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Initial purification of recombinant botulinum neurotoxin fragments for pharmaceutical production using hydrophobic charge induction chromatography

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Abstract

Initial purification of two serotypic variants of recombinant botulinum neurotoxin toxin heavy chain fragment [rBoNT(H_c)], produced intracellularly in the yeast *Pichia pastoris*, using hydrophobic charge induction chromatography (HCIC) is reported. HCIC employs a matrix containing a weakly ionizable ligand that binds proteins through hydrophobic interactions at neutral pH and elutes the proteins by charge repulsion at acidic pH. HCIC optimization led to different purification conditions for each of the proteins even though they have 58% sequence similarity. The HCIC resin has a higher affinity for the fragment of serotype A than that of serotype B. The 10% dynamic breakthrough capacity for the serotype A fragment is >12.5 mg per ml of resin and is ~3.5 mg or the serotype B fragment per ml of resin. Stable elution conditions are also different for the two serotypes. The serotype A fragment is unstable when citrate is used to elute the product. However the serotype B fragment is stable when eluted with citrate buffer, and it is further purified by a overnight precipitation caused by the citrate buffer. This paper reports the development strategy, dynamic capacity breakthrough curves, resin and separation reproducibility, and preliminary scale-up data. The summation of the data demonstrates that HCIC is a scaleable process step for biopharmaceutical production of rBoNT(H_c) proteins.

Keywords: Hydrophobic charge induction chromatography; *Pichia pastoris*; Pharmaceutical analysis; Neurotoxins; Proteins; Recombinant proteins

1. Introduction

Botulinum neurotoxins (BoNTs) are the most potent natural toxins known [1]. Seven serologically distinct BoNT (serotypes A–G), produced by the spore forming bacteria *Clostridium botulinum*, *C. baratti*, and *C. butyricum*, have been identified. The toxins are hetero-dimeric proteins consisting of a light chain of M_r approximately 50 000 and a heavy chain of M_r approximately 100 000 joined by a disulfide bond and non-covalent interactions. The C-terminal portion of the heavy chain is required for toxin binding to nerve cell receptors [2]. The N-terminal portion of the heavy chain is a translocation

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domain that facilitates egress of the toxin from endosomes [3]. The light chain is a zinc-dependent serine protease that hydrolyzes, thereby inactivating, proteins associated with the intracellular transport of acetylcholine-containing synaptic vesicles [4]. Botulinum intoxication is then manifested as a flaccid paralysis resulting from the inhibition of neuroexocytosis.

Several cloned fragments of the neurotoxin have been tested for the ability to elicit protective immunity in mice [5–7]. It has been shown that mice vaccinated with a recombinant C-terminal portion of the BoNT heavy chain from serotypes A [rBoNTA(H_c)] and B [rBoNTB(H_c)] were immune to challenge with substantial doses of native toxin [7]. Smith and co-workers have now extended this observation to also include serotypes C_1, E, and F [7–11].

Burton and Harding [12] recently reported a new type of protein chromatography referred to as hydrophobic charge induction chromatography (HCIC). In general, the method employs chromatographic supports derivatized with weakly ionizable hydrophobic ligands. A HCIC matrix based on the work of Burton and Harding [12], comprised of 4-mercaptoethylpyridine (MEP) linked to a highly cross-linked beaded cellulose, has recently become commercially available. The hydrophobic MEP moiety is present at 70–125 μmol/ml, which is a relatively high ligand density as compared to standard HIC matrices. Therefore, moderately to strongly hydrophobic proteins are adsorbed to the MEP matrix at neutral pH and moderate to low ionic strength. Proteins can be desorbed in aqueous solution, despite the high ligand density, by acidifying the mobile phase, which induces a positive charge on the ligand (pK_a=4.8) concomitant with increasing the protonation state of adsorbed proteins. This charge-induced elution from a strongly hydrophobic surface is the unique feature of HCIC chromatography. This resin has been used previously to purify antibodies [13,14] and recombinant alkaline protease variants [15].

Several methods for purification of rBoNTA(H_c) and rBoNTB(H_c) produced intracellularly in *Pichia pastoris* have been published [7–9,11,16]. In our effort to produce biopharmaceutical-grade rBoNT-(H_c) on an industrial scale, we have found HCIC using MEP to be a superior method for initial purification of the rBoNT(H_c) from crude *P. pastoris* cell extracts. Other methods of chromatography (hydrophobic interaction and cation exchange) eluted product that was less pure and less stable than the product fraction from HCIC (data not shown). The current report summarizes the findings of our method development studies with two related but different proteins and demonstrates the utility of this new separation method for the preparative purification of intracellular recombinant proteins from *P. pastoris*.

2. Experimental

2.1. Materials

rBoNTA(H_c) and rBoNTB(H_c) were produced by fermentation of *P. pastoris* expressing the gene for these proteins. The genes were under the AOX1 promoter and induced with methanol for 40 h. The final biomass was 35–40% of the fermentation volume. MEP HyperCel was acquired from Ciphergen (Fremont, CA, USA) and was packed into XK-16 columns for small-scale work and BPG 140 columns for preliminary scale-up work (Amersham Pharmacia Biotech, Piscataway, NJ, USA). All chemicals were USP grade (or equivalent) and purchased from prequalified vendors.

2.2. Column packing

MEP HyperCel columns were packed at pressures near 3 bar with 20% ethanol until the resin would not compact further. The flow adapter was then lowered onto the resin. After packing, the column was sanitized with 0.5 *M* NaOH until the absorbance at 280 nm was stable and $<$0.01 AU.

2.3. Chemical extraction and clarification

The *P. pastoris* cells were extracted by a proprietary cell membrane permeabilization method [17]. The extract supernatant was then clarified by either centrifugation or depth filtration using diatomaceous earth. If the supernatant was clarified by centrifugation, the extract was separated at 4500 *g* for 30 min in a pre-cooled centrifuge at 2–8 °C.

If the supernatant was clarified by depth filtration,

then 0.3 g of CelPure P1000 diatomaceous earth (Advanced Minerals, Lompoc, CA, USA) was added to the extract per gram of cell mass. The slurry was then filtered using a plate and frame filter press at a feed pressure of 40–50 p.s.i. (1 p.s.i.=6894.76 Pa).

After filtration or centrifugation, the clarified supernatant was further clarified by 0.2-μm filtration. The clarified extract was either loaded directly onto a HCIC column or stored at −80 °C. For rBoNTA(H_c), the clarified extract was produced from either cell pellets that had been stored at −80 °C or fresh cells obtained within 4 h of fermentation harvest. For rBoNTB(H_c), only fresh cells were extracted.

2.4. Chromatography runs

All small-scale (~30-ml bed volumes) chromatography was performed using an Äktaexplorer system (Amersham Pharmacia Biotech). The columns (I.D.=1.6 cm) were run at 300 cm/h (10 ml/min) at ambient temperature. The bed heights were 11–18 cm, except some rBoNTA(H_c) experiments used a 2.5 cm bed height for bed height dependence experiments. Separate columns were used for rBoNTA(H_c) and rBoNTB(H_c) purification. Clarified cell extract was loaded directly onto the MEP HyperCel column. Up to 2.5 mg rBoNTB(H_c) or 12.5 mg rBoNTA(H_c) per ml MEP resin was loaded for experiments other than those conducted for the purpose of generating dynamic capacity breakthrough curves. After the chromatography run the resin was regenerated by washing with five column volumes (CVs) of 8 *M* urea or 6 *M* guanidine·HCl. The column was then cleaned in place (CIP) with 5 CVs of 0.5 *M* NaOH. The column was left in 0.5 *M* NaOH for at least 1 h, and a peak was always observed in the 280 nm UV trace when the NaOH was washed out of the column with purified water. Columns were stored in 0.1 *M* NaOH.

Large-scale (~2.5-l bed volumes) chromatography was performed using a 6 mm BioProcess system (Amersham Pharmacia Biotech) and a BPG 140/500 column (I.D.=14 cm, Amersham Pharmacia Biotech). The column was run at 300 cm/h (767 ml/min) through the product elution step. The column was run at=150 cm/h (383 ml/min) during the regeneration and CIP steps so that the column inlet pressure did not exceed 3 bar. The other conditions were the same as for the small-scale chromatography.

2.5. Breakthrough curves

rBoNTB(H_c) dynamic binding capacity was estimated by generating dynamic capacity breakthrough curves. Columns (14.9 cm×1.6 cm) were loaded with 200 ml of clarified extract. The concentrations of rBoNTB(H_c) in the feed (C_o) and in the unbound fractions (C) were determined by analytical hydrophobic interaction HPLC (HPHIC). A breakthrough curve was then constructed by plotting C/C_o vs. total amount of rBoNTB(H_c) loaded per ml MEP resin. A value of 10% dynamic breakthrough ($Q_{B,10\%}$) was chosen as an arbitrary reference value.

rBoNTA(H_c) breakthrough curves were generated essentially the same as for rBoNTB(H_c) except 686 ml of clarified extract were loaded onto a 26.9 ml column (13.4 cm×1.6 cm).

2.6. Regeneration studies

Several column regeneration agents were evaluated by measuring the dynamic binding capacity for rBoNTB(H_c) after the regeneration agent was used following a purification run. The regeneration agents are listed in Table 7. A complete chromatography cycle, except without a regeneration step, including the 0.5 *M* NaOH CIP step was run on a HCIC column. After the column had been exposed to the NaOH for at least 1 h, it was rinsed with purified water. Then a 5-CV linear gradient to 100% of the regeneration agent was run. The column was then flushed with 5 CVs of the regeneration agent, and then another 5-CV linear gradient was run to 100% purified water. The column was then rinsed with 5 CVs of purified water. A dynamic capacity breakthrough curve was then generated as discussed in the previous section. The resulting $Q_{B,10\%}$ was used as a measure of the regeneration efficiency for the given regeneration agent.

2.7. Protein sequence analyses

The sequences of the two proteins were aligned with BLAST2 [18] using the Blosum62 matrix [19]

and the following parameters: gap open penalty, 9; gap extension penalty, 2; gap x_dropoff, 50; expect threshold, 10; wordsize, 3. BLAST2 was operated from the BCM search launcher (http://searchlauncher.bcm.tmc.edu/) [20].

The molecular mass, theoretical isoelectric point (p*I*), and grand average of hydropathicity (GRAVY) were calculated from the primary structure using the ProtParam program on the ExPASy Molecular Biology Server (http://ca.expasy.org/) [21–24].

2.8. Analytical methods

2.8.1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Samples were analyzed by reducing SDS–PAGE. To reduce the samples, the samples contained 1.2% (v/v) 2-mercaptoethanol, and the samples were heated for 3 min at 85 °C. Tris–glycine polyacrylamide gels (4–20%) were used (Invitrogen, Carlsbad, CA, USA). The gels were run for 55 min at 36 mA/gel. Proteins were detected by colloidal Coomassie blue G-250 staining (Invitrogen), and either Mark12 or SeeBlue Plus2 protein standards (Invitrogen) were used.

2.8.2. Hydrophobic interaction HPLC

rBoNTB(H_c) was analyzed by hydrophobic interaction HPLC (HPHIC). A Phenyl-5PW stainless steel column (Tosoh Biosep, Montgomeryville, PA, USA) with column dimensions of 7.5 cm×7.5 mm, 10 μm particle size and 1000 Å pore size was used. The column was eluted with a complex gradient (Table 1) from 1.5 to 0 *M* ammonium sulfate in 50 m*M* sodium phosphate, pH 6.8. The column temperature was 24 °C, and the samples were held at 4 °C until injection. The separation was controlled and monitored by an Agilent 1100 liquid chromatography workstation (Agilent Technologies, Palo Alto, CA, USA). Chromatograms were integrated, and the concentration of rBoNTB(H_c) was calculated from the area under rBoNTB(H_c) peak using the following equation:

$$\text{Area (mAU min)} = 329.35572 \cdot [\text{rBoNTB}(H_c)]\ (\mu\text{g/ml}) - 321.63162$$

This equation was determined by running a standard curve with purified rBoNTB(H_c), the concentration of which was determined by the absorbance at 280 nm using the theoretical extinction coefficient $E^{0.1\%} = 1.94$, that had five points and a correlation coefficient of 0.998. Integration parameters were as follows: slope sensitivity, 1; peak width, 0.4; area reject, 1; height reject, 1.7; shoulders, off. Sometimes a shoulder at the front of the rBoNTB(H_c) that has been identified as a degradation product of rBoNTB(H_c) had to be manually integrated.

2.8.3. Cation-exchange HPLC

rBoNTA(H_c) was analyzed by cation-exchange HPLC (HPCEX). A SP-5PW stainless steel column (Tosoh Biosep) with column dimensions of 7.5 cm× 7.5 mm, 10 μm particle size and 1000 Å pore size was used. The column was eluted with a complex gradient (Table 2) from 0 to 1.0 *M* NaCl in 20 m*M* sodium acetate, pH 5.0. The column temperature was 23 °C, and the samples were held at 4 °C until injection. The separation was controlled and monitored by an Agilent 1100 liquid chromatography workstation (Agilent Technologies). Chromatograms were integrated, and the concentration of

Table 1
Elution gradients for HPHIC analysis of rBoNTB(H_c)[a]

Time (min)	0	3	10	30	31	32	33	35
Buffer B (%)	10	10	77	89	100	100	10	10

[a] These are the series of linear gradients used to elute rBoNTB(H_c) from the analytical HPHIC column. Each time point is the end of a linear gradient from the previous point and the start of a linear gradient to the next point. The column is run at 0.5 ml/min.

Table 2
Elution gradients for HPCEX analysis of rBoNTA(H_c)[a]

Time (min)	0	4	7	25	26	36	37	47
Buffer B (%)	20	20	28	45	100	100	20	20

[a] These are the series of linear gradients used to elute rBoNTA(H_c) from the analytical HPCEX column. Each time point is the end of a linear gradient from the previous point and the start of a linear gradient to the next point. The column is run at 1.0 ml/min.

rBoNTA(H_c) was calculated from the area under the rBoNTA(H_c) peak using the following equation:

$$\text{Area (mAU min)} = 84.35427 \cdot [\text{rBoNTA}(H_c)]\ (\mu\text{g/ml}) - 14.56305$$

This equation was determined by running a standard curve with purified rBoNTA(H_c), the concentration of which was determined by the absorbance at 280 nm using the theoretical extinction coefficient $E^{0.1\%} = 1.71$, that had five points and a correlation coefficient of 0.999. Integration parameters were as follows: slope sensitivity, 1; peak width, 0.4; area reject, 1; height reject, 1.7; shoulders, off.

3. Results

3.1. Protein sequence analyses

The respective theoretical p*I* values for rBoNTA(H_c) and rBoNTB(H_c) calculated from the primary sequences are 9.11 and 7.48. The respective calculated M_r values are 50 964.0 and 52 963.9, and rBoNTA(H_c) is more hydrophobic than rBoNTB-(H_c) with respective GRAVY values of −0.423 and −0.648.

The BLAST2 sequence alignment of rBoNTA(H_c) and rBoNTB(H_c) (Fig. 1) yielded a result of 40% (178/445) sequence identity and 58% (259/445) sequence similarity (i.e., alignment with same or similar reside, for example Ala with Ser) with 5% (26/445) of the residues in gaps. The score was 319 bits, and the expect value was $4 \cdot 10^{-86}$.

3.2. Optimized chromatographic methods for rBoNTB(H_c) and rBoNTA(H_c)

Although rBoNTA(H_c) and rBoNTB(H_c) are structurally similar [8], they interact differently with the HCIC matrix. In optimizing the HCIC methods, two different processes were developed. Table 3 shows the optimized method for each serotype. Figs. 2 and 3 represent typical chromatograms for serotypes A and B, respectively, using the methods outlined in Table 3.

Fig. 1. BLAST2 alignment of the primary structures of rBoNTA(H_c) and rBoNTB(H_c). Residues that are identical or similar using the BLOSUM62 matrix are highlighted in gray. In the space between the sequences, the one letter code for the amino acid is given if the residues are identical, or a line joins the residues if they are similar. A dash in the sequence indicates a gap in the sequence alignment. The first eight residues in rBoNTA(H_c), which are not aligned, are M–R–L–L–S–T–F–T. The first three residues in rBoNTB(H_c) are M–A–N.

Table 3
Purification steps rBoNTB(H_c) and rBoNTA(H_c) on the MEP HyperCel column

Step	rBoNTA(H_c)		rBoNTB(H_c)	
	Buffer	Column volumes	Buffer	Column volumes
(1) Equilibration	25 m*M* NaPi[a], 20 m*M* NaCl, 5 m*M* EDTA, pH 7.3	5	25 m*M* NaPi, 20 m*M* NaCl, 5 m*M* EDTA, pH 7.3	5
(2) Load	Clarified cell extract	Variable	Clarified cell extract	Variable
(3) Wash 1	25 m*M* NaPi, 20 m*M* NaCl, 5 m*M* EDTA, pH 7.3	5	25 m*M* NaPi, 20 m*M* NaCl, 5 m*M* EDTA, pH 7.3	10
(4) Wash 2	Purified water	5–10	N/A	N/A
(5) Product elution	50 m*M* sodium acetate, pH 5.5	10	50 m*M* sodium citrate, pH 4.0	10
(6) Regeneration	8 *M* Urea	5	8 *M* Urea	5
(7) CIP	0.5 *M* NaOH	5	0.5 *M* NaOH	5

[a] NaPi, Sodium phosphate.

The primary differences between the two methods are the water wash and the product elution conditions. The use of a water wash to elute impurities from the hydrophobic matrix in the rBoNTA(H_c) process helps increase the overall purity of the product fraction. Fig. 4 shows the SDS–PAGE results for the fractions from a representative rBoNTA(H_c) purification. This gel shows that the unbound and water wash fractions contained a number of protein impurities; however, rBoNTA(H_c) was not detected.

A water wash was investigated for rBoNTB(H_c) purification and approximately 20% of the rBoNTB(H_c) loaded onto the column was eluted during the water wash as determined by HPHIC. Therefore, to maximize product recovery a water wash was not incorporated into the optimized rBoNTB(H_c) method.

3.3. Elution buffers

Sodium acetate and sodium citrate were investigated as product elution buffers for both serotypes. Elution buffer selection was based on the purity and stability of the rBoNT(H_c) product fraction. To obtain a similar product recovery, the pH of the elution buffer had to be lower when using citrate to elute the rBoNT(H_c). For rBoNTB(H_c), acetate could be used at pH 4.75 for efficient elution, but the

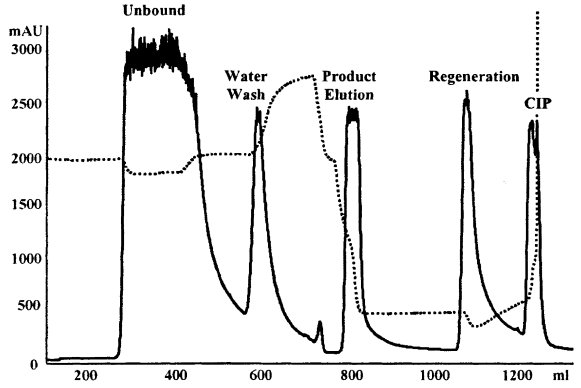

Fig. 2. Chromatogram of an acetate elution of rBoNTA(H_c). Solid line is the 280 nm UV trace and the dotted line is the pH trace. The scale for the pH trace is 3.0–10.0. The 16.2 cm×1.6 cm XK column (Amersham Pharmacia Biotech) was loaded with 130.4 ml of clarified extract. This load contained 5.18 mg rBoNTA(H_c) per ml of MEP resin. The column was run at 300 cm/h at ambient temperature. The unbound peak is the peak from both the load and wash 1 steps.

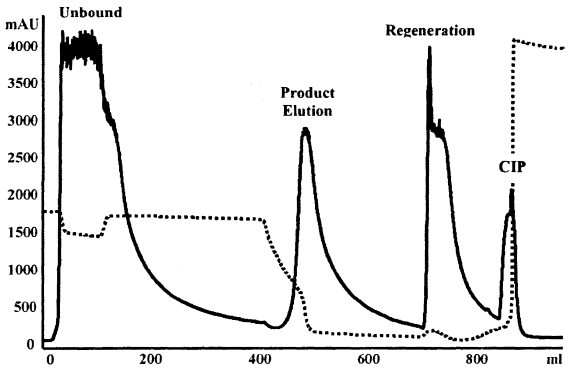

Fig. 3. HCIC chromatogram for a citrate elution of rBoNTB(H_c). Solid line is the 280 nm UV trace and the dotted line is the pH trace. The scale for the pH trace is 4.0–13.0. The 14.6 cm×1.6 cm XK column (Amersham Pharmacia Biotech) was loaded with 75 ml of clarified extract. This load was 1.6 mg rBoNTB(H_c) per ml of MEP resin. The column was run at 300 cm/h at ambient temperature. The unbound peak is the peak from both the load and wash 1 steps.

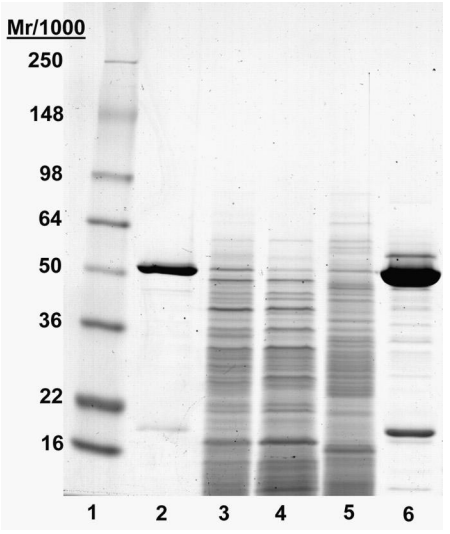

Fig. 4. Reducing SDS–PAGE (4–20%) of fractions taken from a rBoNTA(H_c) MEP run using an acetate elution. Lanes: 1=15 μl of SeeBlue Plus 2 protein standard (Invitrogen); 2=highly purified rBoNTA(H_c) reference sample loaded to 1.4 μg; 3=clarified cell extract loaded to ~5 μg of total protein; 4=unbound fraction ~5 μg of total protein; 5=second (water) wash fraction ~5 μg of total protein; 6=product elution fraction loaded to 10 μg of total protein. The gels were run at 35 mA per gel for 55 min. The gel was stained with colloidal Coomassie G-250 stain (Invitrogen).

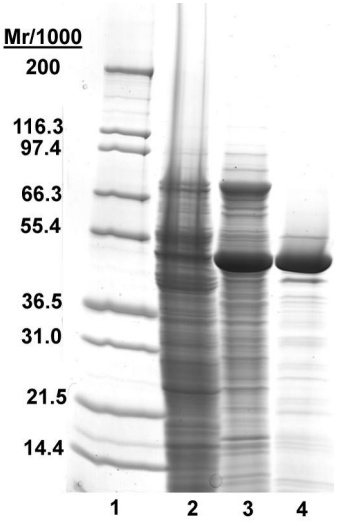

Fig. 5. Reducing SDS–PAGE (4–20%) of rBoNTB(H_c) HCIC purification. Lanes: 1=20 μl of Mark12 wide range protein standard (Invitrogen); 2=clarified cell extract; 3=product elution fraction before precipitation; 4=product elution fraction after precipitation, pH adjustment and clarification. All lanes have 5 μg of rBoNTB(H_c) loaded. The gels were run at 35 mA per gel for 55 min. The gel was stained with colloidal Coomassie G-250 stain (Invitrogen).

pH was 4.0 for efficient elution with a citrate buffer. Sodium citrate also caused the product elution fraction to precipitate. In the case of rBoNTB(H_c), the precipitation resulted in further purification, and the stability of the purified product to proteolysis is much greater for the product of a citrate elution as opposed to an acetate elution. Fig. 5 shows the SDS–PAGE analysis of the citrate product elution fraction before and after precipitation. Table 4 shows the purity and stability of rBoNTB(H_c) after eluting from columns using acetate or citrate elution buffers. As shown in Table 5, the precipitate does not contain rBoNTB(H_c) since the rBoNTB(H_c) concentration is the same before and after precipitation.

There is protease activity in the clarified extract that degrades rBoNTB(H_c) if the protease(s) are active after HCIC. The first degradation product to appear is a rBoNTB(H_c) truncation that is missing the first five N-terminal amino acids. The amount of N-5 degradation product can be followed by HPHIC analysis. The protease activity does not degrade rBoNTB(H_c) in the cell extract and is more active at higher pH in the HCIC eluate. It is critical that the protease activity is removed from the product fraction because it is difficult to remove later, and it is also difficult to separate the N-5 degradation product from the intact protein. The protease activity is

Table 4
Comparison of purity and stability data from rBoNTB(H_c) experiments with acetate and citrate elutions

Elution buffer	rBoNTB(H_c) purity[a] (%)	rBoNTB(H_c) stability[b] (%)
Acetate	41.3	84.3
Acetate	52.4	86.5
Acetate	38.9	50.7
Citrate	63.1	>100
Citrate	72.7	98.0
Citrate	79.2	>100

[a] Percentage of rBoNTB(H_c) peak area relative to total peak area from HPHIC analysis before overnight precipitation for citrate elutions.

[b] Percentage of intact rBoNTB(H_c) remaining after overnight incubation at 4 °C from the amount present immediately after elution.

Table 5
Concentrations of rBoNTB(H_c) in HCIC eluates from representative experiments using citrate buffer before and after precipitation

[rBoNTB(H_c)] before precipitation (μg/ml)[a]	[rBoNTB(H_c)] after precipitation (μg/ml)[b]	Change (%)
148.5	149.7	+0.8
291.8	286.1	−2.0
149.8	156.9	+4.7

[a] rBoNTB(H_c) concentrations were measured by HPHIC. The product elution fraction was immediately filtered through a 0.2 μm syringe filter (Pall Filtron, Northborough, MA, USA), to protect the HPLC system, and analyzed by HPHIC to determine the before precipitation value.

[b] The product elution fraction was allowed to precipitate overnight at room temperature. The pH of the product was then adjusted to 5.5 with 1.5 *M* sodium citrate, filtered through a 0.2 μm filter, and analyzed by HPHIC to determine the after precipitation value.

removed or deactivated, along with other host cell protein impurities, during the citrate induced precipitation. If the citrate eluate is adjusted to a higher pH before the precipitation is complete, then formation of the N-5 degradation product is observed. However, if the eluate is allowed to precipitate completely (⩾9 h at room temperature) then a pH increase will not lead to product degradation. Comparatively, a precipitate was not formed when rBoNTB(H_c) was purified with an acetate elution, and such material was proteolytically degraded at the pH used for product elution, and the rate of N-5 formation increased with increasing pH. Table 6 shows a comparison of the amount of rBoNTB(H_c) degradation observed in acetate and citrate elutions.

For rBoNTA(H_c), acetate is the superior product elution buffer. rBoNTA(H_c) degrades under the citrate elution conditions and is stable under acetate elution conditions. Fig. 6 shows the HPCEX analysis of product elutions using both buffers. The multiple peaks in the analysis of the citrate product eluate is indicative of product degradation, which is minimal for the acetate elution.

3.4. Binding capacity

The dynamic binding capacity of the MEP HyperCel resin for the two serotypes was very different. The $Q_{B,10\%}$ for rBoNTB(H_c) is 3.2–3.5 mg/ml of resin (Fig. 7), and the $Q_{B,10\%}$ for rBoNTA(H_c) is >12.5 mg/ml (Fig. 7).

Table 6
Stability and degradation data for rBoNTB(H_c) representative experiments conducted with acetate and citrate elution buffers

Elution buffer	Overnight stability at 4 °C[a] (%)	[N-5]/[intact protein]	
		$T=0$[b]	$T=$ overnight[c]
Acetate	92	0.04	0.02
Acetate	71	0.11	0.14
Acetate	69	0.04	0.23
Acetate	81	0.16	0.09
Acetate	84	0.05	0.08
Acetate	89	0.08	0.13
Citrate	104	nd[d]	0.03
Citrate	100	nd	nd
Citrate	101	nd	0.01
Citrate	90	0.01	0.02
Citrate	105	nd	0.02

T = time.

[a] The percentage of intact rBoNTB(H_c) remaining after overnight incubation at 4 °C from the amount present immediately after elution from the column.

[b] Concentration of the N-5 truncation of rBoNTB(H_c) divided by the concentration of the intact protein as measured by HPHIC within a few hours of elution from the column.

[c] Concentration of the N-5 truncation of rBoNTB(H_c) divided by the concentration of the intact protein as measured by HPHIC after overnight incubation at 4 °C.

[d] nd, N-5 truncation was not detected.

Dynamic binding capacity was also used to estimate resin reproducibility as a function of cycle number. The $Q_{B,10\%}$ for rBoNTB(H_c) was measured

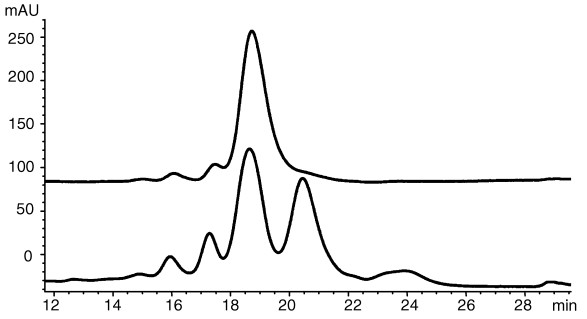

Fig. 6. HPCEX chromatograms of the acetate eluate (top) and the citrate eluate (bottom) for rBoNTA(H_c). The *y*-axis is offset for the two chromatograms for clarity. The peak at 19 min is the desired product. The other peaks are degradation products and misfolds.

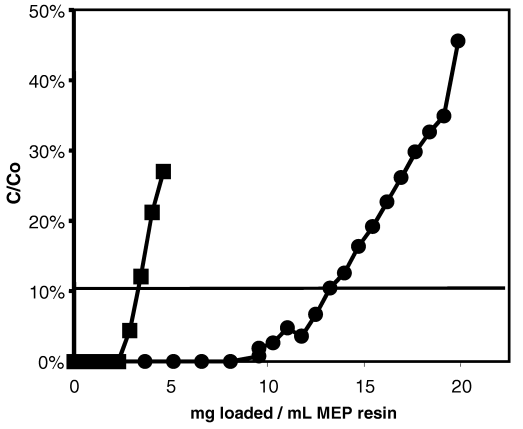

Fig. 7. Comparison of breakthrough curves for rBoNTB(H_c) (squares) and rBoNTA(H_c) (circles).

periodically over the course of 30 cycles on an MEP HyperCel column (Fig. 8). The binding capacity did not systematically change over the 30 cycles.

3.5. Ionic strength of buffers

3.5.1. rBoNTB(H_c) equilibration buffer

The ionic strength of both the equilibration and elution buffers was optimized during method development. For rBoNTB(H_c), several equilibration buffers were tested: (1) 60 m*M* sodium phosphate, 500 m*M* sodium chloride, pH 7.3, (2) 60 m*M* sodium phosphate, 50 m*M* sodium chloride, pH 7.3, (3) 25 m*M* sodium phosphate, 20 m*M* sodium chloride, 5 m*M* EDTA, pH 7.3. The pH was held constant at 7.3 for all the buffers tested to match the pH of the cell extraction buffer.

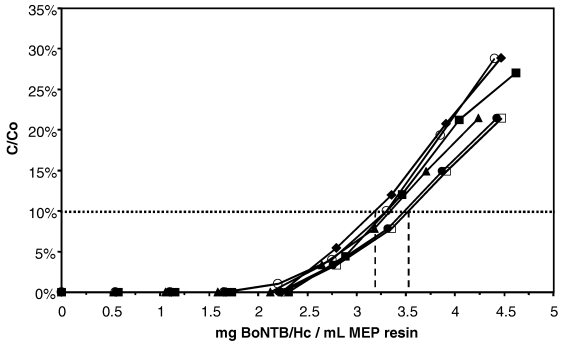

Fig. 8. Breakthrough curves for rBoNTB(H_c) to demonstrate dynamic binding capacity reproducibility for cycles 1 (closed circle), 11 (closed square), 19 (triangle), 20 (diamond), 23 (open circle) and 30 (open square) on the same column. The drop-lines show the range of $Q_{B,10\%}$ for these experiments.

All three equilibration buffers evaluated for rBoNTB(H_c) had similar elution profiles. The purity of the eluates for these elution buffers were consistent with each other (data not shown).

3.5.2. rBoNTB(H_c) low-ionic-strength wash

A low-ionic-strength wash was tested for rBoNTB(H_c). A water wash was evaluated on a column equilibrated with 60 m*M* sodium phosphate, 50 m*M* sodium chloride, 5 m*M* EDTA. After the column was loaded, it was washed with 8 CVs of equilibration buffer. Then it was washed with 5 CVs of half-strength equilibration buffer (30 m*M* sodium phosphate, 25 m*M* sodium chloride, 2.5 m*M* EDTA, pH 7.3) and then 8 CVs of purified water. During the water wash, 20% of rBoNTB(H_c) loaded onto the column was eluted.

3.5.3. rBoNTB(H_c) elution buffer

The ionic strength of acetate elution buffers was also evaluated for rBoNTB(H_c). Two different buffer compositions were tested: 200 m*M* sodium acetate, pH 4.0 and 50 m*M* sodium acetate, pH 4.0. The product elution fractions from both of these elution buffers had similar recovery, stability, and purity profiles.

3.5.4. rBoNTA(H_c) equilibration buffer

Similar ionic strengths were tested for the equilibration buffer for the rBoNTA(H_c) process. The equilibration buffers tested for rBoNTA(H_c) were as follows: (1) 200 m*M* sodium phosphate, 50 m*M* sodium chloride, 5 m*M* EDTA, pH 7.6 (2) 60 m*M* sodium phosphate, 50 m*M* sodium chloride, 5 m*M* EDTA, pH 7.6 (3) 30 m*M* sodium phosphate, 25 m*M* sodium chloride, 2.5 m*M* EDTA, pH 7.3 (4) 25 m*M* sodium phosphate, 20 m*M* sodium chloride, 5 m*M* EDTA, pH 7.3. The buffer composition of the cell extract was also varied testing the same compositions as the equilibration buffer for (1) and (2). The various starting materials and equilibration buffer compositions showed the same general binding capacity and elution pattern. However, the purity and stability of the product elution fraction was highest when the column was equilibrated with 25

m*M* sodium phosphate, 20 m*M* sodium chloride, 5 m*M* EDTA, pH 7.3 and the chemical extract was buffered with 60 m*M* sodium phosphate, 50 m*M* sodium chloride, 5 m*M* EDTA, pH 7.3.

3.5.5. rBoNTA(H_c) low-ionic-strength washes

Low ionic strength washes were also tested for rBoNTA(H_c). When the column was washed with water after loading a large elution peak was observed. This peak did not contain rBoNTA(H_c) but did contain a number of protein impurities (Fig. 4). It was determined that including the water wash produced a rBoNTA(H_c) product elution fraction of higher purity because many protein impurities were eluted from the column during the water wash.

3.6. Regeneration

Estimation of MEP HyperCel resin performance was accomplished by measuring the $Q_{B,10\%}$ of rBoNTB(H_c). Several cleaning agents were investigated for MEP regeneration. The column was cleaned with the different agents after a complete run including the 0.5 *M* NaOH CIP step. Then, a dynamic capacity breakthrough curve was generated to determine the effect of the cleaning agent. Table 7 shows the $Q_{B,10\%}$ of rBoNTB(H_c) after cleaning with each agent.

These data show both guanidine·HCl and 8 *M* urea were more effective cleaning agents than ethanol or hexanediol. The $Q_{B,10\%}$ are lower for these experiments than for the experiments in Fig. 7 because the column had not been properly regenerated prior to establishing proper regeneration conditions. When the column was not properly regenerated the $Q_{B,10\%}$ declined quickly. For subsequent work, either 6 *M* guanidine·HCl or 8 *M* urea was used for column regeneration, and $Q_{B,10\%}$ values were more reproducible, as seen in Fig. 8, which was a column run 30 times and regenerated each cycle with urea or guanidine·HCl.

Table 7
Effect of cleaning agents on MEP performance

Cleaning agent	$Q_{B,10\%}$ (mg/ml)[a]
New column	3.4
70% Ethanol	2.0
8 *M* Urea	2.8
6 *M* Guanidine·HCl	3.0
50% Hexanediol	2.2

[a] Ten percent breakthrough of rBoNTB(H_c) in mg of rBoNTB(H_c) loaded per ml of MEP resin after column regeneration.

Table 8
Effect of bed height and column volume on rBoNTA(H_c) purity

Bed height (cm)	Column volume (ml)	rBoNTA(H_c) purity (%)
2.5	5.0	67.8
16.2	2500	73.8
16.2	2500	78.6
16.3	32.6	79.8
16.3	32.6	82.0
16.3	32.6	84.3
17.1	34.4	73.7
20.0	40.2	79.2

3.7. Column scale and bed height

The processes described in Table 3, that were developed on ~30 ml columns, were scaled-up to 2.5-l columns. The chromatographic profiles obtained during large-scale runs of rBoNTA(H_c) and rBoNTB(H_c) were essentially identical to the profiles of the small-scale runs. The large-scale product recoveries, purities and stabilities were also consistent with those of the small-scale experiments. Product purity, as determined by HPLC, was not affected by column volume or bed height over the ranges studies for rBoNTA(H_c) (Table 8) or rBoNTB(H_c) (Table 9).

Table 9
Effect of bed height and column volume on rBoNTB(H_c) purity

Bed height (cm)	Column volume (ml)	rBoNTB(H_c) purity (%)
18.5	3.6	80
14.6	29.3	73
14.6	29.3	79
14.6	29.3	91
11.6	2200	86

4. Discussion

HCIC was used for the initial purification step for rBoNTA(H_c) and rBoNTB(H_c) from a clarified *P. pastoris* cell extract. Subsequent processing steps were used to produce a purified bulk drug substance for each of these proteins. Although these proteins are structurally similar [8], they interact differently with the HCIC matrix. rBoNTA(H_c) binds much tighter than rBoNTB(H_c) with respective $Q_{B,10\%}$ values of >12.5 and 3.4 mg/ml. Since, rBoNTA(H_c) binds tightly to the HCIC column, a water wash can be employed prior to product elution to remove impurities. The water wash was not effective for rBoNTB(H_c) because a large amount of rBoNTB(H_c) was eluted from the column during the water wash. This reduced product recovery in the product elution fraction to an unacceptable level.

The optimum elution buffer for rBoNTA(H_c) is 50 m*M* sodium acetate, pH 5.5. rBoNTA(H_c) elutes from the HCIC column with high purity and stability when acetate is used as an elution buffer. If sodium citrate is used, then rBoNTA(H_c) degrades rapidly. However, sodium citrate is the best elution buffer for rBoNTB(H_c). Degradation of rBoNTB(H_c) occurs when eluted with sodium acetate. When rBoNTB(H_c) is eluted with sodium citrate, it is stable to proteolysis. It is also further purified in a citrate elution because impurities precipitate in the citrate buffer leaving rBoNTB(H_c) in solution. The citrate precipitation is crucial to the rBoNTB(H_c) process because it removes or deactivates protease(s) and prevents formation of a product related impurity that is difficult to remove in downstream processing.

The high ligand density of the HCIC resin allows proteins to bind through hydrophobic interactions at neutral pH without the addition of high concentrations of preferentially hydrated solutes, and the proteins are eluted through charge repulsion at low pH when both the pyridine ring and the bound proteins are protonated [12]. The ionic strength of the equilibration and elution buffers do not have large effects on the chromatography. Both rBoNTA(H_c) and rBoNTB(H_c) are bound onto columns that have been equilibrated with low- or high-ionic-strength buffers. And rBoNTA(H_c) binds so tightly at neutral pH that washing with purified water does not elute the product. Unfortunately rBoNTB(H_c) does not bind as tightly as rBoNTA(H_c), so the water wash could not be used.

The greater hydrophobic character of rBoNTA(H_c) [GRAVY of -0.423 as compared to -0.648 for rBoNTB(H_c)] allows it to bind more tightly to the MEP HyperCel matrix at neutral pH. However, rBoNTA(H_c) also elutes at a higher pH than rBoNTB(H_c) because it has a higher p*I*: 9.11 and 7.48, respectively. rBoNTA(H_c) elutes from the MEP HyperCel column efficiently with acetate buffer at pH 5.5, but rBoNTB(H_c) will not elute efficiently in an acetate buffer above pH 4.75. Both proteins require a lower pH to elute from the column with sodium citrate than with sodium acetate, so the difference in the required elution buffer pH is exaggerated in the two optimal processes (Table 3).

The MEP HyperCel resin is robust and is not affected by small changes in process variables. The dynamic binding capacity was consistent for >30 cycles, which suggests a resin lifetime of greater than 30 cycles. This lifetime is sufficient for the production of a potent recombinant vaccine. The performance of the column is dependent on the chemical used to regenerate the column. If 8 *M* urea or 6 *M* guanidine·HCl is used, then the binding capacity does not change significantly over the life of the column. If any of the other chemicals are used that we tested or the regeneration step is omitted, then the binding capacity drops quickly over a few cycles. We have also shown that the bed height is not a critical variable for one of the separations. The bed height can be between 11 and 18 cm without any detectable effect on rBoNTB(H_c) purification.

Both rBoNTA(H_c) and rBoNTB(H_c) were purified on at least two different scales. The small-scale used column volumes of approximately 30 ml, and the large-scale used column volumes of approximately 2.5 l. The processes developed on the 30-ml columns transferred directly to the 2.5 l columns, which is large enough to process rBoNT(H_c) at the 15-l fermentation scale.

5. Conclusion

This is the first report where MEP HyperCel has been used to purify recombinant intracellular proteins from *P. pastoris*. HCIC was the best method

investigated for the initial purification of rBoNTA(H_c) and rBoNTB(H_c) since these proteins were not stable after initial purification when using other forms of chromatography (hydrophobic interaction and cation exchange). The optimal conditions had to be found to produce stable product eluates off the HCIC column, and the conditions were different for these similar proteins. The processes developed for the two proteins have been shown to be scalable, so that rBoNTA(H_c) and rBoNTB(H_c) can be purified at a process scale.

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