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Production and Purification of the Heavy Chain Fragment C of Botulinum Neurotoxin, Serotype A, Expressed in the Methylotrophic Yeast *Pichia pastoris*[1]

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A recombinant H_C fragment of botulinum neurotoxin, serotype A (rBoNTA(H_C)), has been successfully expressed in a Mut$^+$ strain of the methylotrophic yeast *Pichia pastoris* for use as an antigen in a proposed human vaccine. Fermentation employed glycerol batch, glycerol-fed batch, and methanol-fed batch phases to achieve high cell density. Induction times were short to maximize rBoNTA(H_C) production while minimizing proteolytic degradation. Concentration of rBoNTA(H_C) in yeast cell lysates was generally 1–2% of the total protein based on ELISA analysis. The H_C fragment was purified from cell lysates using a multistep ion-exchange (IEC) chromatographic process, including SP, Q, and HS resins. The zwitterionic detergent Chaps was included in the buffer system to combat possible interactions, such as protein–protein or protein–DNA interactions. Following IEC was a hydrophobic interaction chromatography (HIC) polishing step, using phenyl resin. The H_C fragment was purified to >95% purity with yields up to 450 mg/kg cells based on ELISA and Bradford protein assay. The purified H_C fragment of serotype A was stable, elicited an immune response in mice, and was protected upon challenge with native botulinum type A neurotoxin.

The bacteria *Clostridium botulinum* produces seven serologically distinct forms (A–G) of botulinum neurotoxin which are similar in structure (1). In humans, these neurotoxins cause acute toxicity, which results in fatal paralysis of respiratory muscles (2). Food poisoning is the familiar source of human botulism. However, the common occurrence of infant botulism (3) and the use of agents such as botulinum toxin in biological warfare (4) have heightened the need for safe, inexpensive, and efficacious vaccine against all seven serotypes. If successful, a recombinant subunit vaccine could potentially replace the current formalin-inactivated pentavalent (types A–E) toxoid vaccine as a safer alternative.

Botulinum neurotoxin is processed from a 150-kDa precursor to a dimer consisting of light chain (~50 kDa) and heavy chain (~100 kDa) subunits, connected by a single disulfide bridge (5,6). The C-terminal half of the heavy chain (the H_C fragment)[2] functions in binding of the toxin (7–9) to specific receptors on peripheral

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[2] Abbreviations used: AOX1, alcohol oxidase 1; ABTS, 2,2′-azinodi-(3-ethylbenzthiazoline-6-sulfonate); BSM, basal salts medium; Chaps, (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate); CV, column volumes; DAB, diaminobenzidine; DO, dissolved oxygen; DV, diafiltration volumes; EBA, expanded-bed absorption chromatography; ED_{50}, efficacious dose to protect 50% of test animals; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FT, flowthrough fraction; H_C, heavy chain C-terminal fragment; H_N, heavy chain N-terminal fragment; HIC, hydrophobic interaction chromatography; HRP, horseradish peroxidase; HS, high-density sulfonic acid; i.d., inner diameter; IEC, ion-exchange chromatography; IgG, immunoglobulin G; LD_{50}, lethal dose to kill 50% of test animals; Mops, 3-(*N*-morpholino)propanesulfonic acid; MW, molecular weight; MWCO, molecular weight cutoff; OD, optical density; PBS, phosphate-buffered saline; PBS-TT, phosphate-buffered saline with Tween 20 and thimerosal; p*I*, isoelectric point; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; Q, quaternary amine; rBoNTA(H_C), recombinant botulinum neurotoxin, serotype A, heavy chain C-terminal fragment; RDB, regeneration dextrose medium with biotin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SP, sulfopropyl; TP, total protein concentration; YNB, yeast nitrogen base medium; YPD, yeast extract peptone dextrose medium.

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cholinergic nerve cells (10). The N-terminal half of the heavy chain (H_N) forms channels in phospholipid membranes (11,12) and facilitates internalization (13) of the light chain, a zinc-endopeptidase (14,15) which targets nerve cells (16). While the complete holotoxin is required for biological toxicity, the H_C fragments alone are nontoxic and antigenic (17,18) and have demonstrated the ability to elicit a protective immune response in animals challenged with native botulinum toxin (19–22). Thus, investigations are ongoing to develop a multivalent vaccine against botulism using the H_C fragments of *C. botulinum* neurotoxin (21–26). A recombinant C fragment botulinum neurotoxin produced in *Escherichia coli* has been shown to elicit an immune response in mice (19). Previously, recombinant H_C fragment of serotypes B and F have been expressed in the methylotrophic yeast *Pichia pastoris* and purified to homogeneity (21,27). This paper similarly describes the successful expression, purification development, and pilot-scale purification of the H_C fragment of botulinum neurotoxin serotype A (rBoNTA(H_C)) from *P. pastoris.*

Methylotrophic yeast can utilize methanol for growth and energy (28–30) and *P. pastoris* has been used extensively as a host system for the expression of foreign genes (31–39). Intracellular expression of a synthetic gene coding the H_C fragment of rBoNTA is possible by integration into the *AOX1* gene of the Mut^+ strain (39–43) of *P. pastoris.* Switching the cell's carbon source from glycerol to methanol activates the *AOX1* promoter (44–46) and induces production of rBoNTA(H_C) in *Pichia.* A complete discussion of a model describing rBoNTA(H_C) production in *P. pastoris* is described elsewhere (47).

MATERIALS AND METHODS

Expression vector construction and cell culture. A recombinant strain of *P. pastoris* [GS115-(*his4*)] was transformed with the integrative plasmid pHIL-D4 (a gift from the Phillips Petroleum Co., Bartlesville, OK) containing a synthetic gene encoding the putative fragment C region (heavy chain fragment) of botulinum toxin type A. Integration was performed with an *Sst*I-digested construct that targets integration by single crossover in the chromosomal *AOX1* locus of *P. pastoris* such that the gene remains intact as the Mut^+ phenotype. Transformants were selected for conversion to His^+ selection on RDB medium (48) containing 20 g/L agar and then further selected for high copy number by their ability to grow on YPD medium (48) containing the antibiotic G418. Seed cultures of the selected transformant, designated as BAC-C-09056-A9 [BoNTA(H_C)/pHIL-D4], were prepared in YPD medium containing 15% glycerol and stored at −80°C.

TABLE 1

Methanol Feed Profile during 60-L Fermentation

Time, h	Methanol feed rate, mL L^{-1} h^{-1}
0–2.5	5.0
2.5–4.9	7.7
4.9–6.3	8.8
6.3–9	11.3

Fermentation. Frozen stock seed culture of *P. pastoris* was grown at 30°C to an A_{600} of five to six in shake flasks containing 200 ml of YNB plus phosphate medium (13.4 g/L yeast nitrogen base without amino acids, 20 g/L glycerol, 0.4 mg/L biotin, in 100 mM potassium phosphate buffer, pH 6.0). The shake flask culture was used to inoculate a 5-L BioFlo III seed fermentor (New Brunswick Scientific, Edison, NJ) containing 4% glycerol in 4 L basal-salt medium (BSM) (48) plus 2 mL/L of PTM_4 trace mineral salts. Dissolved oxygen (DO) was maintained at 40%, and pH was controlled at 5 using 30% ammonium hydroxide. The culture was grown to an OD of 35 at 600 nm. Large-scale fermentation was conducted with a starting volume of 45 L of BSM plus 2 ml/L PTM_1 trace mineral salts, in a 60-L New Brunswick Scientific MPP-80 fermentor. The 60-L fermentor was inoculated with 3.5 L of the seed inoculum. The culture was grown to an OD of 57 at 600 nm, at which time the DO spiked indicating glycerol exhaustion. A 50% (w/v) glycerol solution was then fed into the fermentor at a rate of 20.2 ml L^{-1} h^{-1} for 60 min. The glycerol feed was then linearly reduced to zero over the next 3 h. After 1.5 h, when a glycerol feed rate of 10.0 ml L^{-1} h^{-1} was obtained, the methanol feed was started, beginning the induction phase. A solution of 100% methanol plus 1.2% PTM_1 was added at a rate of 5.0 ml L^{-1} h^{-1}. The methanol feed rate was increased over time to a final induction time of 9 h, according to the profile in Table 1. The final volume of the broth was 55 L with an OD of 165. Cells were harvested by centrifugation using a Westfalia SA-7 disk stack centrifuge (Centrico, Inc., Northvale, NJ) and then stored frozen at −40°C until use.

Cell disruption. Frozen cells were suspended into cold (4°C) lysis buffer (50 mM Mops, 0.25% Chaps, 50 mM NaCl, 5 mM EDTA, 2 mM PMSF, pH 7.5) at 200 g/L and disrupted at 20,000 psig with five passes through a Microfluidics M-110EH homogenizer (Microfluidics, Corp., Newton, MA) equipped with a heat exchanger for cooling. The lysate was centrifuged (Beckman, Palo Alto, CA) at 12,000*g* for 25 min to pellet cell debris. Lysate supernatants were diluted with cold lysis buffer (without PMSF) to a total protein concentration of approximately 5 mg/ml and then filtered through a 0.5-μm filter (Pall, Port Washington,

NY) in preparation for loading onto the SP–Sepharose column, the first chromatography step. For lab-scale evaluations, cells were broken with six 1-min cycles in a bead blender containing 75–80 g cells, 160 ml beads, and 175 ml of lysis buffer. Following centrifugation, the supernatant was diluted with lysis buffer to a protein concentration of approximately 5 mg/ml for loading.

Purification of rBoNTA(H_C) fragment. A 10.0-cm inner diameter (i.d.) BPG chromatography column (Pharmacia Biotech, Uppsala, Sweden), containing 2.0 L of SP–Sepharose Fast Flow cation-exchange resin (Pharmacia), was used in the first purification step. The separation was performed by first equilibrating the resin with 12 column vol (CV) of buffer (50 mM Mops, 50 mM NaCl, 0.25% Chaps, 5 mM EDTA, pH 7.5) at 500 cm/h. The column was loaded with 6.6 L of diluted cell lysate at 5.9 mg/ml total protein concentration, at 100 cm/h. Protein load was 19.5 mg/ml-resin. The column and buffers were maintained at ambient temperature, and the sample was kept on ice during loading. The column was washed with 2.5 CV of equilibration buffer at 100 cm/h, followed by 7.5 CV at 500 cm/h. Product was desorbed by step elution with 6 CV of 300 mM NaCl in equilibration buffer (without EDTA). The column was eluted with 1 M NaCl in buffer and then cleaned with a mixture of 1 M NaOH and 1 M NaCl. The fraction obtained from the SP column was diafiltered at 4°C against 5 vol of 50 mM Mops, 0.25% Chaps, pH 7.5, using 20 ft^2 of 5,000 MWCO PES sheet membrane in a Septaport module (NC SRT, Cary, NC) to decrease the sample conductivity in preparation for the second step.

The second chromatography step was negative purification using Q–Sepharose Fast Flow anion-exchange resin (Pharmacia). A 10.0-cm i.d. BPG column containing 1.4 L of resin was equilibrated with 10 CV of buffer (50 mM Mops, 0.25% Chaps, pH 7.5) at 450 cm/h prior to loading the diafiltered SP product pool. The column and buffers were maintained at ambient temperature, and the sample was kept on ice during loading. After loading was complete, the column was washed with 6 CV of equilibration buffer. The flowthrough fraction containing the rBoNTA(H_C) product was collected. Bound proteins were eluted with a step to 1 M NaCl in Mops buffer. The column was cleaned with a mixture of 1 M NaOH and 1 M NaCl. The Q product fraction was adjusted to 50 mM NaCl in preparation for the third step.

The third chromatography column was a 10.0-cm i.d. column containing 1 L of Poros HS-50 (PE Biosystems, Framingham, MA). The column was equilibrated at 750 cm/h with 50 mM Mops, 0.05% Chaps, 75 mM NaCl, pH 7.5 for 10 CV. The Q product was applied at 750 cm/h. The column and buffers were maintained at ambient temperature, and the sample was kept on ice during loading. A step to 180 mM NaCl in buffer for 8 CV eluted the product. The column was flushed with 1 M NaCl and then cleaned with a mixture of 1 M NaOH and 1 M NaCl.

In the final chromatography step, the HS product fraction was adjusted to 2.6 M NaCl and applied to a 6.0-cm i.d. column containing 700 ml of Phenyl 650C hydrophobic interaction (HIC) resin (TosoHaas, Montgomeryville, PA). The column was preequilibrated with 10 CV of high-ionic-strength buffer (2.6 M NaCl in 50 mM sodium phosphate, pH 7.5) at 500 cm/h. The column and buffers were maintained at ambient temperature, and the sample was kept on ice during loading. After loading was complete, the column was washed with 10 CV of equilibration buffer, and then product was eluted with a step to 50 mM NaCl in phosphate buffer and held for 6 CV. The column was cleaned with a mixture of 250 mM NaOH and 250 mM NaCl.

The HIC product fraction was diafiltered using a 10,000 MWCO, 3-ft^2 spiral-wound cartridge (Millipore, Bedford, MA) against 20 mM sodium phosphate buffer, pH 7.5, to remove residual salt. The product was concentrated to a final concentration of 0.2 mg/mL, aliquotted, and frozen at −70°C for storage.

Total protein analysis. Total protein concentration of samples was determined using the Bio-Rad protein assay, based on the method of Bradford (49) (Bio-Rad Laboratories, Hercules, CA), using bovine serum albumin (Pierce Chemical Co., Rockford, IL) as the reference standard.

SDS–PAGE and Western blot analysis. Samples taken at various stages of purification were analyzed by SDS–PAGE using 4–20% gradient acrylamide gels (Novex, San Diego, CA) based on the method described by Laemmli (50). Samples were mixed with SDS–PAGE sample buffer containing β-mercaptoethanol as a reducing agent. Gels were stained with either Coomassie brilliant blue (Sigma Chemical Co., St. Louis, MO) or silver (Pharmacia). For Western blot analysis, the gels were transferred to a PVDF membrane (Bio-Rad) at 100 V for 1.25 h. Blots were incubated in 5% nonfat dry milk for 1 h at ambient temperature, washed with 0.05% Tween 20 in phosphate-buffered saline, and then treated with the primary antibody, a rabbit polyclonal anti-BoNTA(H_C) in PBS containing 5% goat serum for 1 h at ambient temperature. Blots were again washed and then incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (Kirkegaard & Perry Laboratories, Inc. (KPL), Gaithersburg, MD) diluted 1/5000 in PBS containing 5% goat serum for 1 h. Immunoreactive proteins were visualized by metal-enhanced DAB substrate (Pierce).

Enzyme-linked immunosorbent assay (ELISA). Samples taken at various stages of purification were

TABLE 2

Storage Time and Cell Breakage of *Pichia pastoris* Cells, Lot No. AR-FPP-003-G

Run name	Cell Age		Percentage of disruption					Lysate TP Concn (mg/ml)	TP released (mg/g cells)
	Weeks	Months	Pass 1	Pass 2	Pass 3	Pass 4	Pass 5		
003G	2	0.5	n.d.[a]	n.d.	87%	n.d.	94%	14.2	90
PPP-003	17	4.0	44%	74%	83%	85%	91%	12.9	86
SP-Q	19	4.4	n.d.	n.d.	n.d.	90%	96%	8.8	75
PPP-004	30	7.0	n.d.	n.d.	67%	84%	n.d.	8.7	62
PPP-004.1	30	7.0	42%	51%	58%	72%	81%	7.8	60
PPP-005	34	7.9	41%	54%	56%	66%	66%	5.9	50

[a] n.d., not determined.

analyzed by a sandwich ELISA. Ninety-six-well microtiter plates were coated with a mouse anti-BoNTA(H_C) monoclonal capture antibody (0.5 μg/ml) at 4°C overnight. Plates were washed with carbonate buffer (100 mM sodium carbonate/bicarbonate, pH 9.6) plus 0.05% (w/v) Tween 20 and then blocked with 5% nonfat dry milk in carbonate buffer for 2–4 h at 37°C. Plates were again washed. Samples were diluted with 5% nonfat dry milk plus 0.05% Chaps in carbonate buffer and then added to wells and incubated at 37°C for 1.5 h. Plates were washed, and 2 μg/ml horse anti-BoNTA(H_C) polyclonal antibody was added to wells and incubated at 37°C for 1 h. Plates were washed with phosphate-buffered saline plus 0.05% Tween 20 and 0.01% thimerosal (PBS-TT). Plates were then incubated with 1 μg/ml goat anti-horse IgG(H+L) horseradish peroxidase-labeled antibody (KPL) at 37°C for 1 h. Plates were washed with the PBS-TT solution and incubated with ABTS peroxidase substrate solution (KPL) at 37°C until darkest standard was at 2 AU. A 2% oxalic acid solution was added to stop color development, and color was measured using the MRX plate reader with Revelation software (Dynex Technologies, Inc., Chantilly, VA). Samples were quantified against a standard curve prepared from purified rBoNTA(H_C) fragment.

Dose response. Ten mice per group were vaccinated with purified rBoNTA(H_C). Seven groups were injected with 11 ng per mouse, 33 ng per mouse, 100 ng per mouse, 300 ng per mouse, 900 ng per mouse, 2.7 μg per mouse, or 8.1 μg per mouse. The control group of animals received diluent only (0.2% Alhydrogel in 0.8% saline with 0.8% benzyl alcohol as a preservative). Vaccines were administered intramuscularly in the hind leg muscle in a volume of 100 μl. Animals were challenged at 21 days postvaccination with 10^3 mouse LD_{50}/ml botulinum type A toxin diluted in gel–phosphate buffer (0.4% dibasic phosphate with 0.2% gelatin), delivered intraperitoneally. Mice were observed daily for 5 days postchallenge.

Amino-terminal sequence analysis. The amino-terminal sequence of recombinant rBoNTA(H_C) fragment was determined by automated Edman degradation performed on a Procise Model 491-HT amino acid sequencer (PE Biosystems).

RESULTS AND DISCUSSION

Pilot-scale fermentation and purification processes and supporting lab-scale development work are presented for isolating rBoNTA(H_C) from *P. pastoris.* During fermentation, the rBoNTA(H_C) content in cells increases with induction time, until a plateau is reached, after which the rBoNTA(H_C) concentration decreases quickly (47). The ratio of intracellular expressed rBoNTA(H_C) to total protein drops after 10–12 h of methanol induction, resulting in a shorter induction phase than is typical for expression in *Pichia.* Cells were harvested from the fermentor after 9 h of methanol feeding to maximize yield of the rBoNTA(H_C) product. Expression of rBoNTA(H_C) in *Pichia* was successful in both 5- and 60-L fermentations, and whole cells were stored at −30°C from time of harvest until use. A 20% suspension of frozen cells stored less than 6 months and disrupted at 20,000 psig resulted in 90–95% cell breakage based on microscopic inspection. Cell lysis typically yielded at least 70 mg total protein (TP) per gram of wet cell weight after short-term storage. Several runs (lab and pilot scale) were performed using the same lot of cells over an 8-month period. Analysis of cell lysate protein concentration and percentage of breakage of cells revealed that long-term storage of cells severely affects protein yield and lysis efficiency (Table 2). This is likely due to dehydration of cells and resultant precipitation of protein during frozen storage. Ideally, the cells should be processed immediately for highest rBoNTA(H_C) yield, but at maximum, frozen cells should be processed within 3 to 4 months of harvest.

Purification of rBoNTA(H_C) fragment. The purification scheme described herein has repeatedly pro-

duced product of 95% purity based on silver-stained SDS–PAGE and 98% purity based on Coomassie-stained SDS–PAGE, with final product yields ranging from 100 to 450 mg/kg of wet cell mass based on Bradford protein assay. Product stability increased with subsequent processing steps and cold storage of the Poros HS fraction pool (post-third step) proved acceptable for short periods. Expedient processing of the antigen after cell lysis was found imperative to product integrity. Initially, there was significant loss of product to the pellet upon centrifugation, the flowthrough fraction of the S column, and the bound fraction on the Q column. These observations were opposite to the expected chromatographic behavior of the protein based on its basic isoelectric point (p*I* 9.3). Product loss was ascribed to suspected protein–protein and/or protein–DNA interactions occurring between the positively charged rBoNTA(H_C) fragment and negatively charged cell components; therefore, zwittergent 3-10 and 3-12 detergents (Calbiochem, La Jolla, CA) and the zwitterionic detergent Chaps (Calbiochem) were tested for their ability to break up these interactions. All were successful in combating the unwanted interactions. Of the three, Chaps was reported to be less denaturing to protein, so it was selected as a buffer additive.

During initial screening, several resins of SP functionality were tested, including Streamline (Pharmacia) expanded bed absorption chromatography (EBA). EBA however was not selected as a capture step for this protein because much of the product was lost to the flowthrough under the conditions tested (pH screening from 5 to 7 using various buffers and NaCl concentrations). Therefore, conventional chromatography was employed for capture. Initially, Poros HS resin (PE Biosystems) was used in the first step and the rBoNTA(H_C) product was eluted with a gradient. This resulted in an elution profile of two fused peaks. The two peaks were fractionated and processed separately and determined there was no difference in the final product recovered from each peak. Product was present in both of the peaks, and resolution of these two peaks was not deemed necessary since contaminants were removed downstream. To decrease cost, the process was changed to use SP–Sepharose resin (Pharmacia) with step elution of the product. The SP–Sepharose resin also removed a contaminating 50-kDa band, nonreactive in Western blots.

Loading study. A loading study based on total protein loaded per milliliter of resin was performed using SP–Sepharose FF resin. The SP–Sepharose resin was evaluated for protein load capacity, as determined by breakthrough of rBoNTA(H_C) product in the flowthrough (FT) fraction. A total of 50 mg/ml-resin was loaded onto a 108-ml column (2.7 × 19 cm) at 100 cm/h and fractions were collected, each of which represented approximately 2.5 mg/ml-resin loaded. The running buffer was 50 mM Mops, 50 mM NaCl, 0.25% Chaps, pH 7.5, and column was eluted with a linear gradient to 250 mM. Product that eluted from the SP column was further processed using Q, HS, and HIC resins; final yield was 29.1 mg TP (375 mg/kg cells). The cutoff for minimal loss of product to FT was 20 mg/ml-resin; however, at 50 mg/ml-resin loss was less than 10%. A positive Western signal was detected in all flowthrough fractions, and immunoreactive bands at 50, 36, and 17 kDa were detected, indicating degradation. Under these conditions, 4% of the total protein loaded was recovered in the elution peak, and the product pool was approximately 25% rBoNTA(H_C) fragment based on SDS–PAGE.

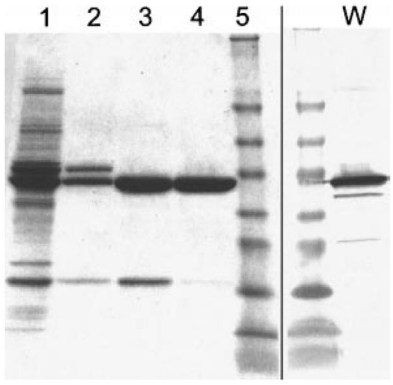

FIG. 1. Coomassie-stained SDS–PAGE gel of the process fractions. Lane 1, SP product; lane 2, Q product; lane 3, HS product; lane 4, final product; lane 5, MW standards, from bottom, 6, 16, 30, 36, 50, 64, 98, and 250 kDa. Lane W, a Western blot of the product fraction probed with polyclonal rabbit anti-rBoNTA(H_C) antibody and detected by HRP–DAB.

SP cation-exchange chromatography efficiently captured the rBoNTA(H_C) fragment and with a large proportion of contaminating proteins flowing through the column. Based on an evaluation of flow velocity and protein concentration of the load material, maximum capture of product was achieved when total protein concentration was less than 5 mg/ml and load velocity was relatively slow at 100 cm/h. At pilot scale, the rBoNTA(H_C) fragment was detected in the flowthrough fraction at less than 5% of the load based on ELISA. The SP product fraction contained approximately 25% H_C fragment based on ELISA and SDS–PAGE (Fig. 1, lane 1).

The second chromatography step was a negative purification step using Q–Sepharose FF (Pharmacia). The rBoNTA(H_C) fragment is a basic protein with a p*I* of 9.3 and is present in the column flowthrough. This step removed many contaminants, some possibly associated with the product (Fig. 1, lane 2). Bound contaminants were eluted from the Q resin with 1 M NaCl for analysis. A small portion of product was present in the Q elution fraction, determined less than 5%.

The third chromatography step utilized a Poros HS column, which removes a prominent higher molecular weight band from the product, as well as other less discernible bands. Analysis of the HS fractions by SDS–PAGE showed approximately 90% purity after this step. The product band (50 kDa) which is immunoreactive by Western blot coelutes with a 17-kDa protein (Fig. 1, lane 3).

Addition of Chaps to buffers. Due to high losses of product to the flowthrough fraction during initial capture from lysate supernatants, it was speculated that there was an interaction occurring between the rBoNTA(H_C) and other proteins or nucleic acid. As mentioned previously, zwitterionic detergents were tested for their effect, and Chaps proved both to be nondenaturing and to have prevented product loss. Thus, Chaps facilitated the increased release of product from the cells and complete capture by the cation-exchange resin. Optimization of the capture step was performed using 0.05% Chaps in lysis and running buffers and included evaluation of lysate protein concentration and load velocity, both at 10 mg/ml resin on a 1.6-ml Poros HS column. There was no difference when 1 or 5 mg/ml lysate protein concentration was loaded onto the column at 10 mg/ml-resin, 400 cm/h load velocity, when comparing loss to flowthrough on the HS column. Both resulted in 17–18% product loss based on ELISA. Load velocities of 100, 200, 300, and 400 cm/h were tested and lower load velocities resulted in increased product yield. Therefore, a load velocity of 100 cm/h was selected.

Chaps concentration was varied in the lysis buffer to see if increasing the Chaps would facilitate release of higher rBoNTA(H_C) levels from cells into the supernatant fraction. Cells were lysed at small scale (1-ml bead beater samples) in Mops lysis buffer at 0.05, 0.1, 0.2, and 0.3% Chaps and supernatants were analyzed by ELISA. Varying concentrations of Chaps were added to the lysis and running buffers. The effect of Chaps on product capture was evaluated using a 1.6-ml Poros HS column, loading at 10 mg/ml-resin, and a load velocity of 100 cm/h. The flowthrough fractions were tested for rBoNTA(H_C) by ELISA and final yield calculated based on total protein. Results are summarized in Table 3.

To confirm that the increase in percentage of rBoNTA(H_C) released from cells was a real phenomenon and not due to enhanced antibody–antigen affinity (detected by ELISA) due to Chaps interference, two complete purification runs were done at lab scale starting with a 100-ml HS column to capture at 10 mg/ml-resin, comparing 0.25% Chaps and 0.05% Chaps. Yield was almost doubled using the higher Chaps to break cells and capture rBoNTA(H_C) from supernatant, confirming that increased Chaps concentrations did facilitate release of more rBoNTA(H_C) from cells during lysis. Based on these results, 0.25% Chaps was selected.

TABLE 3

Effect of the Zwitterionic Detergent Chaps on Capture and Yield of rBoNTA(H_C)

Concentration of Chaps (%)	rBoNTA(H_C) to total protein ratio[a] (%)	rBoNTA(H_C) in flowthrough fraction (%)	Final yield of rBoNTA(H_C)
0.05	2.3	17	71 mg/kg cells
0.10	4.5	8	n.d.[b]
0.20	4.9	2	n.d.
0.25	n.d.	n.d.	123 mg/kg cells
0.30	6.0	3	n.d.

[a] Released in supernatant.
[b] n.d., not determined.

Poros HS-50 resin was compared to Pharmacia SP–Sepharose FF resin for the capture step using 100-ml columns, and Poros HQ-50 was compared to Q–Sepharose FF for the second step (80-ml columns). Two runs were done, both using 0.25% Chaps throughout steps 1 and 2 in the process and then using 0.05% Chaps for step 3 (Poros HS, 28 ml), followed by HIC (no Chaps) as step 4. The purpose of using 0.25% Chaps throughout step 2 was to see if it would decrease the amount of rBoNTA(H_C) lost to the Q elution fraction. At pH 7.5 rBoNTA(H_C) would have an expected net positive charge based on its isoelectric point of 9.3, and should flow through the positively charged Q column. However, probable interactions with negatively charged molecules (in the absence of Chaps) were causing Q "binding." The Pharmacia resins yielded 191 mg/kg of rBoNTA(H_C) versus 132 mg/kg with the Poros resins, and final product was of comparable purity. Therefore, the Pharmacia resins were exchanged for Poros in the first two steps in the process. Poros HS resin was still used in the third step, to take advantage of different selectivity between the two cation-exchange resins.

P17 contaminating protein. A contaminating band of approximately 17 kDa was detected in the product fraction by SDS–PAGE from the initial stages of processing and was present upon cell lysis. It was very difficult to separate P17 from the BoNT-A(H_C) fragment, indicating that it had similar chromatographic properties. Running various NaCl gradients on HS chromatography resulted in elution of P17 in the front shoulder of the rBoNTA(H_C) peak, but the two were not able to be resolved.

Diafiltration through 30K membrane. rBoNTA(H_C) product containing P17 was diafiltered using a 30,000 MWCO filter to see if the P17 would pass through while retaining the rBoNTA(H_C) product. Figure 2 shows the results of permeate and retentate samples taken at various times during the diafiltration process.

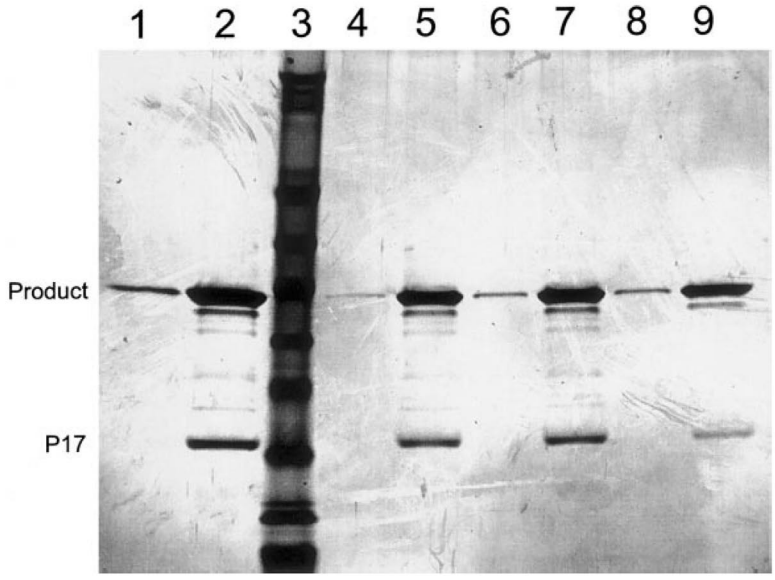

FIG. 2. Silver-stained SDS–PAGE gel where the HS product was diafiltered to test for removal of P17 through a 30-kDa MWCO membrane. Lane 1, permeate at 1 diafiltration volume (DV); lane 2, retentate at 1 DV; lane 3, MW standards, from bottom, 4, 6, 16, 30, 36, 50, 64, 98, and 250 kDa; lane 4, permeate at 2 DV; lane 5, retentate at 2 DV; lane 6, permeate at 3 DV; lane 7, retentate at 3 DV; lane 8, permeate at 4 DV; lane 9, retentate at 4 DV.

The P17 protein was not readily separated, though over time the intensity of the P17 band decreases relative to the product band. P17 that might have passed through the membrane was not detectable by silver-stained SDS–PAGE. There is some, albeit minimal, loss of the product through the membrane as well. The disadvantage of polishing the product to purity by this method was that at higher diafiltration volumes, precipitation of the protein began to occur. Alternately, a fourth chromatography step (HIC) was implemented.

Hydrophobic interaction chromatography. Phenyl HIC was used as a polishing step to separate the 17-kDa protein from the 50-kDa product (Fig. 1, lane 4). Ammonium sulfate and sodium sulfate were both tested and resulted in two resolved peaks at pH 8.5, but due to the volatility of ammonium sulfate at this pH, ammonium sulfate gradients were not reproducible from run to run. A nonvolatile alternative, sodium sulfate, worked well at the high pH; however, clumping and slow dissolution of the salt when mixing buffers was problematic, especially at pilot scale. Sodium chloride was therefore investigated and found to be better at binding the product while selectively excluding P17. Sodium chloride concentrations between 1 and 3 M were tested in 0.2 M increments to find the optimum binding characteristics. At lower NaCl concentrations, some product was lost to the flowthrough, yet at higher concentrations P17 also bound. Optimum concentration was found to be between 2.3 and 2.7 M NaCl. By excluding P17 in the flowthrough fraction, the product

TABLE 4

Pilot Run Purification Table, Lot PPP-005

Purification step	Protein Concn (mg/ml)[a]	Volume (ml)	Total protein (mg)	rBoNTA(H_C) (mg)[b]	rBoNTA(H_C) (%)[c]	Fold purification
Lysate	5.87	6600	38742	371	0.96	1
SP–Sepharose pool	0.294	8650	2543	588	23	24
Diafiltered for Q	0.200	7650	1530			
Q–Sepharose pool	0.055	16800	924	319	35	37
Poros HS pool	0.103	3675	379		90	94
HIC phenyl pool	0.090	2350	212			
Final product pool	0.210	820	180	197	99	103

[a] Based on Bradford protein assay, quantitated from a BSA standard curve.
[b] Mass balance values are affected by an approximate 30% error associated with the ELISA assay.
[c] Based on ELISA and/or SDS–PAGE gel analysis.

could be eluted in a single step to phosphate. Using phosphate as the buffering agent, the eluted product needed only to be concentrated and filtered prior to vialing.

A summary of the recovery process run at pilot scale is presented in Table 4 and Fig. 1. The final yield of pure rBoNTA(H_C) from 785 g of wet cell mass was 172 mg (219 mg/kg). The first SP step was efficient at capturing 95% of rBoNTA(H_C) fragment from cell lysates. As mentioned previously, long-term storage of frozen cells affects yield upon cell lysis. Hence, final rBoNTA(H_C) yield is subsequently affected, compared with a maximum yield of 450 mg of rBoNTA(H_C) per kilogram of wet cell weight (Bradford protein assay) when using fresh cells.

Amino-terminal sequence analysis. Amino-terminal sequencing indicated that the N-terminus of the purified product was 65% intact H_2N-MRLLSTFTEYIK (the expected N-terminal sequence of rBoNTA(H_C)) and 35% H_2N-STFTEYIKNI, a species missing the first four amino acids. The N-terminal sequence of P17 taken off PVDF membrane was H_2N-XVNXQTXKRKDF, which does not match any internal sequence of rBoNTA(H_C). A sequence match for P17 was not found in the database (GenBank) for any catalogued *Pichia* protein, though homology to a chymotrypsin-like protease of *Streptomyces* sp. was suggested.

Final product stability. Several agents were tested as possible storage buffers, including citrate, ascorbate, succinate, phosphate, and glycine. Product, when stored frozen in these buffers ranging in pH from 5 to 10, did not display much banding difference on SDS–PAGE over time (Fig. 3). The purified protein remained stable when frozen at −20°C in sodium phosphate, pH 7.5, buffer. After 4 months, there was no difference in the banding pattern of the product. Stability of the purified antigen did not appear to be significantly dependent upon storage buffer conditions.

The mouse model. An *in vivo* potency assay was necessary to evaluate the effects of manufacture, formulation, storage, and stability of botulinum H_C fragments as vaccine candidates. Since the recombinant product has no catalytic activity, it was assayed by testing the ability of the protein to elicit protective antibodies in mice, and the efficacious dose was (ED_{50}) determined for each batch of rBoNTA(H_C) vaccine. The dose–response ED_{50} curve was reproducible and sensitive to slight changes in potency. Vaccination with one 0.10-μg dose of rBoNTA(H_C) fragment afforded protection from challenge in 60% of the mice (Table 5), 0.30 μg protected 80% of the mice, and 0.90 μg protected 100% of mice challenged. The nontoxic H_C fragment elicits an immune response in mice sufficient to provide protection when challenged with native toxin.

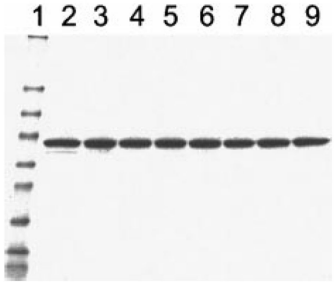

FIG. 3. Coomassie-stained SDS–PAGE gel of purified rBoNTA(H_C) after 8 weeks of storage at −20°C in buffers ranging from pH 5 to 10. Lane 1, MW standards, from bottom, 4, 6, 16, 30, 36, 50, 64, 98, and 250 kDa; lane 2, pH 5; lane 3, pH 6; lane 4, pH 7; lane 5, pH 8; lane 6, pH 9; lane 7, pH 10; lane 8, pH 7 plus NaCl; lane 9, pH 8 plus NaCl.

TABLE 5

rBoNTA(H_C) Challenge Data: Survivors per 10 Mice

Immunization dose (μg/mouse)	Reference Lot 55-153-C (ED_{50} = 0.04961 μg/mouse)	cGMP Lot 022399 (ED_{50} = 0.05272 μg/mouse)
8.1	10/10	10/10
2.7	10/10	10/10
0.90	10/10	10/10
0.30	10/10	8/10
0.10	8/10	6/10
0.033	2/10	4/10
0.011	1/10	2/10

CONCLUSION

Developing a large-scale purification process for the rBoNTA(H_C) was somewhat problematic due to its basic isoelectric point and propensity to interact with negatively charged cell constituents, but incorporating Chaps into the lysis and running buffers alleviated this problem. Using aged cells in later pilot runs resulted in low yield compared to working with fresh cells on lab scale. Information regarding process validation was gained from this project, such as limits on storage of cells and protein stability issues. Despite the laborious purification process, the final pure rBoNTA(H_C) product is stable, elicits protection in mice against native botulinum type A toxin, and is a good candidate for inclusion in a multivalent recombinant subunit vaccine.

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