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## Production and purification of a chimeric monoclonal antibody against botulinum neurotoxin serotype A

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# **Production and purification of a chimeric monoclonal antibody against botulinum neurotoxin serotype A**

## **Abstract.**

Production of recombinant antibodies against botulinum neurotoxin is necessary for the development of a post-exposure treatment. CHO-DG44 cells were transfected with a plasmid encoding the light and heavy chains of a chimeric monoclonal antibody (S25) against botulism neurotoxin serotype A. Stable cell lines were obtained by dilution cloning and clones were shown to produce nearly equivalent levels of light and heavy chain antibody by an enzyme-linked immunosorbent assay (ELISA). In suspension culture, cells produced 35 µg/ml of chimeric antibody after 6 days, corresponding to a specific antibody productivity of 3.1 pg/cell/day. A method for the harvest and recovery of an antibody against botulism neurotoxin serotype A was investigated utilizing ethylenediamine-*N,N'*-tetra(methylphosphonic) acid (EDTPA) modified zirconia and MEP-hypercel, a hydrophobic charge interaction chromatography resin. Purification of the S25 antibody was compared to that achieved using rProtein A–Sepharose Fast Flow resin. After the direct load of culture supernatant, analysis by ELISA and gel electrophoresis showed that S25 antibody could be recovered at purities of 41 and 44%, from the EDTPA modified zirconia and MEP-hypercel columns, respectively. Although the purity obtained from each of these columns was low, the ability to withstand high column pressures and nearly 90% recovery of the antibody makes EDTPA modified zirconia well suited as an initial capture step. Combining the EDTPA modified zirconia and HCIC columns in series resulted in both purity and final product yield of 72%.

## **Keywords:**

Chinese hamster ovary; CHO; Mammalian; Cell culture; Zirconia; HCIC; Botulism; Botulism neurotoxin; BIAcore; Antibody

An increasing number of recombinant monoclonal antibodies are being developed for the treatment of medical conditions such as cancer, arthritis, and autoimmune diseases [1] and [2]. To meet the increased demand for monoclonal antibodies, all aspects of antibody production and purification need to be improved. Another potential use of monoclonal antibodies is the treatment for exposure to toxins, such as botulism neurotoxin (BoNT), one of the most poisonous substances known [3]. BoNT has been classified by the Centers of Disease Control (CDC) as one of the six highest risk threats for use in bioterrorism due to its potency, lethality, and ease of production [4]. BoNT is produced by the spore forming bacteria *Clostridium botulinum* and consists of seven serotypes (A–G) that cause the human disease botulism [5]. Botulism is characterized by flaccid paralysis and often results in death. The paralytic ability of the toxin has led to medical treatments for muscle conditions such as cervical dystonias, cerebral palsy, and posttraumatic brain injury, in addition to its use for cosmetic purposes [6].

The potential use of BoNT in bioterrorism requires either a vaccine or other treatment for exposure. Currently, there are no small molecule drugs available to prevent botulism, although a pentavalent toxoid is available from the CDC. In addition to the pentavalent toxoid a recombinant vaccine is being developed [7] and [8]. Regardless of the availability of a recombinant vaccine, mass vaccination is unlikely due to the rarity of exposure and the fact the vaccination would prevent medical uses of BoNT. Antibodies, however, can be used for the post-exposure treatment of botulism. Equine antitoxin and human botulism immune globulin have been used for the post-exposure treatment of botulism [9] and [10]. Recombinant monoclonal antibodies are currently being developed for the treatment of botulism. Three monoclonal antibodies have been combined to neutralize 450,000 50% lethal doses of BoNT serotype A [3]. Half of the mice treated with a combination of three monoclonal antibodies were able to survive exposure to 450,000 times the amount of BoNT serotype A that would normally kill 50% of mice.

Post-exposure treatment of botulism would consist of a mixture of monoclonal antibodies against each of the seven BoNT serotypes. To produce large quantities of these recombinant monoclonal antibodies, it is necessary to improve production and purification methods. Several mammalian expression systems have been used for the high-level expression of monoclonal antibodies, including the use of dihydrofolate reductase (dhfr) deficient Chinese hamster ovary (CHO) cells [11]. These cells allow for amplification of gene expression upon the addition of methotrexate [12], [13], [14] and [15]. It has been shown that the productivity of CHO cells increases with gene copy number [16]. Upon selection in medium containing stepwise increases in methotrexate, CHO cells with monoclonal antibody productivities (qAb) as high as 100 pg/cell/day have been obtained [17].

After a production cell line is established, it is necessary to develop techniques for purification of the monoclonal antibody. The most common method of antibody purification is affinity chromatography based on Protein A or Protein G [18], [19], [20], [21], [22] and [23]. These purification methods are effective, but the sorbent is expensive and the leakage of Protein A results in the need for further purification processes. The expense and harsh elution conditions of affinity sorbents such Protein A have led to the search for alternative purification processes. These include hydrophobic interaction chromatography [24] and [25], hydroxyapatite [26], and ion-exchange chromatography [27]. Many of these purification techniques require significant treatment of the culture supernatant prior to purification. We have focused our efforts on purification using two different chromatography resins, MEP-hypercel, and ethylenediamine-*N,N'*-tetra(methylenephosphonic) acid (EDTPA) modified zirconia.

In the present study, we have developed and characterized production of a monoclonal antibody in a dhfr deficient CHO cell line and have analyzed a purification scheme that uses EDTPA modified zirconia as an initial capture and purification step followed by a secondary purification using MEP-hypercel, a hydrophobic charge interaction chromatography (HCIC) resin. EDTPA modified zirconia has previously been used for the separation of antibody from bovine serum albumin, a common component of mammalian cell culture medium [28]

and [29]. Zirconia based resins provide excellent thermal and chemical stability compared to more typical resins. The zirconia surface is modified with EDTPA to block direct binding of antibody to the zirconia, which can lead to tailed elution bands and irreversible binding [30]. Hydrophobic charge induction chromatography (HCIC) has been used to purify antibodies directly from cell culture supernatant [31]. HCIC takes advantage of the pH behavior of the ionizable ligands. A decrease in the pH causes both the ligand and the protein to become positively charged, overcoming the hydrophobic interactions [32]. To obtain purified antibody against BoNT serotype A, CHO-DG44 cells were transfected with the genes for the light and heavy chains of the S25 antibody, and a purification scheme utilizing EDTPA modified zirconia and HCIC was compared to that obtained using a Protein A based resin.

## **Materials and methods**

### **Cell line, media, transfection, and expression vectors**

CHO-DG44 cells, which are dhfr negative, were obtained from Dr. Larry Chasin (Columbia University). This host cell line was maintained in  $\alpha$ -MEM media (Invitrogen, Carlsbad, CA) supplemented with 8% fetal bovine serum (FBS) (Invitrogen). The pS25 plasmid (Fig. 1) was constructed by inserting the chimeric light and heavy chain IgG genes against BoNT serotype A, along with the gene for dhfr into the plasmid pcDNA3.1(+) (Invitrogen).

CHO-DG44 cells were transfected with the pS25 plasmid using Lipofectamine 2000 (Invitrogen). Cells were seeded at 0.5 ml in 24-cell plates at a density of  $2 \times 10^5$  cell/ml in  $\alpha$ -MEM media containing 8% FBS and grown overnight. One microgram plasmid DNA and 0.5–2.0  $\mu$ l Lipofectamine 2000 were combined in 0.1 ml Opti-MEM media (Invitrogen) and equilibrated for 20 min. Plasmid DNA was added to the transfection mix either uncut or linearized with *Nru*I placing the amplifiable gene (dhfr) between the heavy and light chains, increasing the likelihood that both the light and heavy chains would be amplified upon methotrexate addition. The

DNA/Lipofectamine 2000 solution was added to the 24-well plates and the plates were incubated at 37 °C overnight. Stably transfected cells were selected in  $\alpha$ -MEM media lacking ribonucleotides and deoxyribonucleotides, which prevent cells lacking dhfr from growing. Cells were passed several times and individual clones were obtained by dilution cloning at 0.5 cells/well in 96-well plates.

## **ELISA**

The concentration of the whole antibody, as well as the concentration of the light and heavy chain portions, was determined using an enzyme-linked immunosorbent assay. Affinity purified rabbit anti-human IgG antibodies were diluted to 5  $\mu$ g/ml in coating buffer (100 mM NaHCO<sub>3</sub>, 100 mM NaCl, pH 9.3). One hundred microliters of diluted antibody was added to 96-well plates (Nunc, Rochester, NY) and incubated overnight at 4 °C. The plates were washed twice with Tris buffer (20 mM Tris-HCl, 50 mM NaCl, pH 7.2) containing 0.1% Tween 20 and then twice with Tris buffer alone. Blocking buffer (Tris buffer containing 0.5% BSA or casein) was added to the 96-well plates and incubated at 37 °C for 1 h. Supernatant samples were diluted in blocking buffer and samples were loaded into the 96-well plates in triplicate. Plates were incubated for 1 h at 37 °C and the washing procedure was repeated. One hundred microliters of a goat anti-human IgG-HRP conjugate antibody diluted to 0.5–2  $\mu$ g/ml in the dilution buffer was added to the plates. The plates were incubated for 1 h at 37 °C and the washing procedure was repeated. Lastly, 100  $\mu$ l of 1 mg/ml ABTS in ABTS buffer (Roche Applied Science, Indianapolis, IN) was added to the plates. The absorbance was determined at 405 nm using an ELx800 plate reader (Bio-Tek, Winooski, VT) after 30 min incubation. This procedure was used for whole antibody, heavy chain (Fc specific), and light chain ( $\kappa$  specific). Whole, Fc, and  $\kappa$  rabbit anti-human IgG coating antibodies and whole, Fc, and  $\kappa$  goat anti-human IgG-HRP conjugated antibodies were used in the ELISAs (Sigma, St. Louis, MO).

## **Transfer to suspension culture**

After screening the clones for antibody production, nine clones that reached 0.5 µg/ml antibody after three days were transferred to suspension culture. Initially, cells were seeded in the spinner flasks at  $2-3 \times 10^5$  viable cells/ml in CHO-S-SFMII media (Invitrogen) containing 1% FBS. The cells were then passed every 2–4 days into fresh media containing decreasing amount of FBS. After 8–10 passages, the cells were frozen in 1.5 ml aliquots in  $\alpha$ -MEM media containing 10% FBS and 10% dimethyl sulfoxide (DMSO) at a cell density of  $10^7$  cells/ml.

### **Growth of cells in suspension culture**

The CHO-DG44 S25 #56 cell line was grown in batch culture to analyze antibody production in suspension culture and to produce a sufficient amount of S25 antibody for purification and analysis. Frozen cells were resuspended in 40 ml CHO-S-SFMII at a seeding density of  $3-4 \times 10^5$  cells/ml. The spinner flasks were incubated at 37 °C and 5% CO<sub>2</sub>. The cells were fed every 3–4 days for several passages and were then seeded at  $2 \times 10^5$  viable cell/ml in 350 ml CHO-S-SFMII media in a 1 L controlled spinner flask. The dissolved oxygen (DO), pH, and temperature were controlled using a Cellferm-Pro control system (DAS-GIP, Julich, Germany). The pH was controlled by addition of CO<sub>2</sub> and 1 M NaOH. Samples were taken every day and viability and cell density were determined by trypan blue exclusion and counting on a hemocytometer. Cell suspensions were centrifuged at 1200 rpm for 5 min and supernatant samples were frozen for later analysis.

### **Protein purification**

EDTPA modified zirconia (Zirchrom, Anoka, MN), MEP-hypercel (Ciphergen, Fremont, CA), and rProtein A–Sepharose Fast Flow (Amersham Biosciences, Piscataway, NJ) resins were compared for the purification of S25 antibody. The supernatant from the CHO-DG44 S25 #56 cells was harvested after 6 days in batch culture. Supernatant was harvested by centrifugation at 300g for 5 min followed by a fivefold diafiltration with PBS

(20 mM Na<sub>2</sub>HPO<sub>4</sub>, 150 mM NaCl, pH 7.4) using a Pellicon XL50 ultrafiltration device containing 0.005 m<sup>2</sup> of a 10 kDa MWCO Biomax membrane.

Supernatant samples were purified using an AKTA FPLC (Amersham Biosciences). Diafiltered samples were purified using a 100 mm × 4.6 mm diameter column containing Protein A–Sepharose Fast Flow resin (Amersham Biosciences). Alternatively, culture supernatant was directly loaded onto the rProtein A–Sepharose Fast Flow column, an MEP-hypercel column (100 mm × 4.6 mm diameter), or an EDTPA modified zirconia column (Zirchrom) (50 mm × 4.6 mm diameter). Prior to loading the EDTPA modified zirconia column, the supernatant was diluted 1:1 in 40 mM Mes buffer containing 8 mM EDTPA (TCI America, Portland, OR).

After loading, the columns were washed with 5 column volumes (CV) of equilibration buffer. The rProtein A–Sepharose Fast Flow resin was loaded using PBS (pH 7.2) and eluted in 50 mM sodium citrate (pH 3.0). The MEP-hypercel column was equilibrated and washed with PBS (pH 7.2) and eluted using 50 mM sodium citrate (pH 4.0). The EDTPA modified zirconia column was equilibrated and washed with Mes buffer (20 mM Mes, 4 mM EDTPA, and 50 mM NaCl, pH 5.5) and eluted in Mes buffer containing 1 M NaCl. Samples were loaded and eluted at flowrates ranging from 0.25 to 0.5 ml/min. The pH of the elution was immediately increased to 7 using 500 mM Tris buffer (pH 9.0). The antibody was later concentrated and transferred into PBS by 10-fold diafiltration using a separate Pellicon XL50 ultrafiltration device. The S25 antibody was quickly frozen in liquid nitrogen at 1 mg/ml (BCA Assay) and was stored at –80 °C for long-term storage.

### **Bradford/BCA assays**

The total protein content for the purified chimeric antibody and the culture supernatant were determined using either a Bradford reagent (Sigma) or BCA reagent (Pierce, Rockford, IL). The Bradford assay was used to determine the S25 antibody concentration in the culture supernatant and flowthrough, while the BCA assay was used to determine the concentration of the eluate. For the Bradford assay, a 1 ml sample was mixed with 1 ml



Bradford Reagent (Sigma). The samples were incubated for 30 min at 37 °C and the absorbance at 595 nm was determined on a spectrophotometer. The BCA assay was used to determine the concentration of the S25 antibody product during the purification. For the BCA assay, 50 µl sample or standard was mixed with 1 ml BCA reagent mixture (Pierce), containing a 1:50 dilution of reagents A and B. The samples were incubated for 30 min at 37 °C and the absorbance at 562 nm was determined on a spectrophotometer. BSA, a major component of the growth media, was used as a standard for both assays. The BCA assay was used to determine the protein content of the eluate samples since IgG and BSA have a similar absorbance/mg in that assay. However, the Bradford assay was used to estimate protein content of other samples since components in the growth media interfered with the BCA assay.

### **SDS–PAGE/Western blotting**

Samples were diluted in phosphate-buffered saline (PBS) to 60 and 20 µl loading buffer (0.5 M Tris–HCl, 20% SDS, 40% glycerol, 10% β-mercaptoethanol, and 0.1% bromophenol blue) was added. For non-reducing gels, the loading buffer lacked β-mercaptoethanol. Samples were boiled for 2 min and resolved on 10–12% Tris–glycine polyacrylamide gels (Invitrogen). The gels were run for 2–4 h at 125 V using an XCell SureLock Mini-Cell (Invitrogen) containing running buffer (50 mM Tris, 300 mM glycine, and 0.1% SDS). The gels were transferred to nitrocellulose in an XCell SureLock Mini-Cell module for 6 h at 25 V in transfer buffer (25 mM Tris, 190 mM glycine, and 20% methanol, pH 8.3). Blots were blocked with 5% nonfat dried milk in TD buffer (140 mM NaCl, 5 mM KCl, 0.4 mM Na<sub>2</sub>HPO<sub>4</sub>, and 25 mM Tris) for 2 h at room temperature. Recombinant chimeric monoclonal antibody was detected by incubating with 0.5 µg/ml goat anti-human IgG (whole molecule specific) (Sigma) in 5% nonfat dried milk in TD buffer for 1 h at room temperature. The protein bands were detected by incubating with ECL (Amersham) and exposing to film. Human IgG (Sigma) was used as a positive control.

### **BIAcore activity assay**

The S25 antibody affinity and binding kinetics were measured by surface plasmon resonance in a BIAcore (Biacore AB, Piscataway, NJ). The method for determination of antibody affinity was previously published [3]. Briefly, purified IgG in 10 mM acetate (pH 3.5–4.5) was coupled to a CM5 sensor chip using *N*-hydroxysuccinimide-*N*-ethyl-*N'*-(dimethylaminopropyl)-carbodiimide chemistry. The association constant ( $k_{\text{on}}$ ) for purified BoNT serotype A Hc was measured under continuous flow of 15  $\mu\text{l}/\text{min}$ . The dissociation constant ( $k_{\text{off}}$ ) was determined at a high buffer flowrate of 30  $\mu\text{l}/\text{min}$  to prevent rebinding. The equilibrium dissociation constant ( $K_{\text{d}}$ ) was calculated as  $k_{\text{off}}/k_{\text{on}}$ .

## Results and discussion

### Isolation of recombinant CHO-DG44 cells with high S25 antibody production

Nearly 200 clones were screened for antibody using an ELISA specific for the Fc portion of the heavy chain of human IgG. Seventeen of the cell lines had antibody titers greater than 0.1  $\mu\text{g}/\text{ml}$ , with one having an antibody titer greater than 2  $\mu\text{g}/\text{ml}$  (Fig. 2). This clone (CHO-DG44 S25 #56) had the highest expression level throughout the selection process. Nine clones showed expression levels greater than 0.5  $\mu\text{g}/\text{ml}$  after three days in adherent cultures (Fig. 3) using an Fc specific ELISA. Fig. 3 shows the concentrations of light, heavy, and whole antibody determined from separate ELISAs for each of the nine high expressing clones. It should be noted that only an Fc specific ELISA was performed for each of clones 160, 180, and 181. Similar light and heavy chain antibody concentrations were determined for each of six clones tested. It is important to have similar expression levels of both the heavy and light chains to ensure full antibody is obtained upon purification. The cell line CHO-DG44 S25 #56 had light and heavy chain concentrations greater than 2  $\mu\text{g}/\text{ml}$  and was therefore used for the initial production of S25 antibody in suspension culture. Each of these nine clones was transferred to increasing levels of methotrexate for gene amplification (data not shown) and into serum-free media (CHO-S-SFM II, Invitrogen) to ensure culture stability and productivity in suspension culture.

## **Production of a S25 antibody in batch culture**

CHO-DG44 cells were transferred to suspension culture in CHO-S-SFM II and were grown in batch culture for the production of S25 anti-BoNT serotype A antibody. The CHO-DG44 S25 #56 cell line reached a maximum cell density of  $4 \times 10^6$  cells/ml after 5 days in suspension culture (Fig. 4A). The viability of the cultures stayed above 90% until day 6 at which point it had dropped to 80% (Fig. 4B). This was confirmed by a corresponding decrease in the oxygen uptake rate. The cells reached a maximum growth rate ( $\mu_{\max}$ ) of  $0.95 \text{ day}^{-1}$ , and the S25 antibody reached a final average concentration of  $35 \mu\text{g/ml}$ , ranging from 21 to  $53 \mu\text{g/ml}$  in four separate runs (Fig. 5). This corresponds to an average specific antibody productivity of  $3.1 \text{ pg/cell/day}$ , which is similar to that found for other recombinant antibodies prior to gene amplification [33].

## **Purification of S25 antibody using rProtein A–Sepharose fast flow resin**

S25 antibody was purified from culture supernatant after a diafiltration step. Two hundred and ninety milliliters of supernatant was concentrated to 40 ml and was then transferred to PBS (pH 7.2) using a Pellicon XL50 ultrafiltration device. Diafiltered sample was loaded onto 2 ml rProtein A Fast Flow resin and eluted by gravity flow. The flowthrough was collected and the column was washed twice with 25 ml PBS (pH 7.2). The S25 antibody was eluted in 50 mM sodium citrate (pH 4), followed by a second elution at pH 3. Samples were analyzed by SDS–PAGE and Western blotting (Fig. 6). Faint bands can be observed for the light and heavy chains of the chimeric S25 antibody (lanes 6 and 7), corresponding to the supernatant from the CHO-DG44 S25 #56 cells and the dialyzed sample, respectively. Little antibody was lost in the flowthrough and wash step. Elution at reduced pH resulted in highly purified antibody. These results were confirmed by Western blotting using a goat anti-human IgG (whole molecule specific) (Sigma) (Fig. 6B).

The purification of S25 antibody with Protein A–Sepharose Fast Flow provided a 76% yield (Table 1). There was little loss of antibody during the ultrafiltration step. The S25 antibody had a purity of greater than 95%, which was confirmed by Coomassie stained SDS–PAGE (Fig. 6A). The total protein concentrations were determined by BCA assay for the eluate samples and a Bradford assay for the rest (Table 1).

Purification of S25 antibody using rProtein A–Sepharose Fast Flow resin

	<b>Volume (ml)</b>	<b>S25 IgG (µg/ml)</b>	<b>Total S25 IgG (mg)</b>	<b>Total protein concentra- tion (µg/ml)</b>	<b>Total protein (mg)</b>	<b>Yield (%)</b>	<b>Purity (%)</b>
Supernatant	290	52.5	15.2	374	112.2	100	14
Diafiltration	39	373	14.5	2689	104.9	96	14
Flowthrough	39	3.6	0.14	1444	56.3	1	0
Wash 1	25	2.6	0.07	207	5.2	0	1
Wash 2	25	0	0.0	4	0.1	0	0
Elution 1 (pH 4) <sup>a</sup>	27.1	347	9.4	370	10.0	62	94
Elution 2 (pH 3) <sup>a</sup>	22.6	96.5	2.2	99	2.2	14	98
Eluate (Total) <sup>a</sup>	49.7	233	11.6	246	12.2	76	95

<sup>a</sup> BCA assay was used to determine the concentration of eluate samples. Absorbance/mg protein was nearly identical for IgG and BSA using the BCA assay, however components in the supernatant interfered with the BCA assay and therefore the Bradford assay was used to estimate protein concentration in the other samples.

## **BIAcore activity assay**

S25 antibody activity was analyzed after purification using the rProtein A–Sepharose Fast Flow resin. The equilibrium binding kinetics were determined by BIAcore to ensure that the chimeric S25 antibody was active and had improved binding kinetics in comparison to the single chain variable fragment from which it was derived. The  $K_d$  of the S25 antibody was  $2.0 \times 10^{-9} \text{ M}^{-1}$ , with a  $k_{\text{on}}$  of  $6.0 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$  and a  $k_{\text{off}}$  of  $1.2 \times 10^{-3} \text{ s}^{-1}$ . This  $K_d$  is much better than that determined for the single chain variable fragment. The previously reported  $K_d$  of the single chain variable fragment was  $7.30 \times 10^{-8} \text{ M}^{-1}$ , with a  $k_{\text{on}}$  of  $1.10 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$  and a  $k_{\text{off}}$  of  $8.10 \times 10^{-4} \text{ s}^{-1}$  [3]. These values are also similar to those previously determined for both the S25 antibody and other antibodies [3], [34] and [35].

## **Comparison of S25 antibody purified using EDTPA modified zirconia, MEP-hypercel, and rProtein A–Sepharose Fast Flow resins**

Culture supernatant was directly loaded onto EDTPA modified zirconia, MEP-hypercel, and rProtein A–Sepharose Fast Flow chromatography columns (Figs. 7A–C). The loading of S25 antibody was well below the binding capacity of the rProtein A–Sepharose resin. The low maximum pressure drop (3 bar) of the rProtein A–Sepharose Fast Flow resin limits the flowrates to 90 cm/h. Approximately 6 mg of total protein containing 1.25 mg S25 antibody was loaded onto the column in 26.4 ml culture supernatant. The S25 antibody was eluted off the rProtein A column with 50 mM sodium citrate (pH 3.0) in 0.6 column volumes (CV) neutralized to pH 7.0 with 0.17 CV of 500 mM Tris base (Table 2). The Protein A–Sepharose Fast Flow column provided a yield of 75% as determined by a whole antibody ELISA. The purity of the antibody was determined to be 99% based on the concentration of IgG in the elution fraction determined by an ELISA divided by the total protein concentration determined by a BCA assay.

Fig. 7. Absorbance (280 nm) of S25 antibody separated on various chromatography columns. (A) rProtein A–Sepharose Fast Flow, (B) EDTPA modified zirconia, (C) MEP-hypercel, and (D) MEP-hypercel (loaded with elution from EDTPA modified zirconia column).

Purification of S25 antibody using various chromatography resins

Sample	Volume (ml)	S25 (µg/ml)	IgG (mg)	Total S25 (mg)	IgG (µg/ml)	Protein (µg/ml)	Total protein (mg)	Yield (%)	Purity (%)
<i>rProtein A–Sepharose</i>									
Supernatant	26.4	47.4	1.25			228	6.03	100	21
Flowthrough	26.4	0	0			81.4	2.15		
Wash	8.3	0	0			42.0	0.35		
Eluate <sup>a</sup>	1.28	731	0.94			738	0.95	75	99
<i>rPEZ</i>									
Supernatant	53.2	23.7	1.26			114	6.08		
Flowthrough	53.2	0	0			42.1	2.24		
Wash	8.3	0	0			20.0	0.17		
Eluate <sup>a</sup>	2.08	542	1.13			1312	2.73	89	41
<i>MEP</i>									

Sample	Volume (ml)	S25 (µg/ml)	IgG (mg)	Total S25 IgG (mg)	Protein (µg/ml)	Total protein (mg)	Yield (%)	Purity (%)
Supernatant	48.5	47.4	2.30		228	11.07		
Flowthrough	48.5	0	0		53.6	2.60		
Wash	8.3	0	0		25.3	0.21		
Eluate <sup>a</sup>	5.4	319	1.72		729	3.94	75	44
<i>rPEZ/MEP</i>								
Diafiltration	8.5	57.5	0.49		283	2.41		
Flowthrough	8.5	0	0		12.9	0.11		
Wash	8.3	0	0		7.8	0.06		
Eluate <sup>a</sup>	2.4	164	0.39		229	0.55	72	72

<sup>a</sup> BCA assay used to determine the concentrations of the eluate samples.

The EDTPA modified zirconia column was loaded with approximately 50 ml solution consisting of a 1:1 dilution of CHO-DG44 culture supernatant and Mes loading solution (40 mM Mes, 8 mM EDTPA) at a pH of 5.5. The EDTPA modified zirconia column has a smaller particle size (40 µm), which resulted in a higher initial pressure drop. However, the EDTPA modified zirconia resin can handle pressure drops exceeding 400 bar. The S25 antibody was eluted off the EDTPA modified zirconia column by increasing the NaCl concentration to 1 M in 20 mM Mes buffer containing 4 mM EDTPA. The S25 antibody eluted in 2.4 CV (Fig. 6B) and neutralized

to pH 7.0 with 80  $\mu$ l of 500 mM Tris base. The elution fraction had a S25 antibody concentration of 542  $\mu$ g/ml and a protein concentration of 1312  $\mu$ g/ml, which corresponds to a purity of 41%, a 1.9-fold increase. The yield for the EDTPA modified zirconia column was 89% (Table 2).

The MEP-hypercel column was loaded with approximately 50 ml supernatant from the CHO-DG44 S25 cells. The column was washed with 5 CV PBS (pH 7.2) and eluted using 50 mM sodium citrate (pH 4.0). The S25 antibody was eluted from the MEP-hypercel column in 2.7 CV (4.5 ml), similar to the EDTPA modified zirconia column. The elution fraction had an S25 antibody concentration of 319  $\mu$ g/ml and a total protein concentration of 729  $\mu$ g/ml, which corresponds to a purity of 44%, a 2.1-fold increase. The yield for the MEP-hypercel column was 75% which is similar to that achieved using the rProtein A column. The loss of S25 antibody appears to be due to irreversible binding onto the resin since there was little antibody in either the flowthrough or the wash fractions.

### **Analysis of purification by Western blotting**

A non-denaturing SDS-PAGE gel was run to compare purity of the EDTPA modified zirconia and MEP-hypercel purified samples to that purified using rProtein A-Sepharose Fast Flow (Fig. 8). Both rProtein A purified samples were very pure with no visible bands corresponding to non-IgG proteins. There are numerous bands that correspond to contaminating proteins from the CHO-DG44 S25 culture supernatant purified using EDTPA modified zirconia column. The S25 elution peak from the MEP-hypercel column is significantly broader than that obtained from the other columns and has a shoulder on the front. The peak was collected in three fractions, which were compared by SDS-PAGE (Fig. 8, lanes 9–11). All three fractions contain a high amount of contaminating proteins and therefore were combined. This shoulder suggests that an improvement in purity could be obtained by elution at several pH steps. Comparing the EDTPA modified zirconia and MEP-hypercel peaks it was observed that the contaminating bands in the MEP-hypercel column were different from



those occurring on the EDTPA modified zirconia column. As a result, the EDTPA modified zirconia column and MEP-hypercel column were run in series to improve the purity of S25 antibody.

Fig. 8. Comparison of S25 antibody purification using rProtein A–Sepharose Fast Flow, MEP-hypercel, and EDTPA modified zirconia resins. (1) Human IgG (10 µg), (2) Human IgG (2 µg), (3) Human IgG (0.4 µg), (4) CHO-S-SFM II media, (5) CHO-DG44 S25 supernatant, (6) rProtein A pooled peak fraction (ultrafiltered load), (7) rProtein A pooled peak fraction, (8) EDTPA modified zirconia (9–11), MEP-hypercel fractions, and (12) See Blue Protein Standard.

### **Combination of EDTPA modified zirconia and MEP-hypercel purification**

The EDTPA modified zirconia column was chosen as the first purification step since it can be operated at higher pressure drops and higher flowrates. In addition, the high antibody recovery makes it the preferred choice for an initial purification step. Neither the MEP-hypercel nor the EDTPA modified zirconia columns achieved purification efficiencies close to that achieved using the rProtein A–Sepharose Fast flow column. Numerous impurities that result in large bands in the S25 antibody elute taken from the EDTPA modified zirconia column were removed in the MEP-hypercel column. As a result, the product from the EDTPA modified zirconia column was loaded onto the MEP-hypercel column. The S25 antibody from the EDTPA modified zirconia column was dialyzed into PBS (pH 7.2) using an 8000 kDa MWCO dialysis membrane (Spectrum Laboratories, Rancho Dominguez, CA). The dialyzed sample was loaded onto the MEP-hypercel column followed by a 5 CV with PBS. The load onto the MEP-hypercel column was much less than the amount of supernatant previously loaded onto the column. The S25 antibody was eluted with 50 mM sodium citrate (pH 4.0).

The flowthrough of EDTPA modified zirconia purified S25 antibody sample loaded onto the MEP-hypercel column had an absorbance of about 70 mAU (Fig. 7D). This corresponds to protein that is being removed using

the MEP-hypercel column. The S25 antibody is eluted at pH 4.0 and a peak height of 370 mAU is observed, which is much lower than those observed in the other columns due to the decreased antibody load. The antibody is eluted in 2 ml volume and 0.4 ml of 500 mM Tris base was immediately added to bring the pH to 7.0. The final S25 antibody concentration was 164 µg/ml and the final total protein concentration was 229 µg/ml, resulting in a final purification of 72%, a significant improvement to the purity obtained using the EDTPA modified zirconia column alone. The final yield for the EDTPA modified zirconia/MEP-hypercel purification was 72% which is just slightly less than that obtained from a single rProtein A column.

The S25 antibody purified using the EDTPA modified zirconia/MEP-hypercel columns in series was run on reducing SDS-PAGE gel, along with the samples purified using the EDTPA modified zirconia, rProtein A-Sepharose Fast Flow, and the MEP-hypercel alone (Fig. 9). Comparison of lanes 6, 7, and 9 shows the improvement in S25 antibody purity obtained after running both columns in series.

Fig. 9. Reducing gel of S25 antibody purified using various EDTPA modified zirconia and MEP-hypercel resins. (1) CHO-S-SFM II media, (2) CHO-DG44 S25 #56 supernatant, (3) rProtein A-Sepharose Fast Flow resin (dialyzed load), (4) rProtein A-Sepharose Fast Flow, (5) EDTPA modified zirconia, (6) MEP-hypercel, (7) EDTPA modified zirconia #2, (8) Dialyzed sample from EDTPA modified zirconia #2, (9) EDTPA modified zirconia/MEP-hypercel, and (10) See Blue Protein Standard.

## Conclusion

An antibody against BoNT serotype A was produced in CHO-DG44 cells and was then purified. The combination of EDTPA modified zirconia and MEP-hypercel provided an initial purification of monoclonal antibodies, but further downstream processing steps or improvements in separation conditions are needed to approach the purity achieved using a single Protein A resin. While EDTPA modified zirconia does not approach Protein A

resins for purity, the ability to operate at increased pressures, the high yield, and the ease of cleaning make it an ideal capture step for the purification of monoclonal antibodies from culture supernatant. In addition, EDTPA modified zirconia and MEP-hypercel prove to be complementary purification steps as demonstrated by the large increase in purity obtained when running these steps in series.

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