Separation of bivalent anti-T cell immunotoxin from *Pichia pastoris* glycoproteins by borate anion exchange

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A major problem encountered in the large-scale purification of the bivalent anti-T cell immunotoxin, A-dmDT390-bisFv(G4S), from Pichia pastoris supernatants was the presence of host glycoproteins exhibiting similar charge, size, and hydrophobicity characteristics. We overcame this problem by employing borate anion exchange chromatography. The borate anion has an affinity for carbohydrates and imparts negative charges to these structures. We found that at a concentration of sodium borate between 50 and 100 mM, the nonglycosylated immunotoxin did not bind to Poros 50 HQ anion exchanger resin, but glycoproteins, including aggregates related to the immunotoxin, did. By using this property of the immunotoxin in the presence of sodium borate, we successfully developed a 3-step purification procedure: (i) Butyl-650M hydrophobic interaction chromatography, (ii) Poros 50 HQ anion exchange chromatography in the presence of borate, and (iii) HiTrap™ Q anion exchange chromatography. The final preparation exhibited a purity of greater than 98% and a yield of greater than 50% from the supernatant. Previously, boronic acid resins have been used to separate glycoproteins from proteins. However, combining borate anion with conventional anion exchange resins accomplishes the separation of the immunotoxin from glycoproteins and eliminates the need to evaluate nonstandard resins with respect to good manufacturing practice guidelines.

INTRODUCTION

The bivalent anti-T cell immunotoxin, A-dmDT390-bisFv(G4S), was developed for treatment of T cell leukemia, autoimmune diseases, and tolerance induction for transplantation. This immunotoxin consists of the first 390 amino acid residues of diphtheria toxin (DT390) and two antigen-binding domains of the anti-CD3ε antibody, UCHT1 (1). Two N-glycosylation sites in the immunotoxin have been removed by introduction of two mutations (2), resulting in a nonglycoprotein with a molecular weight of 96.5 kDa. The immunotoxin is sensitive to pH levels below 6.0, as shown by the fact that low pH induces irreversible conformational change of the translocation domain in the DT390 moiety. The translocation domain mediates translocation of the A chain in the DT390 from the endosomes or the plasma membrane to the cytosol in a proton-dependent manner.

The catalytic A chain is responsible for protein synthesis inhibition by ADP-ribosylation of elongation factor 2 in the cytosol. The pH sensitivity of the immunotoxin restricts the use of cation exchange chromatography and affinity chromatography based on eluting with a low-pH buffer.

The multidomain structure of the bivalent immunotoxin hinders efficient production in *Escherichia coli*, and most eukaryotes are sensitive to the toxin. However, a budding yeast, *Pichia pastoris*, has a certain degree of tolerance to diphtheria toxin (3) and allowed us to obtain the immunotoxin at a level of 40 mg/L in fermentor culture. The immunotoxin was produced by fermentation of genetically engineered *P. pastoris* [JW102, renamed from pJHW#2 (3)] via the secretory route. The immunotoxin is functionally active only in its monomeric form. However, the supernatant harvested from the fermentation run contained monomeric,

dimeric, and higher oligomeric forms of the immunotoxin as well as *P. pastoris* proteins. Among these *P. pastoris* proteins, a glycoprotein species of approximately 45 kDa was present in dimeric form (approximately 90 kDa). The dimeric and higher oligomeric forms of the immunotoxin were relatively easy to separate by the use of conventional hydrophobic interaction chromatography and anion exchange chromatography. However, it was difficult to isolate the pure immunotoxin because the 45-kDa glycoproteins were very similar to the immunotoxin in size, hydrophobicity, and isoelectric point.

Previously immobilized phenylboronate resins have been used for separation of glycoproteins from proteins (4–7). These immobilized resins bind and selectively retard glycoproteins depending on pH, presence of sugar, type of sugar, concentration of sugar, and buffer species. However, we used borate anion exchange chromatography

rather than the immobilized phenylboronate affinity chromatography for the separation of the immunotoxin from the 45-kDa glycoproteins, owing to the poor separating capability of the phenylboronate resin. Borate forms complexes with sugar residues having vicinal *cis*-hydroxyl groups (8), and these complexes are reversible (9). Reversible complex formation of borate with carbohydrate on glycoproteins resulted in an increased negative charge of the glycoproteins. This property allowed us to separate nonglycoproteins and glycoproteins on anion exchange chromatography, which is analogous to methods previously described for small glycopeptides (10,11).

Here we demonstrate that in large-scale purification, conventional anion exchange chromatography by step-eluting with a series of sodium borate buffers enhanced separation of the nonglycosylated immunotoxin from *P. pastoris* glycoproteins. We also describe a scaleable high-yield 3-step purification procedure for the bivalent immunotoxin that incorporates the borate anion exchange step.

MATERIALS AND METHODS

Strains and Strain Maintenance

The JW102 strain whose former name was pJHW#2 was genetically engineered in a previous study (3). This strain was used for the production of the immunotoxin and has been maintained as a frozen stock at -80°C after being expanded from a single colony.

Fermentation

The details and optimization of fermentation conditions will be reported elsewhere (manuscript in preparation). We used a BioFlo® 4500 fermentor (New Brunswick Scientific, Edison, NJ, USA) containing 10 L initial medium, which was inoculated by a seed culture. After cells were grown for glycerol batch phase and glycerol-fed batch phase, the cells were induced by methanol for the production of the bivalent immunotoxin. After collecting the cultures at 22, 44, and 66 (or 67) h after methanol induction, the super-

natant was obtained by centrifugation. EDTA was added to the supernatant to a final concentration of 5 mM to prevent protein degradation for storage of the supernatant at 4°C.

Purification

Butyl-650M hydrophobic interaction chromatography. Approximately 100 mL Butyl-650M resin (Tosoh Biosep LLC, Montgomeryville, PA, USA) was packed in a 5 × 20 cm XK column (Amersham Biosciences, Piscataway, NJ, USA), and the column was equilibrated with Buffer A containing 200 mM Na₂SO₄, 1 mM EDTA, 20 mM Tris-HCl buffer (pH 8.0). This step was carried out at room temperature. Solid sodium sulfate and 1 M Tris-HCl buffer (pH 8.0) were added to 1 L supernatant to a final concentration of 200 and 20 mM, respectively. The sample was filtered with an 802 fluted filter paper (>15 µm particle retention; Whatman, Clifton, NJ, USA) before loading. The flow rate was 44 cm/h (14.4 mL/min). After equilibrating the column, 1 L prepared sample was applied onto the column, and then the column was washed with 6 column volumes of binding buffer A. The bound proteins to Butyl-650M resin were eluted with 6 column volumes of Buffer B containing 5% glycerol, 1 mM EDTA, 20 mM Tris-HCl buffer (pH 8.0). The eluted fractions having the immunotoxin were pooled for the next step (approximately 600 mL). After each run, the column was regenerated according to the manufacturer's protocol. After this first step, all steps were performed in a cold room.

Poros 50 HQ anion exchange chromatography by step elution with sodium borate buffer. Approximately 40 mL Poros 50 HQ resin (Applied Biosystems, Foster City, CA, USA) was packed in a 2.6 × 20 cm XK column, and then the column was equilibrated with Buffer B. The pooled sample from the previous step was diluted with 4.2 L TE buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA). The diluted sample was loaded onto the column at a flow rate of 80.2 cm/h (7.08 mL/min), and then the column was washed with 6 column volumes of Buffer B. The bound proteins were eluted in steps of

sodium borate of 25, 50, 75, and 100 mM in Buffer B (10 column volumes for each step). These eluted fractions were pooled for the next step. The residual protein bound to the resin was stripped with 6 column volumes of 1 M NaCl in Buffer B. After each run, the column was washed with 0.5 M NaOH and then reequilibrated with Buffer B minus 5% glycerol for the next use.

HiTrap™ Q anion exchange chromatography. A prepacked HiTrap Q anion exchange column (5 mL) was purchased from Amersham Biosciences. The pooled sample from the previous step was diluted with 3.6 L TE buffer. The sample was loaded onto the equilibrated column with Buffer B at a flow rate of 221.5 cm/h (7.08 mL/min). The column was washed with 5 column volumes of Buffer B. The bound immunotoxin was eluted with a linear 0–400 mM NaCl gradient in Buffer B (20 column volumes). The flow rate for washing and eluting steps was 2 mL/min, and the fraction size was 5 mL.

Protein Quantification by Comparison Using SDS-PAGE and Cytotoxicity Assay

The concentration of the immunotoxin was quantified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using an immunotoxin standard of known concentration prepared previously (3). Samples to be measured were subjected to SDS-PAGE utilizing Tris-glycine 4%–20% precast gels (Invitrogen, Carlsbad, CA, USA) under nonreducing or reducing conditions.

The specific cytotoxicity of the purified anti-human anti-CD3 immunotoxins was performed as described (12). Briefly, immunotoxins were applied to Jurkat cells, a human CD3ε⁺ T cell leukemia line, (5 × 10⁴ cells/well) in 96-well plates in leucine-free RPMI 1640 medium. After 20 h, a 1-h pulse of [³H] leucine was given. Cells were collected onto filters with a cell harvester (Mach II M; Perkin Elmer Life Sciences, Gaithersburg, MD, USA). After the addition of scintillant, samples were counted in a scintillation counter (model LC5801; Beckman Coulter, Fullerton, CA, USA) using standard liquid scintillation counting (LSC) techniques.

Analytic Gel Filtration

A Superdex™ 200 10/300 GL pre-packed column (dimension 1 × 30 cm) was purchased from Amersham Biosciences. The column was connected to a high-performance liquid chromatography (HPLC) system (HPLC pump, model LC1150 and photodiode array detector, model LC5100; GBC Scientific Equipment, Arlington Heights, IL, USA). Gel filtration buffer consisted of 90 mM sodium sulfate, 10 mM sodium phosphate monobasic, and 1 mM EDTA (pH 8.0). The flow rate was 0.5 mL/min, and the injection volume was 500 µL.

RESULTS

Butyl-650M Hydrophobic Interaction Chromatography

As shown in Figure 1, Butyl-650M hydrophobic interaction chromatography is an efficient capture step for immunotoxin in supernatant. However, glycoproteins were also purified with the immunotoxin during this step. Among these glycoproteins, identified by periodic acid Schiff staining, the glycoprotein species of approximately 45 kDa (Figure 1, arrow) impeded the isolation of the pure immunotoxin. By conventional chromatography such as gel filtration and anion exchange chromatography, these glycoproteins were not separated from the immunotoxin, indicating that these 45-kDa glycoprotein species were present in dimeric form and had similar isoelectric points. Therefore, we concluded that these 45-kDa glycoproteins were very similar to the immunotoxin in size and isoelectric point as well as in hydrophobicity.

We evaluated various hydrophobic resins that complied with good manufacturing practices. Among these resins, Butyl-650M had the best binding-and-eluting profile of the immunotoxin. We also found that 200 mM sodium sulfate was the minimum concentration for binding of the immunotoxin to the Butyl-650M resin.

The fermentor culture normally had approximately 30% wet cell density at the end of the fermentation run. In large-scale production, the supernatant will be obtained by continuous centrifuga-

tion, requiring a 3-fold dilution of the high cell density culture. We confirmed that the immunotoxin in this diluted sample was processed the same way as the immunotoxin in the supernatant that was effectively bound to the Butyl-650M resin at 200 mM sodium sulfate.

Poros 50 HQ Anion Exchange Chromatography by Step-Eluting with Sodium Borate Buffer

By employing borate anion exchange chromatography, we successfully separated the immunotoxin from the *P. pastoris* glycoproteins (Figure 2). We bound the immunotoxin to anion resin by diluting the sample from the previous step, thereby simplifying the purification procedure. In fractions eluted with 50, 75, and 100 mM sodium borate in Buffer B (Figure 2, lanes 9–11), most of the immunotoxin was present in monomeric form. These three fractions were pooled for the next step.

To remove glycoprotein species in the sample obtained from the previous step, we employed sodium borate in anion exchange chromatography because sodium borate increases the negative charge of glycoproteins by binding to the carbohydrate residues of the glycoproteins. We also noted that the immunotoxin was bound to anion ex-

change resins at pH 8.0 from a previous study (3). Based on this information, we first set up preliminary experiments for optimizing binding conditions of the immunotoxin in the presence of sodium borate. Aliquots of the dialyzed sample against Buffer B were mixed with different volumes of 200 mM sodium borate in Buffer B to obtain the designated concentration of sodium borate. The prepared samples were then loaded onto a Poros 50 HQ anion column (40 mL) equilibrated with Buffer B containing a corresponding concentration of sodium borate. We found that at 100 mM sodium borate the immunotoxin did not bind to the Poros 50 HQ anion resin, but the majority of glycoproteins still bound, yet at a concentration of sodium borate below 50 mM, the immunotoxin bound to the Poros 50 HQ anion resin.

Based on these results, we explored the conditions of step elution with sodium borate after binding of the immunotoxin to an anion exchange column. First, the sample dialyzed against Buffer B was bound to the anion column and then eluted in steps of increasing concentration of sodium borate (100, 120, 140, and 200 mM) and 1 M NaCl. The bound immunotoxin was mainly eluted at 100 mM sodium borate, but these eluted fractions also

Figure 1. SDS-PAGE analysis of proteins obtained by Butyl-650M capture step. Lanes 1–4, sample flow-through fraction nos. 1–4; lane 5, pooled sample flow-through fractions; lanes 6–8, wash fraction nos. 1–3; lane 9, pooled wash fractions; lanes 10, 11, and 17, supernatant; lane 12, Mark 12 protein standards (Invitrogen); lanes 13–15, eluted fraction nos. 1–3; lane 16, pooled eluted fractions. IT, immunotoxin.

contained significant amounts of 45-kDa glycoproteins, which were not separable in the next step. The majority of glycoproteins were eluted at 1 M NaCl. After loading the same sample as the first experiment, the bound immunotoxin was eluted in steps of 50, 75, and 100 mM sodium borate and 1 M NaCl. A majority of the bound immunotoxin was eluted at 75 mM sodium borate. However, a protein band corresponding to 21 kDa was included in the fraction eluted with 50 mM sodium borate. Finally, we set up the last experiment for optimizing the conditions of step elution. After binding to the column, the bound immunotoxin was eluted in steps of 25, 50, 75, and 100 mM sodium borate and 1 M NaCl in Buffer B (Figure 2). By washing with 25 mM sodium borate buffer, we reduced the amount of a protein band corresponding to 21 kDa.

Comparison with Phenylboronate Affinity Chromatography

To compare their separation profiles, we also performed phenylboro-

nate affinity chromatography. The eluant from the Butyl-650M hydrophobic interaction chromatography capture step was dialyzed against the low ionic strength buffer (10 mM HEPES, pH 8.2, 0.25 mM EDTA, and 20 mM MgCl₂) for phenylboronate affinity chromatography. This dialyzed sample was applied to a 5-mL bed volume column of phenylboronate agarose (Sigma, St. Louis, MO, USA) and washed with the same buffer, and then the bound proteins were eluted with either 0–100 mM sodium borate gradient or 0–50 mM sorbitol gradient in the same buffer (20 bed volumes). Glycoproteins and the immunotoxin were bound under binding condition of low ionic strength. The glycoproteins and immunotoxin were not separated by phenylboronate affinity chromatography. They were co-eluted with either 0–100 mM sodium borate gradient or 0–50 mM sorbitol gradient (data not shown).

Q Anion Exchange Chromatography

Q anion exchange chromatography was used for concentration of the dilut-

Figure 2. SDS-PAGE analysis of proteins obtained by Poros 50 HQ borate anion exchange step. Lane 1, Mark 12 protein standards (Invitrogen); lane 2, sample obtained from Butyl-650M hydrophobic interaction chromatography step; lanes 3–7, sample flow-through fraction nos. 1–5; lane 8, fraction no. 1 eluted with 25 mM borate in Buffer B; lane 9, fraction no. 2 eluted with 50 mM borate in Buffer B; lane 10, fraction no. 3 eluted with 75 mM borate in Buffer B; lane 11, fraction no. 4 eluted with 100 mM borate in Buffer B; lane 12, fraction no. 5 eluted with 1 M NaCl in Buffer B. IT, immunotoxin.

ed sample that was obtained from the Poros 50 HQ anion exchange chromatography. We noted that at a concentration of sodium borate below 50 mM the immunotoxin was bound to the anion exchange resin. Accordingly, the pooled sample from the previous step was diluted with three sample volumes of TE buffer (20 mM Tris-HCl, pH 8.0, and 1 mM EDTA), resulting in less than 20 mM sodium borate in the diluted sample. As expected, the immunotoxin was effectively bound to the Q anion exchange column. The bound immunotoxin was eluted with 0–400 mM NaCl gradient elution (20 column volumes). The immunotoxin fractions were pooled and then assessed for yield, purity, and toxicity of the final preparation by SDS-PAGE and protein synthesis assay.

Protein Yield, Repeatability of Purification Procedure, and Purity and Function of the Purified Immunotoxin

Table 1 summarizes the immunotoxin yields that were obtained in three batches of the three-step purification runs by using the supernatants taken at 44 h methanol induction from three fermentation runs that were carried out under relatively similar fermentation conditions and had similar expression levels of the immunotoxin. The average yield of this purification batch was 52.8%. By using the three-step purification procedure, we were able to obtain approximately 16 mg purified material from 1 L supernatant. The starting supernatants had different levels of immunotoxin aggregates and monomeric immunotoxin depending on the induction time during fermentation run. Among these immunotoxin aggregates, some portions could be reversible to monomeric form of the immunotoxin during the Butyl-650M hydrophobic interaction chromatography step. Fractionation of supernatant by gel filtration and subsequent SDS-PAGE analysis showed that the supernatants contained more than 50% of the immunotoxin aggregates (data not shown). However, the final yield of the immunotoxin after the three-step purification procedure was 52.8%, indicating that a portion of the aggregates

Table 1. Comparison of Immunotoxin Purification from *Pichia pastoris* Fermentor Cultures^a

Batch No.	Step	Immunotoxin Concentration (μg/mL)	Volume (mL)	Total Immunotoxin (mg)	Yield (%)	Accumulated Yield (%)
1	Supernatant	30.0	1000	30.0	100.0	100.0
	Butyl-650M HIC	45.0	585	26.3	87.8	87.8
	Poros 50 HQ borate AEX	15.0	1200	18.0	67.0	60.0
	Q AEX	400.0	40	16.0	88.9	53.3
2	Supernatant	30.0	1000	30.0	100.0	100.0
	Butyl 650M HIC	40.0	585	23.4	78.0	78.0
	Poros 50 HQ borate AEX	15.0	1200	17.5	74.6	58.2
	Q AEX	400.0	40	16.0	91.6	53.3
3	Supernatant	30.0	1000	30.0	100.0	100.0
	Butyl-650M HIC	40.0	585	23.4	78.0	78.0
	Poros 50 HQ borate AEX	15.0	1200	18.0	67.0	60.0
	Q AEX	450.0	35	15.8	87.5	52.5

Supernatants were obtained from three fermentation runs at 44 h methanol induction.

^aHIC, hydrophobic interaction chromatography; AEX, anion exchange chromatography.

Figure 3. Analytical gel filtration and SDS-PAGE analysis of purified immunotoxin. (A) Chromatogram of Superdex 200 10/300 GL gel filtration. (B) Picture of Coomassie® Brilliant Blue-stained SDS polyacrylamide gel.

could be dissociated into monomeric immunotoxin during purification.

A comparison of our purification procedure applied to three separate fermentation runs that contained similar amounts of supernatant immunotoxin demonstrates good repeatability of the procedure with respect to yields (Table 1).

We assessed the purity of the purified immunotoxin by analytical gel filtration (see the Materials and Methods section). The immunotoxin in the final preparation displayed a single peak corresponding to the monomeric form of the immunotoxin (Figure 3A). The analyses of purity of the final preparations confirmed that the three-step purification yielded an immunotoxin with about 98% purity (Figure 3B).

To investigate the effects of the three-step purification procedure on immunotoxin bioactivity, we carried out a protein synthesis assay for the specific T cell toxicity of the final preparation. The estimated concentration of the immunotoxin in the final preparation coincided with concentration of the immunotoxin standard (data not shown).

DISCUSSION

We successfully developed a three-step procedure for purification of an anti-T cell bivalent immunotoxin used for the treatment of T cell leukemia, autoimmune diseases, and tolerance induction for transplantation. This procedure contained Butyl-650M hydrophobic interaction chromatography, Poros 50 HQ borate anion exchange chromatography, and Q anion exchange chromatography. This procedure has several advantages: (i) it is a relatively simple process without any dialysis or diafiltration step; (ii) it exhibits good repeatability; (iii) the final yield is greater than 50%; and (iv) the final purity is greater than 98%. Development of this procedure was accomplished by the introduction of borate anion exchange chromatography. We used borate anion exchange chromatography for the separation of the immunotoxin from *P. pastoris* glycoproteins.

In a previous study utilizing shake flask culture, we employed a two-step

procedure that involved diethylaminoethyl anion exchange chromatography and Protein L affinity chromatography for purification of the immunotoxin (3). However, the supernatants of high-density fermentor cultures of *P. pastoris* contain materials that severely reduce the capacity of anion exchange resins (unpublished data). In addition, the Protein L affinity step required excessive column size, was expensive, and was not available under good manufacturing practice certification. Consequently, we explored alternative procedures. Hydrophobic interaction chromatography using Butyl-650M not only worked well as a capture step but also concentrated *P. pastoris* glycoproteins having charge, size, and hydrophobicity similar to the immunotoxin. Concanavalin A affinity resin was promising for glycoprotein removal, but bleeding of potentially toxic concanavalin A from the resin resulted in unacceptable contamination of the final product. Finally, we developed the three-step purification procedure for the immunotoxin by introducing sodium borate in anion exchange chromatography.

It is known that borate reversibly associates to hydroxyl groups of many compounds including carbohydrates, vitamins, coenzymes and ribonucleic acids by the formation of complexes that increase the negative charge of the parent compounds (13,14). Based on this property of borate, many applications have been developed. For example, borate was used in capillary electrophoresis for the analysis of the oligosaccharide composition of heparin (15), for the separation of ovalbumin glycoforms (16), for high-voltage paper electrophoresis separation of glycopeptides from ovalbumin (17), for immobilized isolectin affinity chromatography for the isolation of trypsin inhibitor from *Echinodorus paniculatus* seeds (18), and for gel filtration analysis of immunoglobulin G glycopeptides (19). Borate anion exchange chromatography was also developed for separation of glycopeptides by using a linear 0–0.4 M sodium borate gradient (10,11) and for the separation of reduced N-acetylhexosamines by using step elution (20).

On the basis of previous applications of borate, we extended the application of borate anion exchange chro-

matography for the removal of host glycoproteins from *P. pastoris* supernatants. By using Poros 50 HQ borate exchange chromatography, substantial purification of the monomeric form of the immunotoxin was obtained, even though the immunotoxin in the eluted fraction was diluted and a subsequent concentration step by Q anion exchange chromatography was necessary.

In the separation of the immunotoxin from glycoproteins, borate anion exchange chromatography had different binding characteristics from phenylboronate affinity chromatography. In phenylboronate affinity chromatography, glycoproteins as well as the immunotoxin were bound under the condition of low ionic strength and they were co-eluted by either 0–100 mM sodium borate gradient or 0–50 mM sorbitol gradient, suggesting that the immunotoxin physically interacts with at least one of the bound glycoproteins or interacts with the phenylboronate column through an alternate mechanism. The fact that purified bivalent immunotoxin also bound to the phenylboronate column indicates binding through an alternate mechanism.

Borate anion exchange chromatography is likely to be useful for the purification of other *P. pastoris* expressed proteins. *P. pastoris* is being increasingly used as an expression system for therapeutic recombinant proteins (21). Many of these recombinant proteins will have their glycosylation sites removed because of the profound differences in glycosylation patterns between *P. pastoris* and humans. These recombinant proteins will then be amenable to purification using borate anion exchange chromatography.

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