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The Combined Use of Expanded-Bed Adsorption and Gradient Elution for Capture and Partial Purification of Mutant Diphtheria Toxin (CRM 9) from *Corynebacterium diphtheriae*

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ABSTRACT

Expanded-bed adsorption (EBA) is a new approach for performing the initial recovery or capture of proteins from various crude feedstocks. The essence of the method is direct adsorption of the desired protein from the unclarified suspension by passing it through a stable expanded bed of the adsorbent. This type of operation replaces centrifugation, clarification, dialysis, and concentration with one simple unit operation. The recovery is done by pumping the feedstock upward on the expanded column and eluting it downward in a step mode from the packed bed. One of the unique properties of the expanded bed is its behavior as a true plug-flow column, which makes it possible to use gradient elution and to achieve better purification in addition to the other benefits. In this work the ''traditional'' recovery and purification process of the extracellular mutant diphtheria toxin (CRM 9) was replaced with an expanded bed adsorption process in which the protein was eluted using a linear salt gradient in an upward mode instead of the standard downward step elution. This combined procedure is a simpler, shorter process that yielded a purified protein preparation (that only had to pass through a gel filtration column instead of through the next ion-exchange step). Twenty grams of protein suitable for clinical use was prepared using this method.

INTRODUCTION

Diphtheria toxin, an extracellular protein secreted by *Corynebacterium diphtheriae*, has been in pharmaceutical use since the early twentieth century when the inactivated toxin was introduced as a vaccine (1). The production pro-

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cess of the inactivated toxin (toxoid) is relatively simple. The protein secreted by the high producing strain PW8 (2) is inactivated with formaldehyde and then precipitated from the bacterial supernatant with ammonium sulfate (3).

In the last few years there has been growing interest in various diphtheria toxin mutants, which are needed for the preparation of conjugated vaccines (4, 5) and immunotoxins (6). Because of the low concentration of the secreted proteins and the degree of purity needed, the recovery and purification process of these proteins is more complicated than the process used for the preparation of the native diphtheria toxin. After removal of the microorganisms, the supernatant is clarified either by microfiltration or high *g* centrifugation, and then ultrafiltered, diafiltered, and precipitated with ammonium sulfate (Fig. 1). The ammonium sulfate precipitate is then chromatographed on an ion exchanger, followed by gel filtration (7, 8). Because of the relatively high ionic strength of the bacterial supernatant, it is impossible to absorb the protein directly on an ion exchanger, and since dilution will significantly increase the volume, microfiltration and intensive ultrafiltration and diafiltration steps are needed. However, the presence of antifoam in the bacterial supernatant significantly reduces the efficiency of this indispensable preliminary diafiltration process (9). Expanded-bed chromatography is a recently developed process (10) in which a crude feedstock, e.g., a suspension of whole microorganisms or a

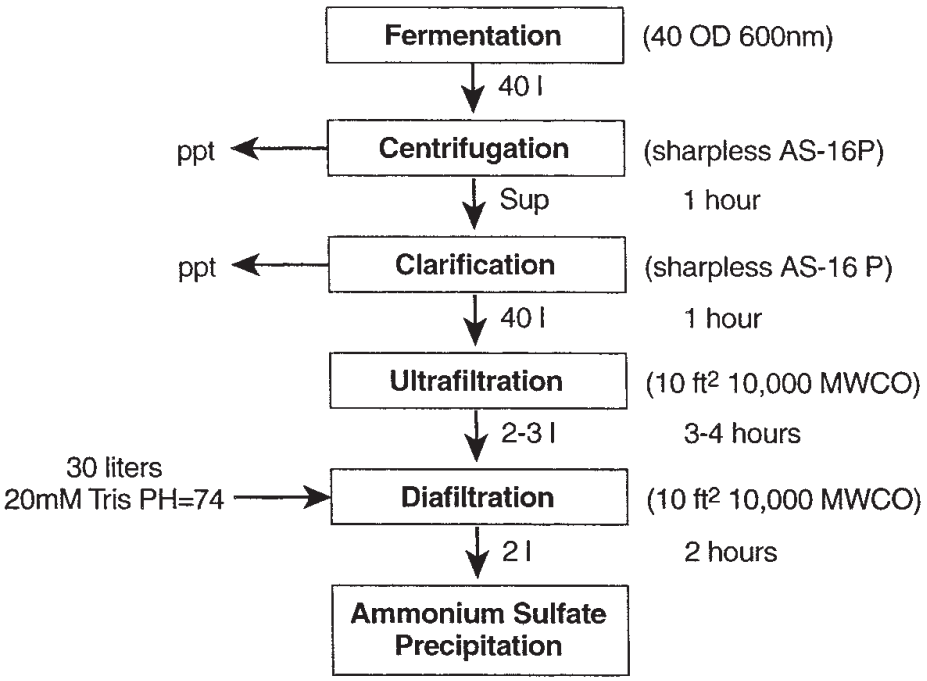

FIG. 1 Flow diagram of the traditional capture process for CRM 9.

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suspension of homogenized microorganisms, can be loaded directly on an expanded adsorbent material such as an ion exchanger. This process utilizes special adsorbent material in a specially designed column and functions as a capture step (11–13), replacing the centrifugation, clarification, and diafiltration, altogether disregarding the presence of antifoam. Because the loading is being done at a high flow rate (400 cm/h), the sample volume does not have a significant effect on the operation. Therefore it is possible to adjust the ionic strength of the loading solution by simple dilution instead of microfiltration and extensive diafiltration. In addition, the ion exchanger used in this capture step can potentially produce purified product. After loading, the matrix is allowed to settle and the elution is done from a packed column, usually in a step elution mode. The reason for the step elution is the large particle size (200 μm) and the nature of the adsorption process. However, gradient elution, if possible, can improve the product quality and perhaps eliminate the next purification step.

Successful operation of the expanded bed depends on achieving a stable bed (12) where the mixing in the column is very limited. Achieving a stable bed was found to be difficult when dealing with intracellular products (11). The column stability depends an several parameters such as percent solids, salt concentration, viscosity, pH, and the linear flow rate. Achieving a stable bed is expected to be less sensitive, and therefore easier, when dealing with extracellular bacterial product, since both the viscosity and the solids concentration are lower.

In this report the expanded-bed adsorption process coupled with gradient elution is introduced as a substitute for both the capture and the initial purification process, replacing several unit operations (centrifugation, clarification, and the first chromatography step) with one unit operation. The expanded-bed operation is compared to the ‘‘traditional’’ recovery and purification process.

MATERIALS AND METHODS

Bacterial Strain and Cultivation Process

The required protein, CRM 9 (14), mutant diphtheria toxin, was produced by *Corynebacterium diphtheriae* C7(β)$^{(tox\text{-}201.tox9)}$ (D. Neville, NIH, NIMH). The growth and production process was done according to Fass et al. (15). The bacterial fermentation was performed in a 100-L bioreactor. The bacteria grew to an OD 600 nm of 40 and the level of the extracellular toxin was between 50 and 80 mg/L.

Analytical Method

CRM 9 analysis was done by measuring the ADP-ribosylation activity using wheat germ extract enriched in Elongation factor 2 (EF-2) as substrate

(16). The toxin was nicked by mild trypsinization (1 μg/mL trypsin at 25°C for 30 minutes) and cleaved by treatment with dithiothreitol in 4 M urea prior to assay. The standard curve was prepared using a nonnicked form of diphtheria toxin (List Lab, CA).

The purity of the various CRM 9 preparations was characterized by 8–16% acrylamide gel electrophoresis in nonreducing SDS buffer at pH 8.0 and by gel filtration using a Zorbax GF-250 column 9.4 × 250 mm (Rockland Technologies, PA), in phosphate-buffered saline pH 7.2 at a flow rate of 1.0 mL/min.

''Traditional'' Capture Process

At the end of the fermentation the culture was centrifuged using a Sharpless AS 16P continuous centrifuge at a rate of 100 L/h. The supernatant was collected and clarified using the same centrifuge at a lower flow rate of 50 L/h. The clear supernatant was ultrafiltered using a Millipore Pellicon cassette system with 10 ft^2 of 10,000 MWCO membrane (PTGC) operating at a circulation rate of 20 $L/m^2/h$. The concentrated solution was dialyzed with 20 mM Tris buffer, pH 7.4 using the same membrane and was stopped when the conductivity was 3 mS/cm. Ammonium sulfate (516 g) was added to each liter of the final concentrated toxin solution before further purification.

Experimental Design

In order to find the maximum binding of the CRM 9 to the anion exchanger with the minimum dilution, several binding conditions were evaluated using the factorial design approach (17). Bacterial supernatant at various conditions (Table 1) were applied on a 1.6 × 10 cm DEAE Sepharose Fast Flow column

TABLE 1
Binding of CRM 9 to DEAE Sepharose. Experiments Were Conducted Using Factorial Design as Described in the Materials and Methods Section

Exp	pH	Dilution	Protein/resin (mg/mL)	CRM9 binding (%)
1	9.0	0.25	50	22
2	7.4	0.12	50	80
3	7.4	0.25	50	8
4	9.0	0.12	50	100
5	7.4	0.12	100	33
6	9.0	0.25	100	25
7	9.0	0.12	100	50
8	7.4	0.25	100	12

at a flow rate of 1 cm/min. The column was eluted with 1 M KCl, and the CRM 9 concentration was measured. The experimental plan as well as the analysis were done using Design-Ease Software (Stat-Ease Inc. Minneapolis, MN).

Breakthrough Curve and Dynamic Capacity Measurement

Diluted bacterial culture (1:8) was pumped directly on a 5-cm expanded-bed column STREAMLINE 50 (Pharmacia Biotech Uppsala, Sweden) containing 100 mL packed STREAMLINE DEAE (Pharmacia Biotech Uppsala, Sweden) at a flow rate of 300 cm/h at pH 8.9 and a conductivity of 2.9 mS/cm. Run-through fractions were collected and analyzed for toxin concentration.

Expanded-Bed Capture Process

At the end of the fermentation the culture conductivity was adjusted to 3.5 mS/cm with 20 mM Tris buffer, pH 9.0 (diluted approximately 6 times) and was pumped upward on equilibrated STREAMLINE DEAE (Pharmacia Biotech, Uppsala, Sweden) expanded in a STREAMLINE 200 column (Pharmacia Biotech, Uppsala, Sweden) at a ratio of 1 L original culture to 80–100 mL packed resin at a flow rate of 300–400 cm/h. After all the culture was applied to the column, the column was washed with the same buffer until the eluent OD at 280 nm was back at the baseline. At this point the upward flow was stopped, the bed was allowed to settle, and the flow adaptor was lowered to the surface of the packed bed. In the step elution case the column was washed in a downward mode with 2 column volumes at a rate of 100 cm/h, and the CRM 9 diphtheria toxin was eluted with 20 mM Tris buffer, pH 7.4 containing 0.175 M KCl. The OD peak at 280 nm was collected and the protein was precipitated by adding 516 g/L ammonium sulfate. In the gradient mode, the column was washed in an upward mode with 2 column volumes and then eluted upward with a gradient of 5 column volumes from 0 to 0.175 M KCl in 20 mM Tris buffer, pH 7.4 at a flow rate of 100 cm/h. After the gradient, the column was washed with 4 column volumes of the 0.175 M KCl buffer. Two-liter fractions were collected and analyzed, the fractions containing the CRM 9 were pooled, and the protein was precipitated by adding 516 g/L ammonium sulfate.

RESULTS

Capture of CRM 9 Using ''Traditional'' Process

The traditional capture process from 40 L bacterial culture is summarized in Fig. 1. At the end of the fermentation process (15), the bacteria were

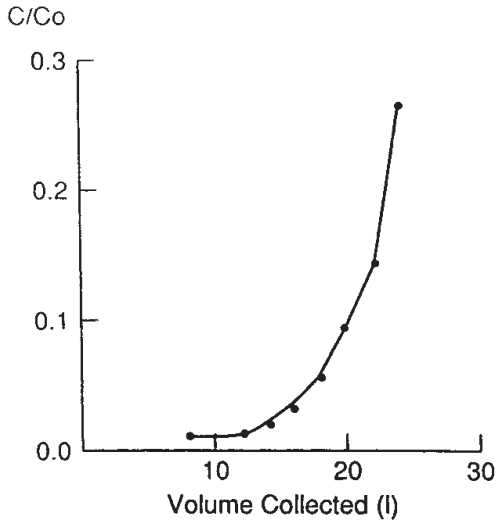

FIG. 2 Breakthrough curve for CRM 9 on Streamline DEAE. Bacterial culture containing 40 mg CRM 9 per liter was diluted 8-fold (2.9 mS/cm) and was applied directly on a 5-cm expanded-bed column containing 100 mL packed STREAMLINE DEAE at a flow rate of 300 $cm \cdot h^{-1}$ at pH 8.9. Run-through fractions were collected and analyzed for toxin concentration.

removed and the supernatant was clarified by centrifugation. The clear supernatant was first ultrafiltered and than diafiltered using 10,000 MWCO membrane, and the protein was precipitated by adding ammonium sulfate to 50% saturation. The overall process took 8 hours and 3.3 gr of the mutant toxin were obtained.

Binding Conditions of CRM 9 to the Expanded-Bed Resin

The essence of the expanded-bed process is direct adsorption of the protein from the bacterial culture on an adsorbent gel in an expanded state. Because the binding of the mutant diphtheria toxin on the DEAE is possible only after intensive dialysis, an optimization of the binding step was required to minimize the dilution needed. The effect of pH, conductivity, and ratio of resin to protein on the binding was analyzed using an experimental factorial design (16) (Table 1). Based on these results, the selected binding conditions were pH 9.0, a ratio of 50 mg protein per 1 mL packed resin, and dilution of 0.12 (conductivity of 3.6 mS/cm). At these conditions, 1 mg toxin was bound to 1 mL resin. The profile of the breakthrough curve on the expanded bed (see Materials and Methods Section) is shown in Fig. 2.

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Capture of CRM 9 Using Expanded-Bed Adsorption Followed by Step Elution

The capture process of CRM 9 on the expanded-bed column using the previously determined conditions is summarized in Fig. 3. At the end of the fermentation process the bacterial suspension (40 L) was diluted with 40 mM Tris buffer, pH 9.0, the conductivity was adjusted to 3.6 mS/cm, and the diluted bacterial suspension (320 L) was then loaded upward on the expanded STREAMLINE DEAE at a flow rate of 400 cm/h. After loading, the column was washed, packed, and then step eluted downward with 0.175 M KCl in 20 mM Tris buffer, pH 7.4 at a flow rate of 100 cm/h. The overall process took 4 hours and 3.0 g of mutant toxin were obtained.

Gradient Elution of CRM 9 from the Expanded Bed

As was indicated earlier, because of the bead size and the adsorption process, the regular operation of the expanded bed calls for loading from the bottom (expanded mode) and step elution from the top (packed mode). In an

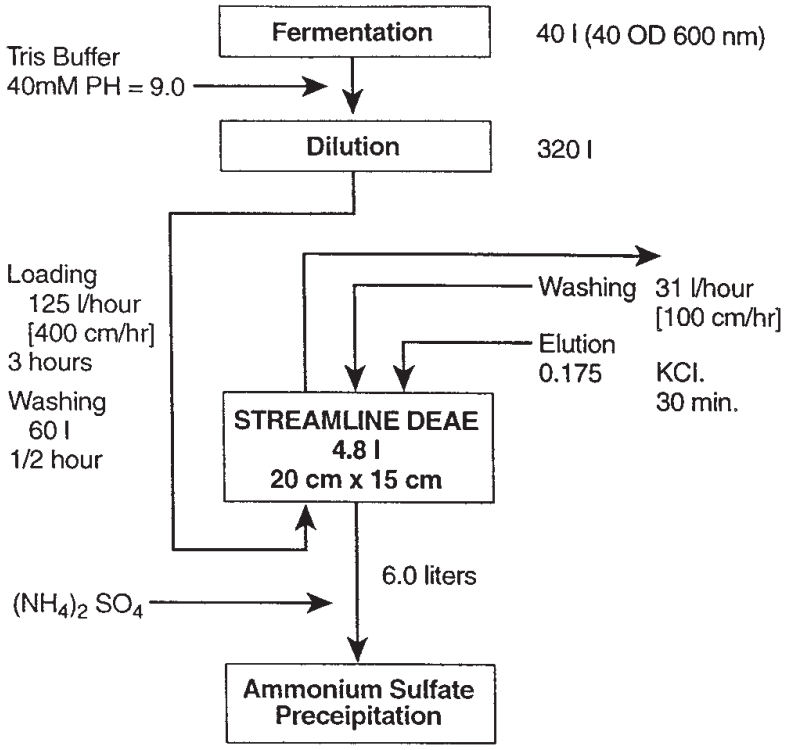

FIG. 3 Flow diagram of the expanded-bed capture process for CRM 9.

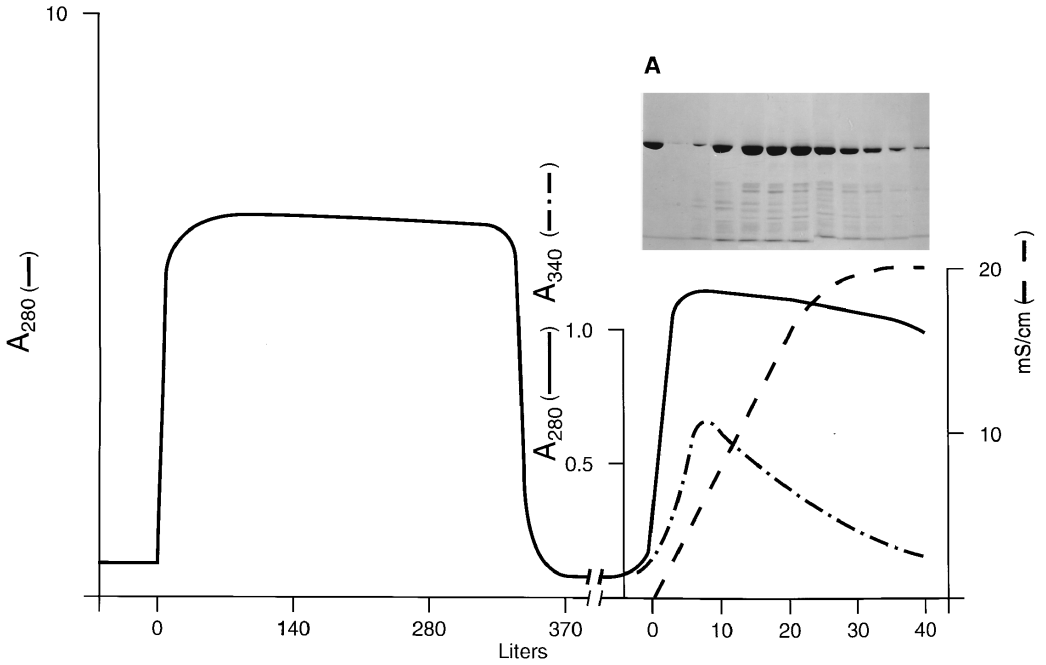

FIG. 4 Capture and purification of CRM 9 on expanded bed with STREAMLINE DEAE: 320 L of diluted bacterial culture (3.2 $mS \cdot cm^{-1}$) was applied on 4.8 L STREAMLINE DEAE in a STREAMLINE 200 column (see Fig. 3). After loading, the column was washed and then eluted using a linear gradient of 5 column volumes followed by wash with 4 column volumes. The elution was done from the packed column in an upward flow of 100 cm/h. Fractions were collected and analyzed using SDS PAGE (see insert) with A indicating pure toxin and the rest of the gel lanes corresponding to the collected fractions. The first section (0–370 L) describes the loading, and the second section (0–40 L) describes the elution.

attempt to obtain purified, colorless product from this capture step, gradient elution (5 column volumes) from 0 to 0.175 M KCl in 20 mM Tris buffer, pH 7.4, followed by washing with 4 column volumes of 0.175 M KCl was performed from the loading direction in packed mode. The fractions collected were analyzed for absorbence at 280 nm (protein), 340 nm (colored material), and gel electrophoresis. The overall chromatography process is shown in Fig. 4, and comparison of the HPLC profiles of the product using traditional process, step elution, and gradient elution are shown in Fig. 5. The gradient-eluted CRM 9 (Fig. 5C) shows one major peak while the CRM 9 step eluted (Fig. 5B) and the CRM 9 prepared in the conventional way (Fig 5A) show broad and multiple peaks. The lower molecular weight compound is very likely the colored material. Because of the gradient operation, the processing time was longer than the step elution (8 hours), but the amount of the protein obtained was the same (3.0 gr).

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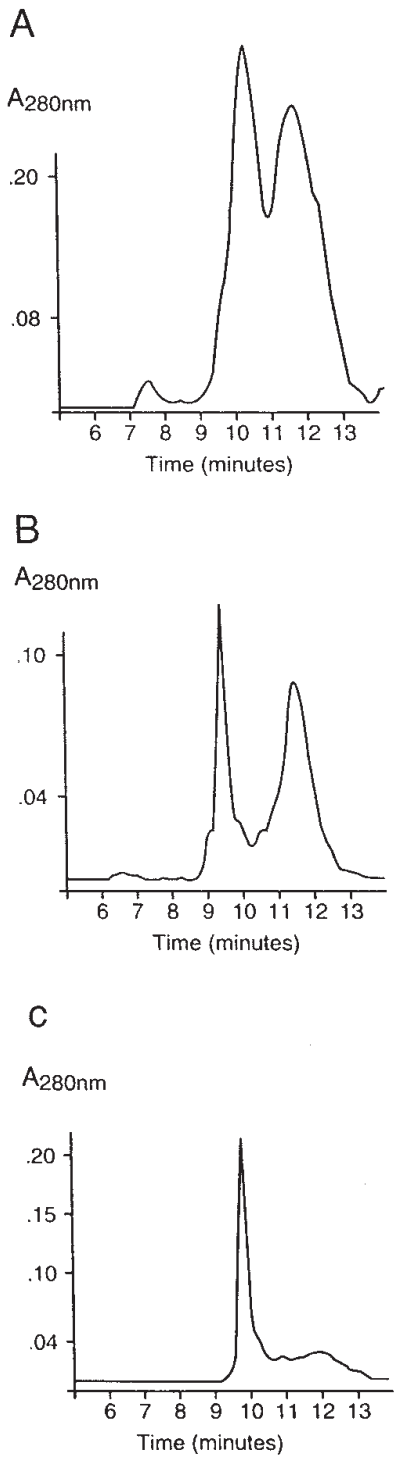

FIG. 5 HPLC analysis of various CRM 9 preparations at the end of the capture step. Fractions were chromatographed on a GF 250 Zorbax column (9.4 × 250 mm) at a flow rate of 1 mL/min. (A) Conventional preparation (50 μg protein), (B) step elution from EBA column (67 μg protein), (C) gradient elution (81 μg protein).

DISCUSSION

The initial recovery process of proteins from microorganisms, whether intracellular or extracellular, involves centrifugation, clarification, concentration, and dialysis before the solution containing the various proteins can be loaded on a packed column, usually an ion exchanger, for further purification. The expanded-bed adsorption concept can significantly simplify the process by replacing the various unit operations (centrifugation, clarification, concentration, and dialysis) with one unit operation. This approach is particularly useful when the recovery process is associated with large volumes of bacterial suspension or bacterial extracts containing compounds such as cell debri, media particles, and antifoam agents. Unlike the known fluidized-bed process (18), the expanded-bed column, because of its limited local mixing, behaves like a true plug-flow column. Therefore it is possible to achieve not only proper concentration but sometimes purification of the required protein.

The working recovery procedure of the extracellular mutant diphtheria toxin CRM 9 is based on the traditional process of centrifugation, filtration, and dialysis before adsorption on DEAE cellulose. The introduction of expanded-bed adsorption followed by the step elution process described here simplified and shortened the recovery of the CRM 9 by eliminating the centrifugation, the clarification, and the extensive dialysis. However, the quality of the product obtained using this approach was not much better than the quality of the product obtained using the traditional approach (Figs. 5A, B), in spite of the fact that the protein was captured by an anion exchanger and not just ultrafiltered as in the traditional process. To obtain the properly purified protein, further purification using an anion exchanger (DEAE) and gel filtration (Zorbax GF 250) steps was needed.

Expanded-bed adsorption is primarily a capture process, and therefore the adsorbed protein is recovered using step elution from the reverse direction of the loading. But, if possible, gradient elution can produce purified product by capitalizing on the fact that the protein is being eluted from an ion exchanger. In general when dealing with a packed column, gradient elution is done from the loading direction, allowing the protein mixture to separate through the column. The unique property of the expanded-bed column is its behavior as a plug-flow column, and therefore the elution should be done from the loading direction and not from the reverse direction as in the step elution mode. Indeed, the gradient elution mode resulted in a better product (Fig. 5C), eliminating the next ion-exchange step.

As was indicated in the Introduction, successful operation of the expanded-bed process depends on achieving a stable bed. Establishing column stability when dealing with a whole cell suspension was not as sensitive as it was

TABLE 2
Capture Step Comparison for Processing 40 L of *Corynebacterium diphtheriae* Culture for Production of CRM 9 Diphtheria Toxin

		Expanded-bed process	
	''Traditional'' process	Step elution	Gradient elution
CRM 9 (gr)	3.3	3.0	3.0
Filtration area (m^2)	0.97	NA	NA
Column volume (L)	NA	4.8	4.8
Processing time (h)	8.0	4.0	8.0
Product volume (L)	2.5	6.0	20.0

when a broken cell suspension was applied on the column, making the capturing process of extracellular product through the expanded bed very attractive.

A comparison between the conventional and the expanded-bed processes is summarized in Table 2. There is a small difference in the amount of protein obtained, but the protein concentration was higher after the conventional process. The reason is the fact that the last step of the conventional process is ultrafiltration, while the last step of the expanded-bed process is column elution. The expanded-bed process took less time. It is important to note here that the traditional process can be shortened by using larger filtration areas for both the ultrafitlration and the diafiltration. The important point, however, is the fact that the expanded-bed gradient elution process produced colorless protein solution, compared with a brownish solution from the traditional process. As a result, only a gel filtration step was needed for final polishing. In this case the main advantage of using the expanded-bed process is not the time or the yield, but the quality of the final product and the simplicity and the reproducibility of the process. The process was operated routinely for production of 20 gr of protein suitable for first-phase clinical trials.

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