas, Montgomeryville, PA). The column was equilibrated with 5 CV of 25 mM succinate, pH 4.0 at a linear velocity of 300 cm/h prior to loading. After loading, a 1 CV wash was performed with the equilibration buffer followed by a 3 CV wash of 35% 25 mM succinate, pH 4.0 and 65% 25 mM succinate 1 M NaCl, pH 4.0 buffer. The elution profile for this column was a 10 CV gradient from 65% 25 mM succinate 1 M NaCl, pH 4.0 buffer to 100% 25 mM succinate 1 M NaCl, pH 4.0 buffer. This was followed by a 5 CV hold step at the end of the gradient to elute the remaining rBoNTC(H<sub>c</sub>).

The CM 650M product was loaded directly onto a second 2.0 cm  $\times$  17.5 cm (Waters Chromatography, Milford, MA) 55 mL MEP HyperCel™ resin (Pall Corp., East Hills, NY) and equilibrated with 5 CV of 50% 25 mM succinate, pH 4.0 and 50% 25 mM succinate 1 M NaCl, pH 4.0 at 300 cm/h. After loading, the column was washed with 2 CV 50% 25 mM succinate 1 M NaCl, pH 4.0 and 50% 25 mM succinate, pH 3.0. rBoNTC(H<sub>c</sub>) was eluted from the column using a 10 CV gradient from the 50% mix wash buffer to 30% 25 mM succinate 1 M NaCl, pH 4.0 and 70% 25 mM succinate, pH 3.0. This was followed by a 3 CV step of 15% 25 mM succinate 1 M NaCl, pH 4.0 and 85% 25 mM succinate, pH 3.0.

The final product was dia-filtered with 15 mM succinate, pH 4.0 using 10 kDa Molecular Weight Cut Off (MWCO) Amersham Hollow fiber 5.2 ft<sup>2</sup> membrane (part #UFP-10-C-6A) (GE Healthcare Piscataway, NJ) until the final product reached a pH of 4.0. The purified drug substance was sterile filtered with an AcroPak 20, with a 0.2  $\mu\text{m}$  Fluorodyne II membrane (Pall Corp., East Hills, NY) and stored at –80 °C.

#### Pilot-scale purification

All chromatographic separations were performed using a North Carolina SRT (Apex, NC) pilot-scale chromatography skid. All chromatographic conditions were the same as the bench-scale work, i.e., linear velocities, equilibration, washing and elution methods. The MEP HyperCel™ capture step was performed using a 1.08 L (10 cm  $\times$  20 cm) Quickscale 100 column (Millipore,

Bedford, MA). The CM 650M step was performed on a 1.02 L (10 cm  $\times$  20 cm) BPG 100/500 column (GE Healthcare Piscataway, NJ). A polishing step was performed using a 650 mL MEP HyperCel™ (7 cm  $\times$  10 cm) QS 70/500 column (Millipore, Bedford, MA). Final product was dia-filtered to pH 4.0 using 15 mM succinate, pH 4.0 at 5 °C with a North Carolina SRT Model 10 filtration unit equipped with a 10 kDa Amersham Hollow fiber 5.2 ft<sup>2</sup> membrane (part #UFP-10-C-6A) (GE Healthcare Piscataway, NJ). Final product was sterile filtered with an AcroPak 200, with a 0.2  $\mu\text{m}$  Fluorodyne II membrane (Pall Corp., East Hills, NY) and stored at 2–8 °C.

#### Protein analysis

Total protein concentrations were determined using the BCA (Pierce Chemical, St. Louis, MO) Standard Assay, using Bovine Serum Albumin (BSA) as a standard. Final product protein concentration was determined by A280 absorbance with an extinction coefficient of 1.75 [(mL/mg)  $\times$  A280] based on amino acid analysis. Purity was determined by a reducing SDS–PAGE using 10% Bis-Tris gels with MOPS buffer system (Novex, San Diego, CA) stained with Coomassie blue. Western blot analysis was performed using polyclonal rabbit anti-rBoNTC(H<sub>c</sub>) antibody (produced by USAMRIID) incubated at 0.05  $\mu\text{g}/\text{mL}$  for 1–3 h at room temperature. The secondary antibody was a peroxidase labeled affinity-purified goat anti-rabbit IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) incubated at 0.025  $\mu\text{g}/\text{mL}$  for 1 h at room temperature. The SDS–PAGE-separated proteins were transferred to PVDF membranes (BioRad, Hercules, CA) at 25 V for 20 min, blocked with 5% non-fat dry milk for 30 min and washed with tris base-buffered saline (TBS) prior to treatment with antibodies. Blots were then visualized by chemiluminescence using the ELC plus Detection Kit (Amersham Pharmacia Biotech, Piscataway, NJ). N-Terminal sequencing was performed by the University of Nebraska-Medical Center Protein Core Facility using an ABI Procise 494 Sequencer (Applied Biosystems, Foster City, CA).

The Biological Process Development Facility (BPDF) Quality Control Laboratory (QCL) estimated quantity of rBoNTC(H<sub>c</sub>) from fermentation samples by HPLC using hydrophobic interaction chromatography (HIC) with a 50 mm length TSK-gel Ether-5PW, 5 mm HIC column (Tosoh Bioscience, Montgomeryville, PA). Samples of 0.1 g of wet cell paste were suspended in 0.99 mL of cold 2 M sodium chloride with 5  $\mu\text{L}$  of 4 °C 0.5 M EDTA in MilliQ adjusted to pH 8.3 with NaOH and 5  $\mu\text{L}$  of 4 °C 0.2 PMSF in methanol in a 2 mL centrifuge tube. To this mixture was added 3.8 g of cold 0.5 mm zirconia/silica beads (BioSpec Products, Bartlesville, OK). The 2 mL centrifuge tube containing the mixture was loaded into a Mini-Beadbeater (BioSpec Products, Bartlesville, OK) and mechanically disrupted using five cycles for 1 min with 5 min rest in a cold room. After the 5th cycle a small hole was poked into the bottom of the 2 mL centrifuge tube using a hot 25G needle so as to retain the beads. The 2 mL microcentrifuge tube was placed in a 15 mL conical tube and spun for 5 min at  $5000 \times g$  recovering the lysate. The supernatant was transferred to a 2 mL microcentrifuge tube and clarified at  $18,000 \times g$  for 10 min. The supernatants were analyzed using a 0.8 mL/min gradient elution from 2 M sodium chloride (in 50 mM sodium succinate, pH 4.5) to 0 M sodium chloride, over 11 min at 23 °C. Peak areas (280 nm) were compared to that of a standard to estimate amounts in each injection. The BPDF QCL estimated purity and quantity of rBoNTC(H<sub>c</sub>) by HPLC using Reverse Phase chromatography (RP) with a 75 mm length Bio-Suite pPhenyl 1000, 10 mm RPC column (Waters, Milford, MA). Samples were analyzed using a 1 mL/min gradient elution from 97% mobile phase A (HPLC grade water with 0.1% formic acid) and 3% mobile phase B (acetonitrile with 0.1% formic acid) to 3% mobile phase A and 97% mobile phase B, over 40 min at 25 °C.

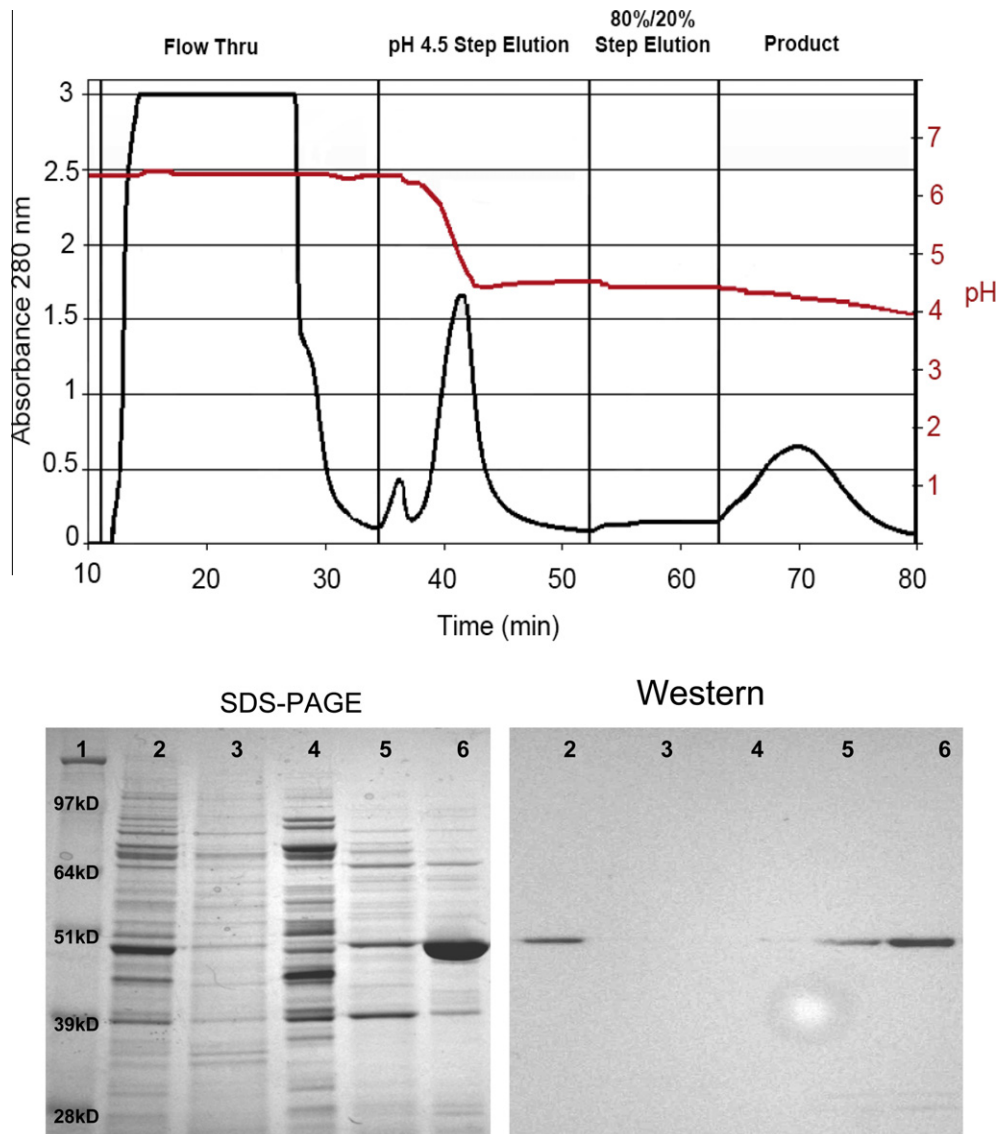
### Mouse potency bioassay

The potency of purified rBoNTC(H<sub>c</sub>) was determined using a mouse potency bioassay [25]. Groups of 10 female mice (strain Crl:CD-1 (Charles River, NC)) were intramuscularly vaccinated twice (0 and 2 weeks) with the following doses of antigen, i.e., 8.1, 2.7, 0.9, 0.3, 0.1, 0.033, and 0.011 µg rBoNTC(H<sub>c</sub>)/mouse for a total of 70 mice per assay. The antigen was formulated in 25 mM sodium succinate, 15 mM sodium phosphate, 0.2% Alhydrogel™ (HCl Bio sector, Frederikssund, Denmark), 5% mannitol, pH 5.0. Mice were challenged intramuscularly 3 weeks after the final vaccination with 1000 mouse i.p. LD<sub>50</sub> BoNT/C toxin complex. Mice were observed twice daily for 5 days post-challenge. Results were tabulated and analyzed using Probit analysis (SPSS, Chicago, IL). These studies were conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adhere to principles stated in the Guide for the Care and Use of Laboratory Animals of the National Research Council (38a). The facility (USAMRIID) where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

### Results and discussion

#### Process development strategy

The strategy for developing a process to produce rBoNTC(H<sub>c</sub>) started by finding an optimal cell lysis buffer by screening different pH and ionic strength buffers for release and solubility of rBoNTC(H<sub>c</sub>). After the optimal cell lysis buffer was identified, purification development began by screening resins to capture the rBoNTC(H<sub>c</sub>). HIC and MEP HyperCel™ resins were selected for initial screening based on the characteristics of this protein and the optimal lysis buffer developed for rBoNTC(H<sub>c</sub>), i.e., high NaCl concentration (2 M). After the initial capture step was established subsequent chromatographic steps were developed based on speed of processing, ease of scale-up, avoiding the requirement for buffer exchange between chromatography steps, and the potential for eventual cGMP scale-up. Given the susceptibility to proteolysis and the desire for a rapid process with a minimum number of steps, a significant effort was made to identify chromatography steps that were amenable to the previous elution buffers. A final diafiltration step exchanged the rBoNTC(H<sub>c</sub>) into its final storage buffer.



**Fig. 1.** SDS-PAGE and Western blot (reduced) of HCIC equal volume column fractions. Lane 1, see blue plus 2 MW marker; lane 2, HCIC load; lane 3, HCIC flow thru; lane 4, HCIC pH 4.5 elution; lane 5, HCIC 80%/20% step elution; lane 6, HCIC elution product.



### Bench and pilot-scale fermentation

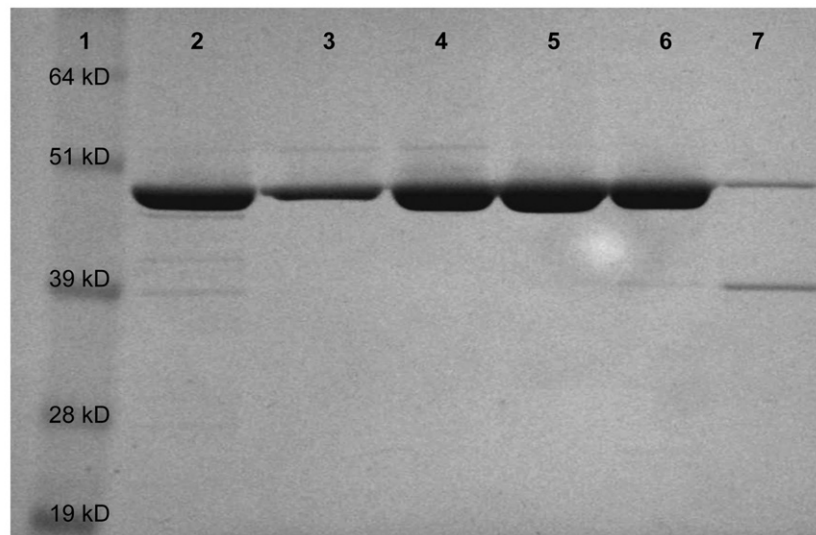
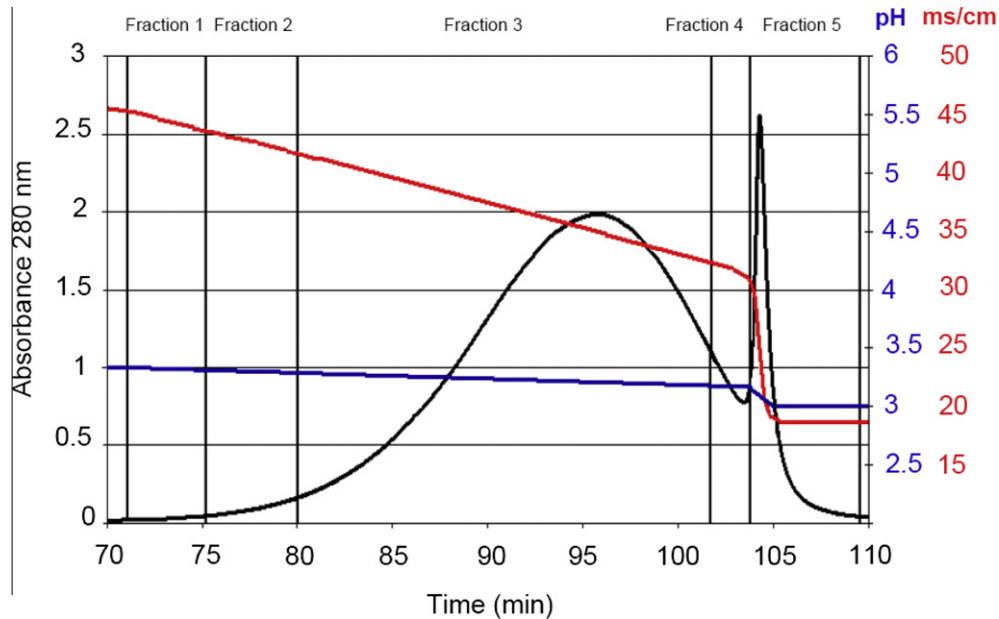
The fermentation at both the 5 and 19 L scale produced rBoNTC(H<sub>c</sub>) at 7–10 mg rBoNTC(H<sub>c</sub>)/g WCW as determined by HIC HPLC as described above. Cells were stored at –80 °C prior to use.

### Bench-scale purification

Initially 2 mM phenylmethylsulfonyl fluoride (PMSF) was added to the cell suspension just prior to disruption to inhibit protease activity and prevent the formation of degradation products. This helped to produce a non-degraded product, but after 24 h degradation product products appeared when the purified bulk material was stored at 2–8 °C. To further reduce the amount of degradation another protease inhibitor, pepstatin, was added during the second purification step, which halted the degradation of rBoNTC(H<sub>c</sub>). N-Terminal sequencing was performed on the purified intact rBoNTC(H<sub>c</sub>) and the purified degradation product.

N-Terminal sequencing of the purified full length rBoNTC(H<sub>c</sub>) indicated that the N-terminal methionine was missing and the purified degraded product was missing the first 22 amino acids from the N-terminus (Table 1). Both of these products were evaluated in a mouse bioassay and were shown to be equally protective with an ED<sub>50</sub> (theoretical effective dose protecting half the mice) of approximately 102 ng. Based on the potency results and the desire not to use protease inhibitors in a cGMP manufacturing process, a new strain was constructed that incorporated the truncated rBoNTC(H<sub>c</sub>) gene excluding the first 22 N-terminal amino acids.

Cell lysis buffer optimization studies evaluated different pH and ionic strength concentrations. The optimal pH was between 6 and 7.5 and the amount of soluble rBoNTC(H<sub>c</sub>) released from the cell increased with increasing amounts of NaCl concentrations up to 2 M. The final cell disruption buffer was 50 mM sodium phosphate 2 M NaCl, pH 6.5 buffer, which is ideal for binding to either a HIC or a MEP Hypercel™ resin. Initially three passes through the Microfluidizer were performed as described in the section 'Materials and



**Fig. 2.** The final HIC column chromatogram and SDS-PAGE (reduced) showing the separation of high molecular weight contaminants and degradation product are removed across the elution of the rBoNTC(H<sub>c</sub>) peak. Lane 1, see blue plus 2 MW marker; lane 2, HCl load; lane 3, HCl fraction 1; lane 4, HCl fraction 2; lane 5, HCl fraction 3 (product peak); lane 6, HCl fraction 4; lane 7, HCl fraction 5.

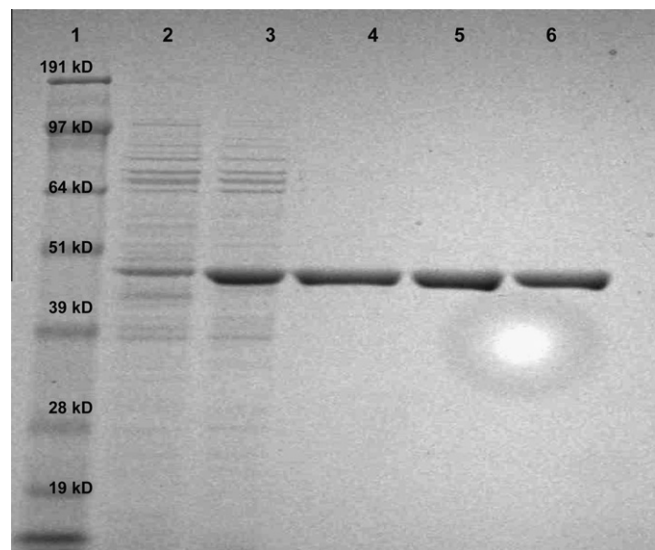
methods', but BCA, SDS-PAGE and Western blot results indicated that two passes through the Microfluidizer yielded the same amount of rBoNTC(H<sub>c</sub>) as three passes. Two passes were also advantageous since this reduced the total processing time of the homogenization step, reducing the potential for proteolytic degradation of rBoNTC(H<sub>c</sub>).

The rBoNTC(H<sub>c</sub>) was purified at the bench-scale using ion exchange and hydrophobic charge induction chromatography techniques. Initially, an HIC resin was investigated to capture the rBoNTC(H<sub>c</sub>). Ether, butyl, octyl, and phenyl HIC resins were evaluated and only phenyl sepharose was able to bind rBoNTC(H<sub>c</sub>) using 2 M NaCl. The rBoNTC(H<sub>c</sub>) eluted continuously over a 10 CV gradient from 2 to 0 M NaCl and continued to elute at 0 M NaCl with the peak not reaching baseline after an additional CV. Fifteen percent glycerol was added to elution buffers, which did sharpen the rBoNTC(H<sub>c</sub>) peak slightly, but it still eluted from approximately 1.5 M NaCl to 0 M NaCl. Phenyl Sepharose did bind all of the rBoNTC(H<sub>c</sub>) at a load of 30 mg of total protein/mL resin with BoNTC(H<sub>c</sub>) being approximately 5–10% of total protein. The phenyl HIC product pool was subjected to further process development using ion exchange, but regardless of the efforts lower MW degradation products and higher MW proteins were seen in downstream purification steps that proved to be very difficult to separate from rBoNTC(H<sub>c</sub>). Based on previous experience of purifying BoNTC(H<sub>c</sub>) with MEP HyperCel™ resin [18] we investigated capturing the rBoNTC(H<sub>c</sub>) from the cell lysate with MEP HyperCel™ resin, which is based on hydrophobic charge induction chromatography (HCIC). The rBoNTC(H<sub>c</sub>) was captured from the clarified cell lysate, which is in 50 mM sodium phosphate, 2 M NaCl, pH 6.5, by loading directly on the MEP Hypercel™ resin. The rBoNTC(H<sub>c</sub>) does not elute off the HCIC resin until a pH of 4.2 in a 50 mM succinate buffer (Fig. 1). A step elution of 50 mM succinate at pH 4.5 prior to elution conditions was added and this enabled removal of 30% of the total protein loaded onto the column with no rBoNTC(H<sub>c</sub>) present based on Western blot. This was followed by a 3 CV wash of 80% 50 mM succinate, pH 4.5, 20% 25 mM succinate, pH 3.0, which further separated some higher molecular weight proteins from the rBoNTC(H<sub>c</sub>). Elution was performed by a 5 CV gradient from 70% 50 mM succinate, pH 4.5, 30% 25 mM succinate, pH 3.0 to 20% 50 mM succinate, pH 4.5, 80% 25 mM succinate, pH 3.0. A gradient elution was chosen to elute the rBoNTC(H<sub>c</sub>) instead of a step elution because the product pool during step elution had a high protein concentration which caused some precipitation of the rBoNTC(H<sub>c</sub>). The maximum total protein loaded onto the capture HCIC column was 30 mg/mL resin. The chromatogram from a bench scale run along with the corresponding SDS-PAGE and Western blot are shown in Fig. 1.

Product from the capture column was directly loaded onto a CM 650M column which removed a majority of the larger molecular weight *Pichia* host cell proteins that are present after the capture step. Initially a resin screen was performed using cation and anion exchange resins. It was found that rBoNTC(H<sub>c</sub>) bound tightly to strong cation exchange resins and could not be eluted at low pH, and raising the pH across the pI, while the protein was bound to the resin, caused some precipitation. It was also noticed that an increase in pH above the pI during dialysis (buffer exchange) caused precipitation of rBoNTC(H<sub>c</sub>). Thus, cation exchange methods were no longer evaluated. A Toso Haas CM 650S (35 μm bead diameter) anion exchange resin was determined to be most suitable for this step, but due to process scale-up concerns, CM 650M (75 μm bead diameter) was used instead because of the larger resin particle size. In addition, there were only slight differences between the CM 650M and the CM 650S resin elution profile. The elution gradient was optimized from a 40 CV gradient from 100% 25 mM succinate, pH 4.0 to 100% 25 mM succinate, 1 M NaCl, pH 4.0 to a 3 CV wash step of 35% 25 mM succinate, pH 4.0 and 65% 25 mM succinate and

1 M NaCl, pH 4.0 and a 10 CV gradient from the above wash step to 100% 25 mM succinate and 1 M NaCl, pH 4.0 buffer. It was determined that increasing the column length from 15 to 20 cm improved separation of lower degradation product, but did not remove all of them. Total protein load for this chromatography step was optimized for 4 mg of total protein/mL of resin.

A second HCIC step was the most effective polishing step to remove degradation products of rBoNTC(H<sub>c</sub>) after numerous ion exchange resins and conditions were attempted. The product from the CM 650 step contained several degradation product of rBoNTC(H<sub>c</sub>) that are faintly seen (lane 2, Fig. 2). Several bands were transferred from an SDS-PAGE gel, transferred to a PVDF membrane and submitted for sequencing. A fragment of the last 230 amino acids of the rBoNTC(H<sub>c</sub>), with a theoretical pI of 6.3 and a MW of 27 kDa and the first 200 amino acids of the rBoNTC(H<sub>c</sub>), with a theoretical pI 5.04 and a MW of 22 kDa were identified. The pI of rBoNTC(H<sub>c</sub>) is 5.5, making it difficult to remove the fragments by ion exchange. Different types of pH gradients and steps were attempted to purify away the remaining contaminants with the HCIC resin but this did not prove effective. Since the HCIC resin separates by both charge and hydrophobicity a gradient from 25 mM succinate 1 M NaCl buffer at pH 4.0 to a 25 mM succinate, pH 3.0 buffer, which combined both a pH and ionic strength gradient was tried. This turned out to be very effective in separating the remaining contaminants (Fig. 2). A total protein load of 5 mg/mL resin resulted in no rBoNTC(H<sub>c</sub>) detected in the flow through and



**Fig. 3.** (A) SDS-PAGE (reduced) of the product peaks from each step of a pilot run with an equal total protein load based on BCA. Lane 1, lysate; lane 2, HCIC column 1 product; lane 3, CM 650M product; lane 4, HCIC column 3 product; lane 5, UF/DF product. (B) SDS-PAGE (reducing) of the final product from pilot scale run 2 at 0.5–5 μg loads which are based on an experimental extinction coefficient.

**Table 2**  
Yield from bench-scale purification of rBoNTC(H<sub>c</sub>).<sup>a</sup>

Step (product pool)	Volume (mL)	Protein conc. (mg/mL)	Total protein (mg)
Lysate	490 ± 5	5.1 ± 0.9	2500 ± 400
HCIC column 1	575 ± 35	0.79 ± 0.07	456 ± 56
CM 650M	750 ± 135	0.41 ± 0.05	302 ± 46
HCIC column 3	478 ± 68	0.54 ± 0.10	240 ± 30
Diafiltration	707 ± 113	0.25 ± 0.07	171 ± 28

<sup>a</sup> Average of four bench-scale purification runs starting from 80 g cells wet weight.



**Table 3**  
Yield from second pilot-scale purification of rBoNTC(H<sub>c</sub>).<sup>a</sup>

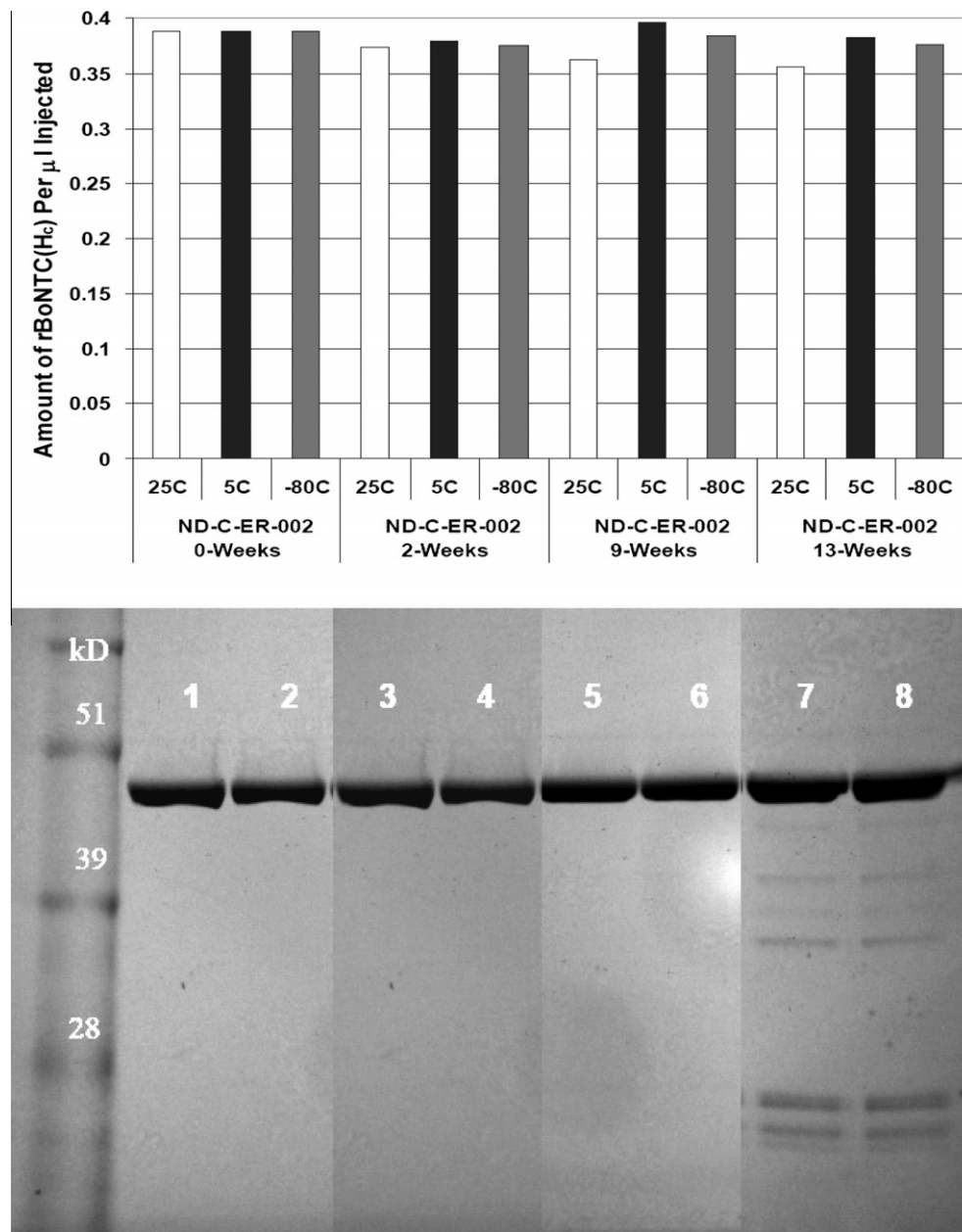
Step (product pool)	Volume (mL)	Protein conc. (BCA) (mg/mL)	Total protein (mg)	Est. purity (%)	rBoNTC(H <sub>c</sub> ) (mg)	Step yield (%)	Total yield (%)
Lysate	5000	6.82	34,100	12	4092	100	100
HCIC column 1	9800	0.58	5684	70	3979	97	97
CM 650M	13,000	0.19	2470	95	2347	59	57
HCIC column 3	5700	0.38	2166	99	2144	91	52
Diafiltration	4200	0.50	2100	99	2079	97	51

<sup>a</sup> Purification is from 1.0 kg cells wet weight.

by collecting most of the elution peak (Fig. 4) it was possible to separate the degraded rBoNTC(H<sub>c</sub>) from the intact rBoNTC(H<sub>c</sub>).

After the last chromatography step the HCIC product pool was dia-filtered with 15 mM succinate, pH 4.0 using a 10 kDa GE Healthcare hollow fiber 5.2 ft<sup>2</sup> membrane. This step was used to

raise the pH and reduce the conductivity of the product. The HCIC elutant is at pH 3.5 with a conductivity of 25–30 mS/cm. SDS-PAGE of each product pool from each step of the process is presented in Fig. 3. The Bulk Drug Substance (BDS) is at pH 4.0, conductivity of 1 mS/cm<sup>2</sup>, protein concentration of 0.5 mg/mL, and by visual



**Fig. 4.** (A) Bar chart of RP-HPLC results from the 13 week stability study showing that the BDS from the second scale-up run showed little loss at all temperatures based peak integration. (B) SDS-PAGE (reduced) lanes 1 and 2 – time = 0 BDS; lanes 3 and 4 – 13 weeks at –80 °C; lanes 5 and 6 – 13 weeks at 5 °C; lanes 7 and 8 – 13 weeks at 25 °C.

**Table 4**  
Potency results of a 24-month stability study of BDS stored at  $-80^{\circ}\text{C}$ .

Time point (months)	ED <sub>50</sub> (ng)	95% Confidence limits (ng)
0	102	58–175
3	117	76–195
6	118	7–691
24	66	32–120

inspection of an SDS–PAGE is  $>98\%$  pure (Fig. 3A – lane 5). The entire purification process from cell washing to final sterile-filtering of the BDS at the bench scale was performed within 7 h.

Four bench-scale purification runs were performed to determine the robustness of the process. The BDS from these runs were very similar in purity and elution profiles. The yields ranged from 1.7 to 2.5 g of purified rBoNTC(H<sub>c</sub>)/kg WCW (Table 2). The yield varied based on how the product peak was collected from the final HCIC column, with a conservative “cut” for the first run which was increased for the second run resulting in a higher yield. Analysis by N-terminal sequencing showed the rBoNTC(H<sub>c</sub>) to have an intact N-terminus (MYFNNIN) with a N-terminal Methionine, and is identical to the gene sequence of the N-22 form.

#### Pilot-scale purification

Two 19 L fermentations and two pilot-scale purifications runs were performed. The fermentations resulted in final WCW of 254 and 248 g/L with final volumes of 12 and 11.7 L, respectively. The pilot-scale purification process from cell washing to bulking of the BDS took just under 12 h.

The first pilot-scale purification produced 1.4 g BDS/kg wt cell mass and was  $>98\%$  pure based on SDS–PAGE (Fig. 3) with the N-terminal sequence intact (Table 3). A second pilot-scale purification generated a  $>98\%$  pure product based on SDS–PAGE (Fig. 3B), with a N-terminal sequence intact (MYFNNIN) and produced 2.08 g/kg WCW (Table 3). A residual DNA test was performed on the final product (AppTec, Philadelphia, PA), and was below the limit of detection ( $<20$  pg DNA/100  $\mu\text{g}$  of rBoNTC(H<sub>c</sub>)).

#### Biophysical stability

Stability testing was performed on the final product from the second pilot scale run. This material was put into 15 mL aliquots and placed at three different temperatures (25, 5 and  $-80^{\circ}\text{C}$ ). Analysis by RP–HPLC and SDS–PAGE were performed at time points 0, 2, 9, and 13 weeks. Fig. 4A shows the amount of intact rBoNTC(H<sub>c</sub>) over the course of the stability study by RP–HPLC. Based on these assays the rBoNTC(H<sub>c</sub>) was stable for at least 3 months at 5 and  $-80^{\circ}\text{C}$ . Protein decreases were seen in the 25  $^{\circ}\text{C}$  sample beginning at 9 weeks. This was confirmed by the degradation bands appearing in the SDS–PAGE (Fig. 4B). These results show the BDS remained intact for at least 3 months at both 5 and  $-80^{\circ}\text{C}$ .

#### Mouse potency assay and stability

A mouse bioassay was used to determine rBoNTC(H<sub>c</sub>) BDS potency and stability over time. Material from the second pilot scale run was used for vaccination and a 24-month stability study. Potency assay results showed protection to be centered, with an ED<sub>50</sub> (theoretical effective dose protecting half the mice) of 102 ng, having 95% confidence limits of 58–175 ng (Table 4). The potency of this rBoNTC(H<sub>c</sub>) preparation is roughly equivalent to potencies of other rBoNT vaccines, which range from 89 to 116 ng [26]. A 24-month potency stability study of material stored at  $-80^{\circ}\text{C}$  was performed and the results are presented in Table 4.

Based on these results the potency of the bulk material is stable for 24 months. Biophysical analysis (SDS–PAGE and RP–HPLC) was not performed past 3 months.

#### Conclusion

The recombinant heavy chain fragment C of botulinum neurotoxin serotype C was produced from *P. pastoris* X-33 and purified using only MEP HyperCel™ and the CM 650M resins. The process was scaled-up and resulted in similar product quality at both the bench and pilot scales. The rBoNTC(H<sub>c</sub>) was stable, based on SDS–PAGE and RP–HPLC when stored at a pH of 4.0 in 15 mM succinate and 0.25–0.5 mg/mL and showed biophysical stability for at least 3 months at 5 and  $-80^{\circ}\text{C}$ . The product remained potent when stored at  $-80^{\circ}\text{C}$  for up to 24 months. The purification process required three sequential chromatographic steps with no ultrafiltration steps between and with the bulk formulation step requiring membrane diafiltration to a protein concentration of 0.25–0.5 mg/mL. The process was easy to scale up with a production time under 12 h at the pilot scale. The product was intact and comparable in potency to other rBoNT vaccines and ready to be transitioned into cGMP manufacturing.

#### Acknowledgments

This publication was made possible by Contract Number 1U01 AI 056514-01 from National Institute of Allergy and Infectious Disease and its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Institute of Allergy and Infectious Disease. This work was also supported by The Medical Research and Materiel Command Contract No. DAMD17-02-C-0107. We would like to thank the BPDF's Quality Control Group and Analytical Methods Laboratory for sample analysis through out this project. Also we would like to thank the BPDF's Purification Development Laboratory staff for process development support throughout this project.

#### References

- [1] C.L. Hatheway, Toxigenic *Clostridia*, Clin. Microbiol. Rev. 3 (1990) 66–98.
- [2] C. Lamanna, E.R. Hart, Relationship of lethal toxic dose to body weight of the mouse, Toxicol. Appl. Pharmacol. 13 (1968) 307–315.
- [3] S.S. Arnon, R. Schechter, T.V. Inglesby, D.A. Henderson, J.G. Barlett, M.S. Ashcer, E. Eitzen, A.D. Fine, J. Hauer, M. Layton, S. Lillibridge, M.T. Osterholm, T. O'Toole, G. Parker, T.M. Perl, P.K. Russell, D.L. Swerdlow, K. Tonat, Botulinum toxin as a biological weapon: medical and public health management, JAMA 285 (2001) 1059–1070.
- [4] K.K. Hill, T.J. Smith, C.H. Helma, L.O. Ticknor, B.T. Foley, R.T. Svensson, J.L. Brown, E.A. Johnson, L.A. Smith, T.R. Okinaka, P.F. Jackson, J.D. Marks, Genetic diversity among Botulinum neurotoxin-producing clostridial strains, J. Bacteriol. 189 (2007) 818–832.
- [5] R.P. Webb, T.J. Smith, P.M. Wright, V.A. Montgomery, M.M. Meagher, L.A. Smith, Protection with recombinant *Clostridium botulinum* C1 and D binding domain subunit (H<sub>c</sub>) vaccines against C and D neurotoxins, Vaccine 25 (2007) 4273–4282.
- [6] C. Montecucco (Ed.), Clostridial neurotoxins: the molecular pathogenesis of tetanus and botulism, Curr. Top. Microbiol. Immunol. 195 (1995) 1–278.
- [7] B. Poulain, U. Weller, T. Binz, H. Neimann, B. De Pavia, O. Dolly, C. Leprince, L. Tauc, Functional roles of the domains of clostridial neurotoxins: the contribution from studies on *Aplysia*, in: B.R. Das Gupta (Ed.), Botulinum and Tetanus Neurotoxins: Neurotransmission and Biomedical Aspects, Plenum Press, New York, 1993, pp. 34–360.
- [8] C.C. Shone, P. Hambleton, J. Melling, Inactivation of *Clostridium botulinum* type A neurotoxin by trypsin, purification of two tryptic fragments. Proteolytic cleavage near the COOH-terminus of the heavy subunit destroys toxin-binding activity, Eur. J. Biochem. 151 (1985) 75–82.
- [9] L.L. Simpson, Molecular pharmacology of botulinum toxin and tetanus toxin, Annu. Rev. Pharmacol. Toxicol. 26 (1986) 427–453.
- [10] L.A. Smith, Development of recombinant vaccines for botulinum neurotoxin, Toxicon 36 (1998) 1539–1548.
- [11] L.A. Smith, M. Byrne, Vaccines for preventing botulism, in: M.F. Brin, J. Jankovic, M. Hallett (Eds.), Scientific and Therapeutic Aspects of Botulinum Toxin, Williams & Wilkins, Philadelphia, Lippincott, 2002, pp. 427–440.

- [12] L.A. Smith, Botulism and vaccines for its prevention, *Vaccine* 27 (2009) D33–D39.
- [13] V. Chiruvolu, J.M. Cregg, M.M. Meagher, Recombinant protein expression in an alcohol oxidase-defective strain of *Pichia pastoris* in fed-batch fermentations, *Enz. Microbiol. Technol.* 21 (1997) 277–283.
- [14] S.K. Johnson, W. Zhang, L.A. Smith, K.J. Potter, S.T. Swanson, V.L. Schlegel, M.M. Meagher, Scale-up and the fermentation and purification of the recombinant heavy chain fragment C of botulinum neurotoxin serotype F, expressed in *Pichia pastoris*, *Prot. Exp. Purif.* 32 (2003) 1–9.
- [15] T. Kubota, T. Watanabe, N. Yokosawa, K. Tsuzuki, T. Indoh, K. Moriishi, K. Sanda, Y. Maki, K. Inoue, N. Fuji, Epitope regions in the heavy chain of *Clostridium botulinum* type E neurotoxin recognized by monoclonal antibodies, *Appl. Environ. Microbiol.* 63 (1997) 1214–1218.
- [16] K.J. Potter, M.A. Bevins, E.V. Vassilieva, V.R. Chiruvolu, T. Smith, L.A. Smith, M.M. Meagher, Production and purification of the heavy-chain fragment C of botulinum neurotoxin, serotype B, expressed in the methylotrophic yeast *Pichia pastoris*, *Prot. Exp. Purif.* 13 (1998) 357–365.
- [17] K.J. Potter, W. Zhang, L.A. Smith, M.M. Meagher, Production and purification of the heavy chain fragment C of botulinum neurotoxin, serotype A, expressed in the methylotrophic yeast *Pichia pastoris*, *Prot. Exp. Purif.* 19 (2000) 393–402.
- [18] M.P. Dux, R. Barent, J. Sinha, M. Gouthro, T. Swanson, A. Barthuli, M. Inan, J.T. Ross, L.A. Smith, T.J. Smith, R. Webb, B. Loveless, I. Henderson, M.M. Meagher, Purification and scale-up of a recombinant heavy chain fragment C of botulinum type neurotoxin serotype E in *Pichia pastoris* GS115, *Prot. Exp. Purif.* 45 (2006) 359–367.
- [19] J. Sinha, B. Plantz, M. Inan, M.M. Meagher, Causes of proteolytic degradation of secreted recombinant proteins produced in methylotrophic yeast *Pichia pastoris*: case study with recombinant ovine interferon- $\tau$ , *Biotech. Bioeng.* 89 (2004) 102–112.
- [20] X.S. Zhou, Y.X. Zhang, Decrease of proteolytic degradation of recombinant hirudin produced by *Pichia pastoris* by controlling the specific growth rate, *Biotechnol. Lett.* 24 (2002) 1449–1453.
- [21] W. Zhang, L.A. Smith, B.A. Plantz, V.L. Schlegel, M.M. Meagher, Design of methanol feed control in *Pichia pastoris* fermentations based upon a growth model, *Biotechnol. Prog.* 6 (2002) 1392–1399.
- [22] J. Mehmedalija, W. Fredrik, B. Monika, G. Percival, E. Sven-Olof, Temperature limited fed-batch technique for control of proteolysis in *Pichia pastoris* bioreactor cultures, *Microb. Cell Fact.* 2 (2003) 6.
- [23] M. Inan, V. Chiruvolu, K.M. Eskridge, G.P. Vlasuk, K. Dickerson, S. Brown, M.M. Meagher, Optimization of temperature-glycerol-pH conditions for a fed-batch fermentation process for recombinant hookworm (*Ancylostoma caninum*) anticoagulant peptide (AcAP-5) production by *Pichia pastoris*, *Enz. Microbiol. Technol.* 24 (1999) 438–445.
- [24] J. Stratton, V.J. Chiruvolu, M.M. Meagher, High cell-density fermentation, in: D.R. Higgins, J.M. Cregg (Eds.), *Pichia Protocols*, Humana Press, Totowa, 1998, pp. 107–120.
- [25] M.P. Byrne, R.W. Titball, J. Holley, L.A. Smith, Fermentation, purification and efficacy of a recombinant vaccine candidate against botulinum neurotoxin type F from *Pichia pastoris*, *Prot. Exp. Purif.* 18 (2000) 327–337.
- [26] M.P. Byrne, L.A. Smith, Development of vaccines for prevention of botulism, *Biochimie* 82 (2000) 955–966.