2006

Purification and Scale-Up of a Recombinant Heavy Chain Fragment C of Botulinum Neurotoxin Serotype E in *Pichia Pastoris* GS115

Michael P. Dux
University of Nebraska - Lincoln

Rick Barent
University of Nebraska - Lincoln

Jayanta Sinha
University of Nebraska - Lincoln

Mark Gouthro
University of Nebraska - Lincoln

Todd Swanson
University of Nebraska - Lincoln

See next page for additional authors

Available online at www.sciencedirect.com
SCIENCE DIRECT
Protein Expression and Purification 45 (2006) 359–367
Protein Expression & Purification
www.elsevier.com/locate/yprep

Purification and scale-up of a recombinant heavy chain fragment C of botulinum neurotoxin serotype E in *Pichia pastoris* GS115

Michael P. Dux [a], Rick Barent [a], Jayanta Sinha [a], Mark Gouthro [a], Todd Swanson [a], Ardis Barthuli [a], Mehmet Inan [a], John T. Ross [b], Leonard A. Smith [c], Theresa J. Smith [c], Robert Webb [c], Bonnie Loveless [c], Ian Henderson [b], Michael M. Meagher [a,*]

[a] *Biological Process Development Facility, Department of Chemical Engineering, University of Nebraska-Lincoln, Lincoln, NE 68588-0466, USA*
[b] *DVC, LLC, A CSC Company, 64 Thomas Johnson Drive, Frederick, MD 21702, USA*
[c] *United States Army Medical Research Institute of Infectious Diseases, Toxinology Division, Fort Detrick, Fredrick, MD 21702-5011, USA*

Received 17 May 2005, and in revised form 17 August 2005
Available online 20 September 2005

Abstract

A recombinant C-terminus heavy chain fragment from botulinum neurotoxin serotype E (BoNT/E) is proposed as a vaccine against the serotype E neurotoxin. This fragment, rBoNTE(H_c), was produced intracellular in *Pichia pastoris* GS115 by a three-step fermentation process, i.e., glycerol batch phase and a glycerol fed-batch phase to achieve high cell densities, followed by a methanol fed-batch induction phase. The rBoNTE(H_c) protein was purified from the soluble fraction of cell lysates using three ion-exchange chromatography steps (SP Sepharose Fast Flow, Q Sepharose Fast Flow, Sp Sepharose High Performance) and polished with a hydrophobic charge induction chromatography step (MEP HyperCel). Method development at the bench scale was achieved using 7–380 mL columns and the process was performed at the pilot scale using 0.5–3.1 L columns in preparation for technology transfer to cGMP manufacturing. The purification process resulted in greater than 98% pure rBoNTE(H_c) based on HPLC and yielded up to 1.01 g of rBoNTE(H_c)/kg cells at the bench scale and 580 mg vaccine/kg cells at the pilot scale. N-terminal sequencing showed that the purified rBoNTE(H_c) N-terminus is intact and was found to protect mice against a challenge of 1000 mouse intraperitoneal LD_{50}'s of BoNT/E.

Keywords: Botulinum; *Pichia pastoris*; Ion-exchange chromatography; Hydrophobic charge induction chromatography; Homogenized; Filtration; Recombinant protein; Scale-up

Botulinum neurotoxin, a National Institute of Allergen and Infectious Disease class A agent, has been weaponized [1]. There is a need for a safe, well characterized, and efficacious recombinant vaccine that will protect against all seven known serotypes of the toxin. Such a product is needed to protect US military personnel, first-responders in the civilian environment, and, if necessary, civilians who may be at risk because of their geographical locations in the United States. *Clostridium botulinum* produces seven antigenically distinct neurotoxins differentiated serologically by specific neutralization [2]. These have been designated as serotypes A through G and are the most toxic substances known [3]. Thus, seven monovalent vaccines are required if full immunologic protection against botulinum neurotoxins is to be achieved. The botulinum toxin is expressed as a single, 150-kDa polypeptide

* Corresponding author. Fax: +1 4024724985.
E-mail address: mmeagher1@unl.edu (M.M. Meagher).

1046-5928/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2005.08.015

chain that is posttranslationally nicked, forming a simple two-chain polypeptide that consists of a C-terminal 100-kDa heavy chain joined by a single disulfide bond to the 50-kDa light chain [4]. The toxin has three distinct regions: a translocation domain, a binding domain, and a catalytic domain. The carboxy terminus of the heavy chain binds to a nerve cell receptor at the neuromuscular junction [5] and the amino terminus of the heavy chain is capable of forming channels in the cell membrane, allowing internalization of the toxin [6,7]. Finally, the toxin's light chain, a Zn^{2+}-containing endoprotease, is released into the cell cytoplasm, cleaving a specific synaptic protein involved in the docking of acetylcholine-containing vesicles from binding to the synaptic membrane. This blocking prevents the vesicles from releasing acetylcholine into the synapse at the neuromuscular junction, resulting in flaccid muscle paralysis [8]. In humans, botulism is characterized by a descending flaccid paralysis, starting in the head region and descends to the muscles controlling breathing.

The H_c fragments of BoNT (A–F) were shown to be non-toxic, antigenic [9], and capable of eliciting a protective immunity in animals challenged with homologous BoNT [10,11]. These results prompted an effort to develop a recombinant botulinum vaccine against all seven serotypes using the H_c fragments as vaccine antigens [4,12–17]. As mentioned earlier, the seven serotypes are distinct antigenitically, requiring the vaccine to be comprised of seven distinct antigens. To add to the complexity, the heavy chain fragments for each of the seven serotypes are distinct proteins with variable degrees of homology. By way of illustration, the isoelectric point of the recombinant fragments calculated from the known sequences are as follows: serotype A: 9.3, B: 7.1, C: 5.6, D: 6.0, E: 8.3, F: 9.1, G: 7.9, underscoring the fact that fermentation and purification of each antigen will be unique. It was the hope back in 1994 when the The University of Nebraska-Lincoln Biological Process Development Facility (BPDF) began working with Dr. Leonard Smith of the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) on this project that once a fermentation and purification process was developed for BoNTB(H_c) [13] that all subsequent serotypes would follow suite. This was found not to be the case for BoNTA(H_c) [14] and continues to be the case for each of the serotypes we have worked on. The experiences we learned from previous purification research are applied to the next serotype, but the amount expressed during fermentation, the methods of purification, and the characteristics of the molecules are different for each serotype. There in lies the challenge of a recombinant botulinum vaccine, seven different processes to produce seven different proteins, with all seven *distinct* H_c fragments formulated into one vial.

This paper describes research, development, and scale-up of a process to produce a recombinant botulinum neurotoxin serotype E, heavy chain C-terminal fragment (rBoNTE(H_c))[1] candidate vaccine suitable for transfer to a current good manufacturing practice (cGMP) facility for production of clinical material. The rBoNTE(H_c) fragment is expressed intracellularly in *Pichia pastoris* using a three-phase high-cell density fermentation incorporating a 9 h methanol induction to minimize proteolytic degradation of the rBoNTE(H_c). Initial process development was performed at the bench scale resulting in a four-step process and concluded with four bench-scale demonstration runs to determine process reproducibility. Two pilot-scale runs were performed to demonstrate the scalability of this process for continuation towards the goal of cGMP manufacturing.

Materials and methods

Molecular biology

rBoNTE(H_c) gene was originally obtained from *C. botulinum* NCTC 11219. At the Division of Toxicology and Aerobiology of USAMRIID, rBoNTE(H_c) codon optimized gene was inserted into pHILD4 expression vector at *Eco*RI site. After transformation into *P. pastoris* GS115 (*his4*), cells were screened with increasing amount of Geneticin (G418) to determine a multi-copy expression vector. The multi-copy clone developed at USMRIID was then tested for viability and restriction map of the insert by Southern blot.

Fermentation

Pichia pastoris GS115 harboring three copies of the rBoNTE(H_c) encoding gene with expression under the control of AOX1 promoter was grown to high cell density on defined medium. The fermentation was carried out in three different phases, i.e., glycerol batch phase, glycerol fed-batch phase, and the methanol fed-batch phase. Fermentations were performed in 5 L fermentor (Bioflo 3000, New Brunswick Scientific Edison, NJ) or in 19 L fermentor (NLF, Bioengineering AG, Sagenrainstrasse 7 CH-8636 Wald, Switzerland) at pH 5.0 and 30 °C. The cells were grown to desired cell density on glycerol and then induced on methanol for expression as previously described

[1] *Abbreviations used:* BCA, bicinchoninic acid; rBoNT, recombinant botulinum neurotoxin; rBoNTE(H_c), recombinant botulinum neurotoxin serotype E, heavy chain C-terminal fragment; BSA, bovine serum albumin; CV, column volume; FF, fast flow; HP, high performance; H_c, heavy chain C-terminal fragment; kDa, kilo Dalton; MW, molecular weight; PVDF, polyvinylidene difluoride; Q, quaternary amine; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SP, sulfopropyl; HCIC, hydrophobic charge induction chromatography; WCW, wet cell weight; TBS, Tris base-buffered saline; PMSF, phenylmethylsulfonyl fluoride; IEC, ion-exchange chromatography; cGMP, current good manufacturing practices; SEC, size exclusion chromatography; SCX, strong cation exchange chromatography; HIC, hydrophobic induction chromatography; PDS, purified drug substance; NaCl, sodium chloride; i.p., intraperitoneal.

[13,14,17]. The cells were grown exponentially on methanol containing 12 mL/L of PTM1 salts [18] and induced for 9 h. Samples were withdrawn for analyses to determine the wet cell weight and rBoNTE(H_c) 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 either frozen at −80 °C or processed immediately. Two hundred grams of cell paste was suspended in 50 mM sodium phosphate mono basic, 10 mM sodium chloride (NaCl), and pH 7.4, to 20% (w/v) solids to wash the fermentation medium away. The suspension was centrifuged at 11,300*g* at 4 °C for 15 min. Cell paste was resuspended in 50 mM sodium phosphate mono basic, 10 mM NaCl, pH 7.4 to 20% (w/v) solids and homogenized using a Microfluidizer M-110EH (Microfluidics, Newton, CA) set at 22,000 psi with the lysate chilled to <10 °C before processing. Two passes were performed to obtain at least 85% cell disruption determined with a hemocytometer. The homogenate was clarified at 11,300*g* for 30 min at 4 °C followed by filtration through a Pall AcroPak 200 with a 0.2 μm Fluorodyne II membrane (Pall, 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 placed on ice. A 5.0 cm × 19.5 cm column (Waters Chromatography, Milford, MA) packed with 383 mL of strong cation exchange SP Sepharose Fast Flow resin (Amersham Biosciences Piscataway, NJ) was equilibrated with 5 column volumes (CV) of 50 mM sodium phosphate mono basic, 10 mM NaCl, pH 7.4. Supernatant was loaded onto the column at a linear velocity of 300 cm/h followed by a 10 CV wash with the equilibration buffer. The product was eluted using a 5 CV step of 50 mM sodium phosphate mono basic and 100 mM NaCl, pH 7.4.

The SP Sepharose FF product was loaded directly onto a 2.7 cm × 19.8 cm column (Applied Biosystems, Foster City, CA) packed with 113 mL strong anion exchange Q Sepharose Fast Flow resin (Amersham Biosciences Piscataway, NJ). The column was equilibrated with 5 CV of 50 mM sodium phosphate mono basic, pH 7.4 at a linear velocity of 300 cm/h. The flow through fraction was collected from the Q Sepharose FF column as the rBoNTE(H_c) does not bind and passes through the column.

The Q Sepharose FF product was diluted with an equal volume of 25 mM succinate, pH 4.0 to bring down the pH to approximately pH 6.0. The diluted product was loaded onto a 2.7 cm × 13.1 cm (Applied Biosystems, Foster City, CA) 75 mL strong cation exchange SP Sepharose HP resin (Amersham Biosciences Piscataway, NJ) that was pre-equilibrated with 5 CV of 25 mM succinate, pH 6.0 at 200 cm/h. After loading, the column was washed with 5 CV of 25 mM succinate, 200 mM NaCl, pH 5.0 buffer. The rBoNTE(H_c) was eluted from the column using a 25 CV linear gradient from 25 mM succinate, 200 mM NaCl, pH 5.0 to 25 mM succinate, 300 mM NaCl, pH 5.0.

Table 1
Bench-scale purification of rBoNTE(H_c)[a]

Step	Volume (mL)	Protein concentration (mg/mL)	Total protein (mg)
Lysate	1300 ± 130	16 ± 3	21,000 ± 4100
SP Sepharose FF	800 ± 100	0.9 ± 0.2	700 ± 150
Q Sepharose FF	1400 ± 480	0.40 ± 0.15	520 ± 130
SP Sepharose HP	370 ± 120	0.65 ± 0.17	230 ± 48
HCIC	275 ± 72	0.60 ± 0.12	163 ± 45
Diafiltration	504 ± 123	0.30 ± 0.02	153 ± 42

[a] Average of four bench-scale purification runs starting from 200 g cells wet weight.

The SP Sepharose HP product was diluted with an equal volume of 50 mM sodium phosphate mono basic, pH 7.4 to adjust the pH up to 6.0. The diluted product was loaded onto a 2.7 cm × 13.6 cm (Applied Biosystems, Foster City, CA) 77 mL hydrophobic charge induction chromatography column, BioSepra MEP HyperCel resin (Ciphergen, Fremont, CA) and equilibrated with 5 CV of 25 mM succinate, pH 6.0 at 300 cm/h. After loading, the column was washed with 5 CV of 25 mM succinate, pH 5.0 buffer. rBoNTE(H_c) was eluted from the column using a 15 CV linear gradient from 25 mM succinate, pH 5.0 to 25 mM succinate, pH 4.0.

The final product was dia-filtered with 15 mM succinate pH 4.0 using Millipore Pellicon XL Filter, with a 50 cm^2 10K regenerated cellulose membrane (Millipore, Bedford, MA) 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 μm Fluorodyne II membrane (Pall, East Hills, NY) and stored at 2–8 °C (Table 1).

Pilot-scale cell harvest and disruption

Cells harvested from a 19 L fermentation were centrifuged at 11,300*g* at 4 °C for 30 min using a Sorvall Evolution RC Centrifuge (Kendro Laboratory Products, Asheville, NC) and processed immediately. Thousand three hundred to thousand five hundred grams of cell paste were suspended in 50 mM sodium phosphate mono basic, 10 mM NaCl, pH 7.4 to 20% (w/v) solids to remove fermentation medium. The suspension was centrifuged at 11,300*g* for 15 min and resuspended in 50 mM sodium phosphate mono basic, 10 mM NaCl, pH 7.4 to 20% (w/v) solids. Cells were homogenized using a Microfluidizer M-110EH (Microfluidics, Newton, CA) set at 22,000 psi. Two passes were performed to obtain at least 85% cell disruption determined with a hemocytometer. The homogenate was centrifuged at 11,300*g* for 30 min at 4 °C and filtered through a Pall 0.2 μm Fluorodyne II membrane (Pall, East Hills, NY) prior to loading onto the first column.

Pilot-scale purification

All chromatographic separations were performed using a North Carolina SRT (Cary, 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 SP Sepharose FF step was performed using a 3.14 L (20 cm × 10 cm) BPG 200/500 column (Amersham Pharmacia Biotech, Piscataway, NJ). The Q Sepharose FF step was performed on a 1.02 L (10 cm × 13 cm) BPG 100/500 column (Amersham Biosciences Piscataway, NJ). The SP Sepharose HP step was performed using a 557 mL (7 cm × 10 cm) QS 70/500 column (Millipore, Bedford, MA), and the BioSepra MEP HyperCel step was performed using a 531 mL (6 cm × 18.8 cm) Vantage 60A column (Millipore, Bedford, MA). Final product was diafiltered to pH 4.0 using 15 mM succinate, pH 4.0 at 5 °C with a North Carolina SRT Optisep 3000 bench filtration unit equipped with two 2 ft^2 of 10 kDa regenerated cellulose, part number 52-D5B-0010 (North Carolina SRT Cary, NC). Final product was sterile filtered with an AcroPak 20, with a 0.2 μm Fluorodyne II membrane (Pall, East Hills, NY) and stored at 2–8 °C (Table 2).

Protein analysis

Total protein concentrations were determined using the BCA (Pierce Chemical, St. Louis, MO) standard assay, using BSA as a standard. Purity was determined by 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 chicken anti-rBoNTE(H_c) antibody incubated at 0.33 μg/mL for 1 h at room temperature. The secondary antibody was a peroxidase labeled affinity-purified goat anti-chicken IgG (Kirkegaard & Perry Laboratories, Gainsburg, MD) incubated at 0.15 μg/mL for 1 h at room temperature. The SDS–PAGE-separated proteins were transferred to PVDF membranes (Bio-Rad, Hercules, CA) at 25 V for 20 min, blocked with 5% non-fat dried 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). Protease detection was performed with an EnzChek Protease Assay Kit (Molecular Probes, Eugene, OR) with Bodipy FL Casein as the substrate. N-terminal sequencing was performed by the University of Nebraska-Medical Center Protein Core Facility using a ABI Procise 494 Sequencer (Applied Biosystems, Foster City, CA) (Table 3). The Biological Process Development Facility (BPDF) Quality Control Laboratory estimated purity and quantity of rBoNTE(H_c) by HPLC using strong cation exchange chromatography (SCX). SCX analysis was performed with a 50 mm length PolyLC Polysulfoethyl A column (PolyLC, Columbia, USA). For sample analysis a

Table 2
Second pilot-scale purification of rBoNTE(H_c)[a]

Step	Volume (mL)	Protein concentration (mg/mL)	Total protein (mg)	Estimated purity[b] (%)	rBoNTE(H_c)[c] (mg)	Step yield (%)	Total yield (%)
Lysate	7,500	14	105,000	3	3150	100	100
SP Sepharose FF	12,800	0.32	4045	70	2831	90	90
Q Sepharose FF	12,500	0.22	2700	80	2160	76	69
SP Sepharose HP	3,750	0.33	1253	92	1152	53	37
HCIC	2,400	0.33	780	99	772	67	25
Diafiltration	2,720	0.27	734	99	727	94	23

[a] Purification is from 1.3 kg cells wet weight.
[b] Purity estimated by SCX-HPLC.
[c] Determined by multiplication of total protein and purity.

Table 3
N-terminal sequence of purified rBoNTE(H_c)

Sample	N-terminal sequence
Intact rBoNTE(H_c)	p*I* of protein: 8.8 Protein MW: 52220 Amino acid composition: A9 C4 D29 E14 F21 G21 H6 I44 K34 L32 M7 N71 P6 Q12 R15 S37 T26 V24 W8 Y29 MGESQQELNSMVTDTLNNSIPFKLSSYTDDKILISYFNKFFKRIKSSSVLNMRYKNDKYVDTSGYDSNININ GDVYKYPTNKNQFGIYNDKLSEVNISQNDYIIYDNKYKNFSISFWVRIPNYDNKIVNVNNEYTIINCMRDNN SGWKVSLNHNEIIWTLQDNAGINQKLAFNYGNANGISDYINKWIFVTITNDRLGDSKLYINGNLIDQKSILN LGNIHVSDNILFKIVNCSYTRYIGIRYFNIFDKELDETEIQTLYSNEPNTNILKDFWGNYLLYDKEYYLLNV LKPNNFIDRRKDSTLSINNIRSTILLANRLYSGIKVKIQRVNNSSTNDNLVRKNDQVYINFVASKTHLFPLY ADTATTNKEKTIKISSSGNRFNQVVVMNSVGNNCTMNFKNNNGNNIGLLGFKADTVVASTWYYTHMRDHTNSNGCFWN FISEEHGWQEK
Bench-scale rBoNTE(H_c)-ND-DP-010	GESQQ
Pilot-scale ND(E)-ER-001	GESQQ
Pilot-scale ND(E)-ER-002	GESQQ

1 mL/min gradient elution from 65% mobile phase A (20 mM Tris at a pH 7.7) and 35% mobile phase B (20 mM Tris, 2 M NaCl at a pH 7.7) to 60% mobile phase A and 40% mobile phase B, over 6 min.

Mouse potency bioassay

The potency of purified rBoNTE(H_c) was determined using a mouse potency bioassay [19,20]. Groups of 10 female mice (stain Crl:CD-1 (Charles River, NC)) were intramuscularly vaccinated twice with threefold dilutions of antigen (8.1, 2.7, 0.9, 0.3, 0.1, 0.033, and 0.011 μg rBoNTE(H_c)/mouse). The antigen was formulated in 25 mM succinate, 15 mM phosphate, pH 5.0, and diluted into final concentrations of 0.2% Alhydrogel (HCI Biosector, Frederikssund, Denmark), 5% mannitol, 0.9% NaCl, pH 5.5. Vaccinations were at 2-week intervals, and the mice were challenged 3 weeks after the final vaccination with 1000 mouse i.p., LD_{50} BoNT/E toxin complex. Mice were observed twice daily for 5 days post challenge. Results were tabulated and analyzed using probit analysis (SPSS, Chicago, IL).

Results and discussion

Fermentation

It was determined that after 9 h of methanol induction the amount of rBoNTE(H_c)/unit cell did not increase and that intracellular protease levels increased by nearly 50% as the induction time increased to 26 h. *P. pastoris* GS115 rBoNTE(H_c) induced on methanol for 26 h produced increased level of proteases that were detrimental to the stability of rBoNTE(H_c) during the purification process and under final product storage conditions. The 9 h induction time was chosen.

Bench-scale purification

Initial cell disruption experiments consisted of four passes through the Microfluidizer as described in Material and methods section. It was found by bicinchoninic acid (BCA), Western blot assays, and the hemocytometer, that after two passes the protein concentration and the intensity of Western blot bands on subsequent passes were the same. Two passes were also advantageous since this reduced the total processing time of the homogenization step, reducing the potential for proteolytic degradation of rBoNTE(H_c). If homogenate was stored at 2–8 °C for 24 h, the rBoNTE(H_c) would degrade to multiple fragments based on Western blot. The length of the methanol induction had an impact on the quality of the product as mentioned above. 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. From a cGMP perspective, PMSF is not desirable, and should be removed prior to scale-up. Adding 10 mM NaCl to the breaking buffer reduced the binding of proteases to the SP Sepharose Fast Flow resin during the capture step and optimizing chromatography conditions resulted in additional protease activity eluting during the wash step (Fig. 1). A combination of 9 h methanol induction time, a short processing time for harvested cells, and optimization of the separation of proteases from the rBoNTE(H_c) during the capture step eliminated the need for PMSF in the homogenization buffer.

The rBoNTE(H_c) was purified at the bench scale using ion-exchange and hydrophobic charge induction chromatography techniques. Batch binding studies evaluated both anion and cation exchange resins over a pH range from 4 to 8 for capture of rBoNTE(H_c). Clarified cell lysate at a protein/resin ratio of 5 mg/mL was incubated at 4 °C for 3 h. Optimum binding was based on the least amount of rBoNTE(H_c) present in the supernatant. Batch and dynamic binding were compared at a total protein load of 20 mg/mL of resin followed by a step elution using 300 mM NaCl. Surprisingly, 78.2 mg/g total protein of rBoNTE(H_c) was recovered from dynamic binding to the capture column, while only 33.9 mg rBoNTE(H_c)/g of total protein was recovered from batch binding. This was over a twofold increase in the amount of rBoNTE(H_c) recovered from the first step.

Initial elution of the rBoNTE(H_c) from the SP Sepharose FF column was achieved using a 300 mM NaCl step elution in 50 mM sodium phosphate buffer at pH 7.4. Further optimization determined that rBoNTE(H_c) eluted off at 100 mM NaCl. This decreased the amount of contaminants and eliminated a major contaminant at the same MW as the rBoNTE(H_c) (Fig. 2). The maximum total protein loaded onto the SP Sepharose FF column was 55 mg/mL resin.

Product from the capture column was negatively purified through a Q Sepharose FF column removing a majority of the larger molecular weight *Pichia* proteins that are present after the capture step (Fig. 3). Analysis of the eluted contaminants by Western blot found that rBoNTE(H_c) was not present at a protein load of 5.5 mg/mL resin at the bench scale. This protein load was used for future processing runs and scale-up.

The flow through from the Q Sepharose FF column was loaded directly onto a SP Sepharose HP resin. Initially a Source 15 S resin was used for this step, but due to scalability problems and the cost of this resin, a resin screen was performed using Poros 50 HS, SP Sepharose HP, CM 650 M, Source 30 S, and Fractrogel EMD. Based on the results of the resin screen and factoring in cost, SP Sepharose HP resin was selected. The SP Sepharose HP resin increased the purity of rBoNTE(H_c) to 92% based on strong cation exchange (SCX)-HPLC. The elution gradient was optimized from a 45 CV gradient of 100–400 mM NaCl in 25 mM succinate, pH 5.0 to a 25 CV gradient of 200–300 mM NaCl in 25 mM succinate, pH 5.0.

Hydrophobic charge induction chromatography (HCIC) was used as a polishing step to remove a degradation

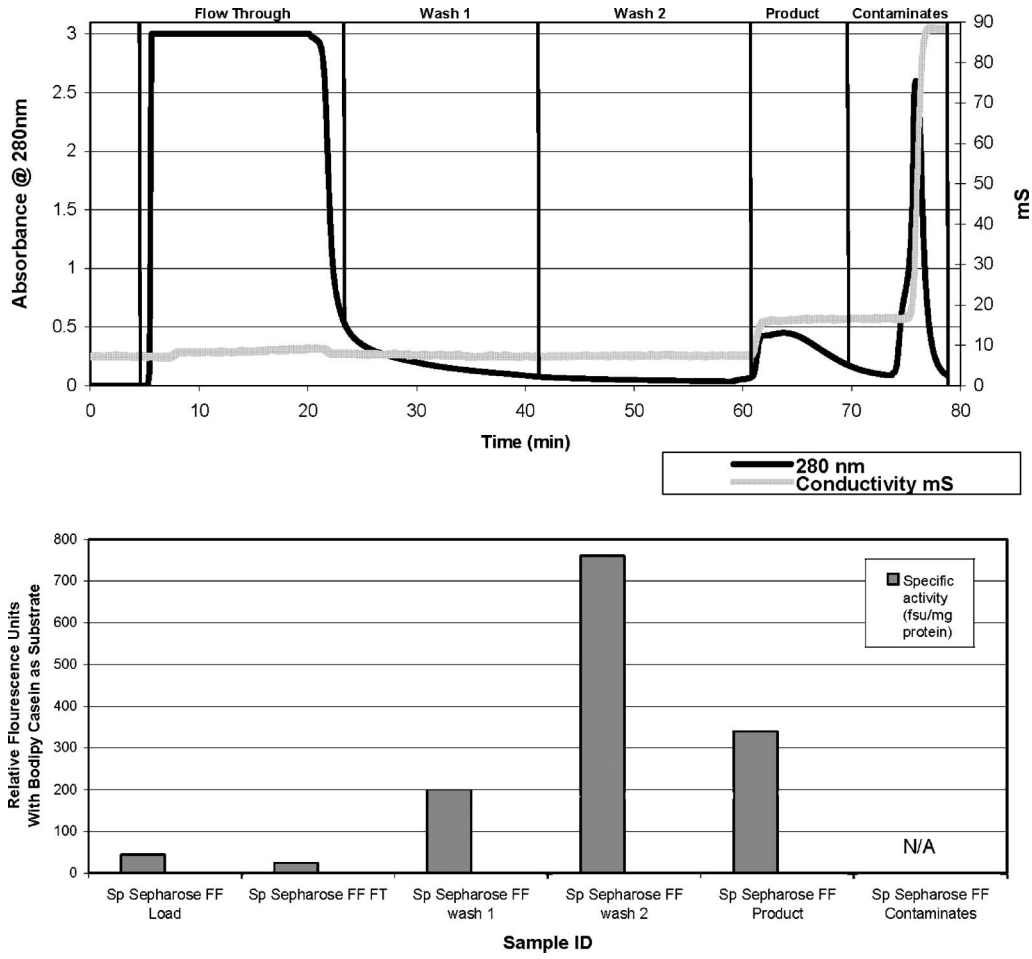


Fig. 1. Protease detection during the capture of rBoNTE(H_c). A chromatogram of the capture step for a bench-scale purification of rBoNTE(H_c) with no PMSF in the cell lysate shown with the corresponding specific activity's of proteases in each of the fractions taken during the separation with bodipy casein used as the substrate.

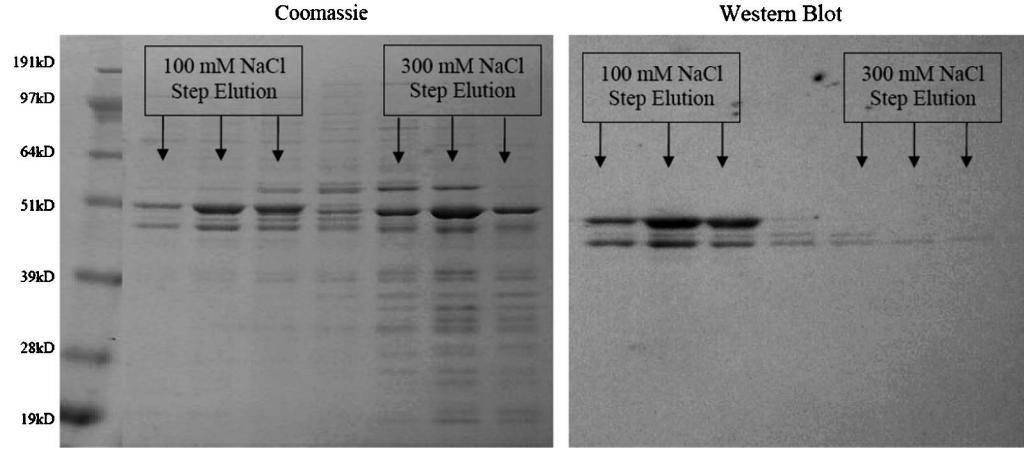


Fig. 2. Capture step optimization. Coomassie-stained SDS–PAGE and Western blot of fractions taken across the elution peaks of the SP Sepharose FF capture step, used for determining the optimal NaCl step elution.

product of rBoNTE(H_c) (Fig. 4 and Table 4). A total protein load of 3.5 mg/mL resin resulted in no rBoNTE(H_c) detected in the flow through. By collecting only the back half of the elution peak (Fig. 4) it was possible to separate the degraded rBoNTE(H_c) from the intact rBoNTE(H_c), the degraded rBoNTE(H_c) comes from the omission of PMSF in the lysis buffer. The purified drug substance (PDS) was shown to be a single band on SDS–PAGE

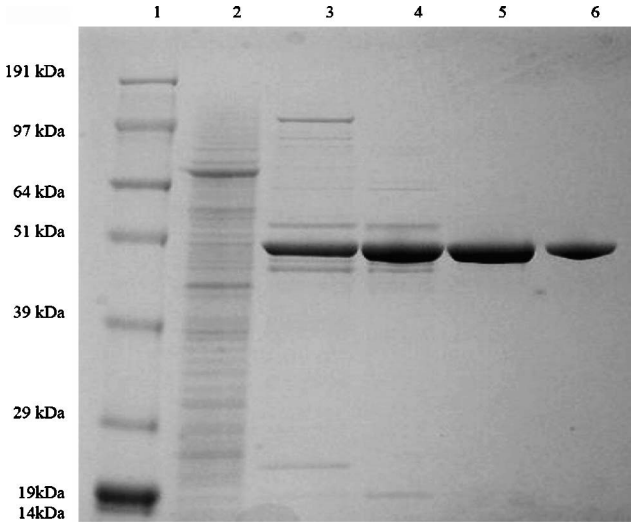


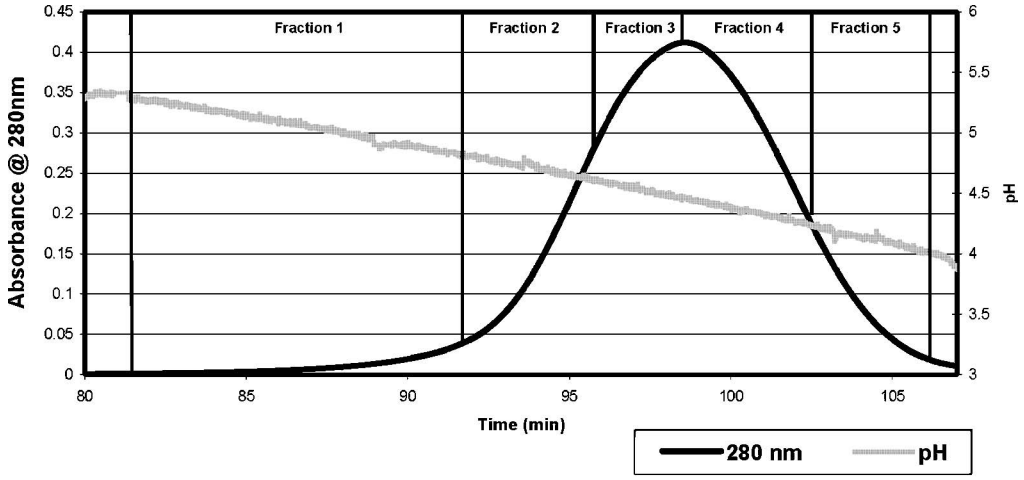
Fig. 3. SDS–PAGE of chromatography products. SDS–PAGE of the different product peaks from all the chromatography steps in the rBoNTE(H_c) purification process with an equal total protein load across the gel. Lane 1, See Blue Plus 2 MW Marker; lane 2, cell lysate; lane 3, Sp Sepharose FF product; lane 4, Q Sepharose FF product; lane 5, SP Sepharose HP product; and lane 6, HCIC product.

Table 4
HPLC-SCX of HCIC fractions

Sample ID	Retention time	Area of intact rBoNTE(H_c) (%)
HCIC load	8.78	90.16
HCIC fraction 1	8.88	
HCIC fraction 2	8.83	86.62
HCIC fraction 3	8.79	90.31
HCIC fraction 4	8.77	96.14
HCIC fraction 5	8.76	100.00

(Figs. 3 and 4) and >98% by SCX-HPLC (Table 4). The pH of the PDS as it eluted from the HCIC column is approximately pH 4.7. By SDS–PAGE, it has been demonstrated that the PDS will degrade over a 4 day period at 4 °C if the pH of the PDS is above a pH 4.7. However, the protein has been shown to be stable over the same storage period at 4 °C following reduction of the pH to pH 4.0 prior to storage (Figs. 5 and 6). The entire purification process from harvesting to final sterile-filtering at the bench scale was performed with in 10 h.

Four bench-scale purification runs were performed to determine the robustness of the process. The PDS from these runs were very similar in purity and elution profiles.



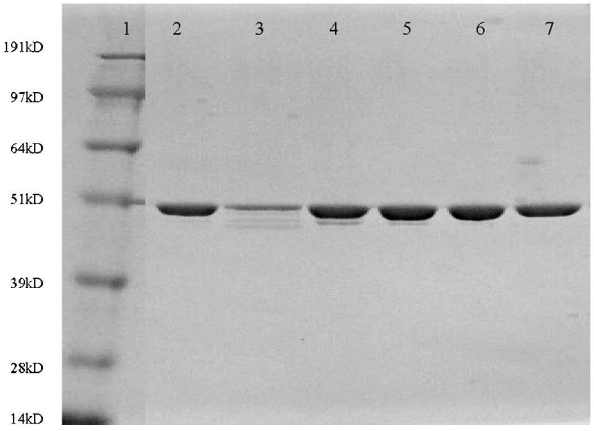


Fig. 4. HCIC chromatogram and SDS–PAGE. Chromatogram and SDS–PAGE of the HCIC column step showing how the degradation product is remove across the elution of the rBoNTE(H_c) peak. Lane 1, See Blue plus 2 MW Marker; lane 2, HCIC load; lane 3, HCIC fraction 1; lane 4, HCIC fraction 2; lane 5, HCIC fraction 3; lane 6, HCIC fraction 4; and lane 7, HCIC fraction 5.

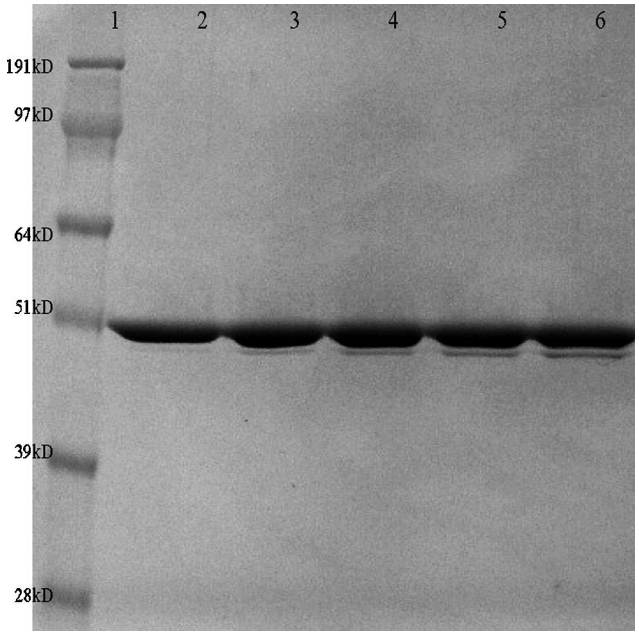


Fig. 5. Stability of rBoNTE(H_c) at pH 5.0. The stability of rBoNTE(H_c) in a pH 5.0 buffer stored at 4 °C for 4 days shown by SDS–PAGE. Lane 1, See Blue plus 2 MW Marker; lane 2, rBoNTE(H_c)-DP-007 Day 0; lane 3, rBoNTE(H_c)-DP-007 Day 1; lane 4, rBoNTE(H_c)-DP-007 Day 2; lane 5, rBoNTE(H_c)-DP-007 Day 3; and lane 6, rBoNTE(H_c)-DP-007 Day 4.

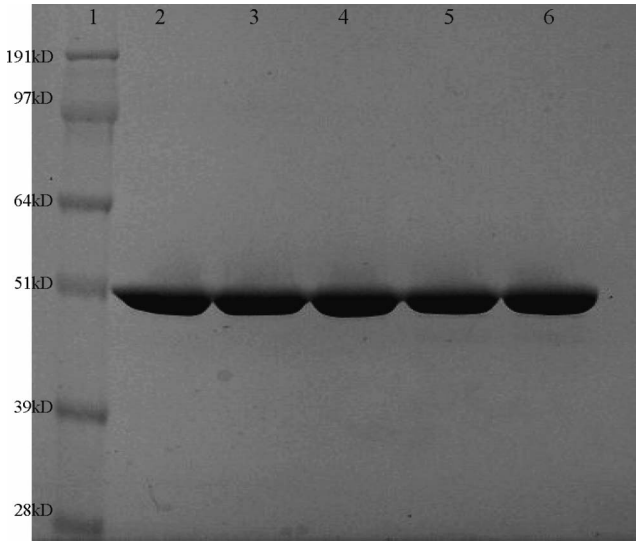


Fig. 6. Stability of rBoNTE(H_c) at pH 4.0. The stability of rBoNTE(H_c) in a pH 4.0 buffer stored at 4 °C for 4 days shown by SDS–PAGE. Lane 1, See Blue plus 2 MW Marker; lane 2, rBoNTE(H_c)-DP-007 Day 0; lane 3, rBoNTE(H_c)-DP-007 Day 1; lane 4, rBoNTE(H_c)-DP-007 Day 2; lane 5, rBoNTE(H_c)-DP-007 Day 3; lane 6, and rBoNTE(H_c)-DP-007 Day 4.

The yields ranged from 577 mg of purified rBoNTE(H_c)/kg cell mass to 1 g of purified rBoNTE(H_c)/kg cell mass (Table 1), and yield varied based on the cut on the final HCIC column. Analysis by N-terminal sequencing showed the rBoNTE(H_c) to have an intact N-terminus (Table 2).

Pilot-scale purification

Two 19 L fermentations and two pilot-scale purifications runs were performed. These fermentations resulted in final wet cell weights of 175 and 139 g/L with final volumes of 10.2 and 11.8 L, respectively.

The first pilot-scale purification was performed using freshly harvested cell mass and produced 462 mg PDS/kg cell mass. Upon completion of the run, analysis showed the final product to contain a minor, lower molecular weight form of rBoNTE(H_c) by SDS–PAGE and was 97% pure by SCX-HPLC. This contaminant was due to fraction selection during the HCIC column step. N-terminal sequencing determined the PDS had an intact N-terminus (Table 2).

A second pilot-scale purification generated a 100% pure product based on SCX-HPLC and no lower molecular weight form of rBoNTE(H_c) was seen by SDS–PAGE. The second pilot-scale run also produced a higher over all yield (Table 3). The product was collected from the HCIC chromatography step just before the apex of the peak, consistent with the bench-scale runs. The HPLC analysis indicated (data not shown) that the lower molecular weight contaminant elutes from the column at the beginning of the peak and the pure rBoNTE(H_c) elutes at the end of the peak. A residual DNA test was performed on the final product (AppTec, Philadelphia, PA) and was below the limit of detection (<5 pg DNA/100 μg of rBoNTE(H_c)). N-terminal sequencing determined the PDS had an intact N-terminus (Table 2).

Mouse potency assay

A mouse bioassay was used to determine rBoNTE(H_c) vaccine potency. Material from the second production runs was used for vaccinations. Potency assay results (Fig. 7) showed protection to be centered, with an ED_{50} (effective dose protecting half the mice) of 190 ng, having 95% confidence limits of 38–678 ng. The potency of this rBoNTE(H_c)

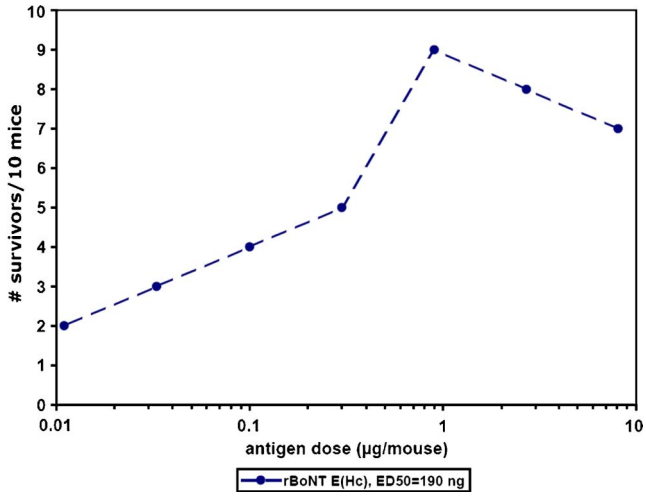


Fig. 7. Potency plot. Plot of the number of surviving mice vs. the microgram dosage of rBoNTE(H_c).

preparation is roughly equivalent to potencies of other rBoNT vaccines, which range from 89 to 116 ng [19].

Conclusion

The production and purification of recombinant heavy chain fragment C of botulinum neurotoxin serotype E from *P. pastoris* GS115 using the process described above has been scaled-up and resulted in similar product purity at both the bench and pilot scales. The rBoNTE(H_c) was most stable stored at a pH of 4.0 in 15 mM succinate. During the HCIC purification step, in the future it is recommended that SCX-HPLC should be used as an in-process assay to adequately determine the proper fraction selection, to avoid including the lower molecular contaminant in the PDS. During these studies our objective was to use peak shape to determine fraction selection. Though this works, to optimize yield, in-process analysis is recommended. The purification process required four chromatographic steps with no ultrafiltration steps between each chromatography step. The formulation step required a membrane diafiltration. The product was intact and comparable in potency to other rBoNT vaccines.

Acknowledgments

Research was supported by The Medical Research and Materiel Command Contract No. DAMD17-02-C-0107 and National Institute of Allergy and Infectious Disease Contract No. 1U01 AI 056514-01. We thank the BPDF's Quality Control Group and Analytical Methods Laboratory for sample analysis through out this project. Also, we thank the BPDF's Purification Development Laboratory staff for process development support through out this project.

References

[1] 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.

[2] C.L. Hatheway, Toxigenic clostridia, Clin. Microbiol. Rev. 3 (1990) 66–98.

[3] C. Lamanna, E.R. Hart, Relationship of lethal toxic dose to body weight of the mouse, Toxicol. Appl. Pharmacol. 13 (1968) 307–315.

[4] L.A. Smith, Development of recombinant vaccines for botulinum neurotoxin, Toxicon 36 (1998) 1539–1548.

[5] C.C. Shone, P. Hambleton, J. Melling, Inactivation of *Clostridium botulinum* type A neurotoxin by trypsin and 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.

[6] L.L. Simpson, Molecular pharmacology of botulinum toxin and tetanus toxin, Annu. Rev. Pharmacol. Toxicol. 26 (1986) 427–453.

[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. DasGupta (Ed.), Botulinum and Tenanus Neurotoxins: Neurotransmission and Biomedical Aspects, Plenum Press, New York, 1993, pp. 334–360.

[8] C. Montecucco (Ed.), Clostridial Neurotoxins: The Molecular Pathogenesis of Tetanus and Botulism, Curr. Top. Microbiol. Immunol. 195 (1995) 1–278.

[9] L.A. Smith, M. Byrne, Vaccines for preventing botulism, in: M.F. Frin, M. Hallett, J. Jankovic (Eds.), Scientific and Therapeutic Aspects of Botulinum Toxin, Lippincott, Williams & Wilkens, Philadelphia, 2002, pp. 427–440.

[10] M.A. Clayton, J.M. Clayton, D.R. Brown, J.L. Middlebrook, Protective vaccination with a recombinant fragment of *Clostridium botulinum* neurotoxin serotype A expressed from a synthetic gene in *Escherichia coli*, Infect. Immun. 63 (1995) 2738–2742.

[11] 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.

[12] C.L. Hathaway, Toxoid of *Clostridium botulinum* type F: purification and immunogenicity studies, Appl. Environ. Microbiol. 31 (1976) 234–242.

[13] 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.

[14] 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.

[15] T. Kubota, T. Watanabe, N. Yokosawa, K. Tsuzuki, T. Indoh, K. Moriishi, K. Sanda, Y. Maki, K. Inoue, N. Fujii, Epitope regions in the heavy chain of clostridium botulinum type E neurotoxin recognized by monoclonal antibodies, Appl. Environ. Microbiol. 63 (1997) 1214–1218.

[16] V. Chiruvolu, J.M. Cregg, M.M. Meagher, Recombinant protein expression in an alcohol oxidase-defective strain of *Pichia pastoris* in feed-batch fermentations, Enzyme Microbiol. Technol. 21 (1997) 277–283.

[17] 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 *Picha pastoris*, Prot. Exp. Purif. 32 (1) (2003) 1–9.

[18] J. Stratton, V.-J. Chiruvolu, M. Meagher, in: D.R. Higgens, J.M. Cregg (Eds.), Pichia Protocols, Humana Press, Totowa, NJ, 1998, pp. 107–120.

[19] M.P. Byrne, T.J. Smith, V.A. Montgomery, L.A. Smith, Purification, potency, and efficacy of the botulinum neurotoxin type A binding domain from *Pichia pastoris* as a recombinant vaccine candidate, Infect. Immun. 66 (10) (1998) 4817–4822.

[20] J.H. Anderson, G.E. Lewis, Clinical evaluation of botulinum toxoids, in: G.E. Lewis (Ed.), Biomedical Aspects of Botulism, Academic Press, New York, 1981, pp. 233–246.