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Journal of Chromatography A, 798 (1998) 73–82

JOURNAL OF CHROMATOGRAPHY A

Accelerated recombinant protein purification process development Automated, robotics-based integration of chromatographic purification and analysis

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Abstract

Recovery of recombinant proteins from endogenous, host molecules can be an experimentally intensive and time-consuming task. Often the time to analyze material during development of recovery procedures is the rate-limiting step. Nowadays, modern techniques and equipment are being specifically engineered to make this effort much more efficient. We present a case study to illustrate how a new, automation tool, designed for easy, systematic methods development, can be used for very rapid process and analytical optimization. This tool uses robotics to integrate process development with rapid LC-based analysis requiring no user intervention. The methods and procedures described can be generalized to any recombinant protein recovery campaign. © 1998 Elsevier Science B.V.

Keywords: Automation; Instrumentation; Method development; Process monitoring; Proteins; Recombinant proteins; Protein A

1. Introduction

Chromatography plays an important role in downstream processing of protein-based therapeutics and is exploited for its tremendous selectivity and relatively high throughput characteristics. Since each protein product has unique physicochemical properties and originates in highly variable mixtures of contaminating solutes, depending on the expression system used, development of chromatographic purification processes is inherently an iterative, trial and error procedure. The number of variables that can influence a chromatographic separation are quite large—ranging from column chemistry to buffer make up and sample preparation. Not all variables have equal influence, with the dominant ones subject to the mode of chromatography employed. During development and optimization of chromatographic purification steps, it is common to run experimental campaigns to assess the impact of the dominant variables, an iterative process that can take several months to complete.

Chromatographic methods development is driven by the integration of purification experiments with assays to determine the results of the separation. Historically, chromatographic runs and subsequent fraction analysis were each extremely labor intensive and required several hours to days to perform a single experiment. Recent developments in high-speed chromatography [1–6] combined with automated instrumentation [7–12] have accelerated the execution of purification experiments, with the ability to execute separations in minutes and experimental campaigns in hours. These advances in purification efficiency without similar acceleration of frac-

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0021-9673/98/$19.00
PII S0021-9673(97)01205-3

tion analysis methodologies will not result in acceleration of the overall development process.

Currently, several analytical techniques are employed during process development, with heavy reliance on electrophoresis (PAGE), immunoassay (ELISA) and HPLC-based techniques. To accelerate the development process, rapid assay methodologies should be emphasized, especially during the initial stages, where semiquantitative or yes/no type answers are sufficient. HPLC-based assays are excellent candidates especially in light of recent developments in high-speed techniques, including the use of nonporous particles [13,14], and perfusion chromatography [15,16]. In addition, the utility of HPLC as a rapid analytical technique goes beyond the conventional reversed-phase chromatography approach, taking advantage of other separation modes including affinity [3,9–11] and ion-exchange [17,18].

Once rapid HPLC-based assays are instituted, automation strategies can further increase purification development efficiency. This paper describes an automated development environment for execution of protein purification and subsequent HPLC analysis of collected fractions. The approach combines a computer-aided, automated system for performing experimental campaigns around key chromatographic variables as a means of accelerating purification with On-Board Analysis (OBA) of collected fractions. It utilizes a fully integrated sample-handling robot, for unattended fraction analysis. A case study, describing development of an efficient, scalable method for purification of recombinant protein A from crude *E. coli* lysate, is presented and discussed.

2. Experimental

2.1. Methods and materials

2.1.1. Cell lysis

Fifty grams of lyophilized *E. coli* cells (Sigma, P/N EC-11303) were resuspended in 500 ml of distilled water and spun at 500*g* for 15 min. The supernatant was removed and the pellet was resuspended with 300 ml of lysis buffer (comprised of 50 m*M* Tris·Cl, pH 8.0, 1 m*M* EDTA, and 100 m*M* NaCl). To the solution was added 80 μl of phenylmethylsulfonyl fluoride (PMSF) and 800 μl of lysozyme (10 mg/ml). The mixture was stirred for 20 min. Then 40 mg of deoxycholic acid and 200 ml of DNAase I (1 mg/ml) were added with constant stirring. Membranes were sheared using a dounce and the material was centrifuged at 500*g* for 15 min. The supernatant was removed and diluted to 1000 ml total volume.

2.1.2. Sample preparation

E. Coli lysate prepared above was spiked with 460 mg of protein A (Repligen, P/N RPA-100). The sample was portioned into several 20-, 50- and 100-ml aliquots and frozen at −20°C.

2.1.3. Anti-protein A ImmunoDetection (ID) cartridge preparation

Approximately 2.5 mg of human IgG (h-IgG, Sigma, P/N A-1653) were dissolved in 2.5 ml of phosphate-buffered saline (PBS). The solution was added to 0.5 ml of POROS AL/M resin (PerSeptive Biosystems, P/N 1-6029-03) and gently mixed for 10 h at 25°C. The mixing was stopped, the resin allowed to settle, and the liquid was removed. The Schiff's base formed between the aldehyde on the resin and primary amines on the antibody was reduced by adding 5 ml of 0.5 mg/ml sodium borohydride to the resin and gently mixing for 2 h at 25°C. Again, the mixing was stopped, the resin allowed to settle, and the liquid was removed. To quench any remaining aldehyde groups on the beads, the resin was resuspended in 5 ml of 0.2 *M* Tris buffer at pH 7 with 0.5 mg/ml sodium borohydride and gently mixed for 2 h.

An ID cartridge was prepared by reducing the volume of the h-IgG resin prepared above to 1 ml (50% slurry), connecting an ID Self Pack device (PerSeptive Biosystems, P/N 1-9544-00) to a 30× 2.1-mm polyether ether ketone (PEEK) cartridge fitted with a 0.2-μm outlet frit with cap, placing approximately 150 μl of the slurried resin into the barrel, securing the SelfPack lid to the barrel, and using the Vision Workstation to flow 0.2 *M* Tris through the cartridge while ramping the flow-rate from 0.5 ml/min to 5 ml/min. After flowing at 5 ml/min for approximately 1 min, the flow was stopped, the SelfPack device was removed from the cartridge, and a 0.2-μm frit and cap were affixed to

the cartridge inlet. Two additional cartridges were packed in the same fashion.

2.1.4. Anti-protein A ID assay

The ID assay consisted of equilibrating a 30×2.1-mm (100 μl bed volume) anti-h-Ig G ID cartridge prepared as described above with PBS at 3 ml/min for 10 column volumes (CVs), loading 150 μl of sample, washing contaminants through with 15 CVs of PBS, and eluting with 12 CVs of 12 m*M* HCl. Total assay time was 1.5 min.

2.1.5. Reversed-phase assay

Solvents used for the reversed-phase assay were 0.1% HCl as buffer A and acetonitrile as buffer B. A 100×4.6-mm (1.67 ml bed volume) POROS R2/M (PerSeptive Biosystems, P/N 1-1024-26) was equilibrated with 15% buffer B for 8 CVs at 10 ml/min. Next 400 μl of sample were loaded onto the column and eluted with a 5-CV gradient from 15 to 30% buffer B. The column was cleaned at the completion of each run with a 5-CV gradient from 30 to 80% buffer B.

2.1.6. pH mapping buffers

The buffer solution used in pH mapping studies for cation-exchange chemistries consisted of 33.3 m*M* 4-(2-hydroxyethyl)-1-pipeazineethanesulfonic acid (HEPES), 33.3 m*M* 2-(*N*-morpholino)ethanesulfonic acid (MES) and 33.3 m*M* sodium acetate. A volume (typically 2 l) of this material was prepared and split into two equal parts. The pH of one of the solutions was adjusted to 4.5 and the other was adjusted to pH 7.5. Solvent line A was placed in the pH 4.5 buffer and solvent line B was placed into the pH 7.5 buffer. Solvent line C was placed into a container of distilled water, and solvent line D was placed into a 3 *M* NaCl solution.

The buffer solution used in pH mapping studies for anion-exchange chemistries consisted of 50 m*M* Tris and 50 m*M* bis-Tris propane. A volume (typically 2 l) of this solution was prepared and split into two equal parts. The pH of one of the solutions was adjusted to 6.0 and the other was adjusted to pH 9.0. Solvent line A was placed in the pH 6.0 buffer and solvent line B was placed into the pH 9.0 buffer. Solvent line C was placed into a container of distilled water, and solvent line D was placed into a 3 *M* NaCl solution.

2.1.7. Purification columns

Three 20-μm diameter particle sized, strong cation-exchange, purification columns; POROS HS, SP, and S (PerSeptive Biosystems); were examined for optimal retention and purification factor characteristics. HS and SP contain sulfopropyl binding ligands with the difference being that HS has higher ligand density than SP. POROS S contains sulfoethyl binding ligands. Development column sizes were 100× 4.6 mm. The scale-up column size was 100×10 mm.

3. Results and discussion

Development of a chromatographic purification process to recover recombinant Protein A from *E. coli* lysate served to demonstrate an accelerated development strategy based on (a) robotics for automation and (b) rapid analytical methods for timely feedback at each step. In order to guide the development effort, several design goals were specified as follows:

1. Protein A must be greater than 95% pure before formulation.
2. Overall process yield must be greater than 60%.
3. The purification method must be scalable to accommodate large-scale production.

3.1. Assay development

To accelerate the entire development process, the first objective was to develop fast assays to measure product purity and concentration. For this purpose, perfusion chromatography columns were selected in two different modes of operation. The first was an assay based on immobilized h-IgG (ID Assay) to detect protein A breakthrough during purification development and to identify the location of protein A during elution from the purification column. Protein A in the sample was extracted by the immobilized antibody and then eluted from the analytical cartridge. This assay was used to identify and quantitate functional protein A since only active, intact molecules were able to bind to the h-IgG immobilized on

the column. The assay was linear between 0.5 μg/ml and 1.2 mg/ml (data not shown).

The second assay utilized a 100×4.6-mm column packed with 20-μm POROS R2 reversed-phase material. This assay served to confirm product quantities calculated using the ID assay and to provide purity estimates. Only this second assay could be used for purity determination since a highly absorbing yellow pigment artificially increased the size of the flow-through peak (contaminant peak) in the ID assay leading to incorrect purity determinations. In the reversed-phase assay, the absorber came through in the void and its peak area was excluded from the total integrated peak area, leading to purity measurements that correlated well with other measurements (e.g. electrophoretic gels).

Fig. 1 shows examples of the two assays performed on crude sample. Execution times were approximately 1.5 min for the ID cartridge (left panel) and 3.5 min for the reversed-phase column (right panel). Given the greater speed (shorter assay time) of the ID assay, it was used as the primary assay during process development (On-Board Analysis) to quickly screen fractions for functional product. Product purity in crude sample, as estimated by the reversed-phase assay at 220 nm, was 12.3%.

3.2. On-Board analysis

The Vision Workstation has been designed to combine purification with subsequent injection of collected fractions for HPLC analysis. Two independent methods, one for purification the other for analysis, were programmed on the system and then linked together for automated analysis. If several methods were queued up for unattended operation, fractions could be analyzed at the completion of all preparative runs or between each run. In addition, to reduce the number of assays performed, the system could be programmed to analyze only fractions having absorbance values above a threshold or to analyze every *n*th (1st, 2nd 3rd, etc.) fraction.

The On-Board Analysis technique was used in combination with automated method development software templates such as column chemistry, pH, gradient and loading studies for accelerated method development.

3.3. Column chemistry screening

Three strong cation-exchange chemistries were examined using automated column and pH screening features of the development equipment in conjunction with On-Board Analysis. Cation-exchange resins were chosen as the first chromatographic purification step to eliminate, as early as possible, negatively-charged nucleic acids derived from the host *E. coli* bacteria. Fig. 2 shows the hardware configuration for this work. Each column was run under four different

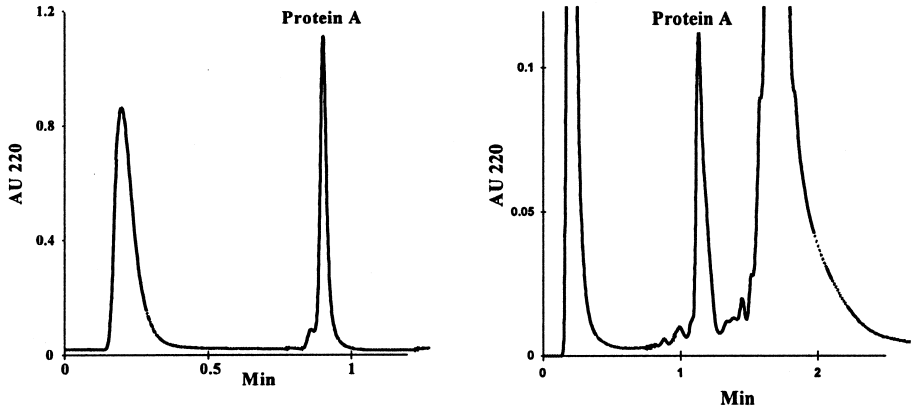

Fig. 1. Anti-hIgG ImmunoDetection (ID; left) and POROS R2/M reversed-phase (right) assays performed on crude *E. coli* lysate. Total assay time for the ID assay was approximately 1.5 min. Total assay time for the reversed-phase assay was approximately 3.5 min. See Sections 2.1.4 and 2.1.5 for specific assay conditions.

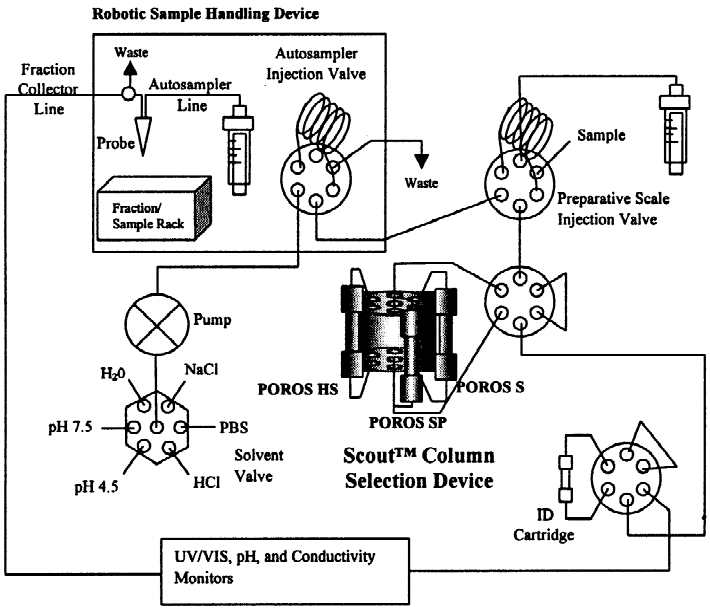

Fig. 2. Plumbing diagram of the Vision Workstation as used in cation-exchange pH mapping studies. Four of the solvents (pH 4.5, pH 7.5, water and NaCl) were used to automatically generate the buffers for the preparative runs. The two remaining buffers (PBS and HCl) were used to execute the ID assays. Typically, preparative samples were introduced by loading the loop on the Preparative Scale Injection Valve using the syringe pump. The sample was then directed over the Scout Column Selection Device connected to the first column valve while the second column valve was placed in the bypass position. The Scout device provided a convenient means for automatically selecting different chemistries during the investigation. Fractions from each run were collected into tubes placed in the Robotic Sample-Handling Device. At the completion of each preparative run, the autosampler probe of the Robotic Sample Handling Device injected aliquots of each fraction into the Autosampler Injection Valve loop for analysis. The sample bypassed the columns on the first column valve and was directed onto the ID cartridge connected to the second column valve. After all fractions were analyzed in this manner, the next preparative run was executed and the process continued until all queued preparative methods and their assays were completed.

pH conditions ranging from 4.5 to 6.5. The software, from a base method written by the operator, automatically generated all twelve methods and the purification runs and their associated assays were executed completely unattended. The effect of pH on separations using the selected HS chemistry is shown in Fig. 3. At pH 4.5, protein A was highly retained and required high salt conditions for elution (shown by the shaded area in the figure). As the pH increased, the product possessed less positive charge and began to elute earlier in the salt gradient. Finally, at pH 6.5, protein A did not bind to the column and eluted in the column void fraction.

A pH of 5.0, was selected for continued development since, under those conditions, the protein A eluted in the middle of the gradient and sample loads could be increased without losing significant product in the flow through. Also, protein A retention was not so strong that extremely high salt concentrations were required for elution.

3.4. Optimization

Once the base chromatographic conditions were determined for the initial separation of protein A from crude lysate, optimization of this step was continued by systematically varying other chromatographic parameters one at a time and examining their effect on the separation.

3.4.1. Gradient optimization

The next step was to investigate whether adjusting gradient slope would increase product resolution. The method, running at pH 5.0 on POROS HS, generated by the system in the previous study was used as the new base method and gradient studies

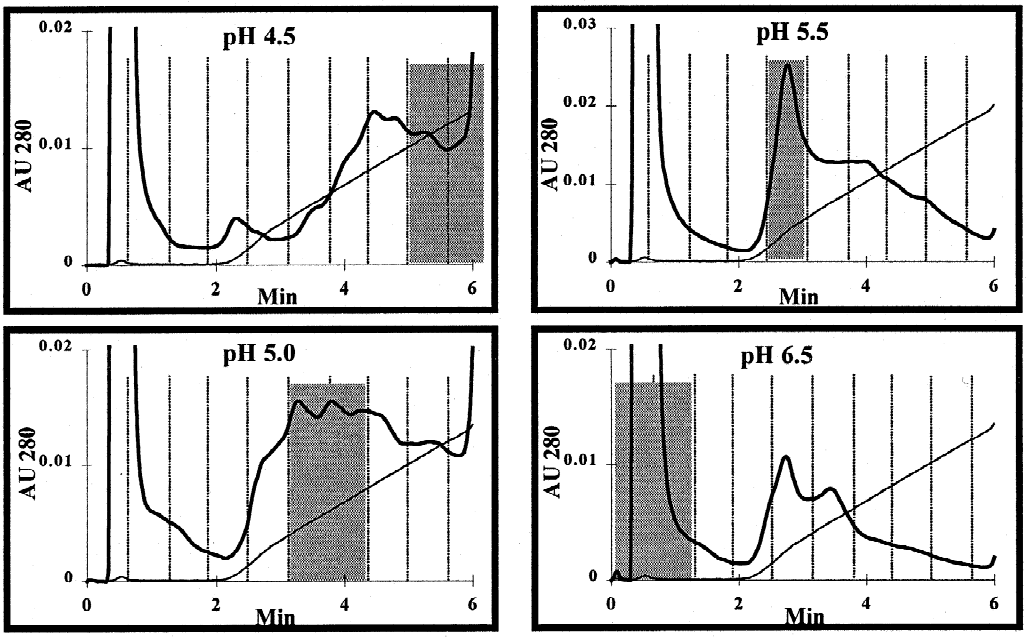

Fig. 3. Effect of varying pH on protein A binding to POROS HS resin. At low pH, protein A bound tightly to the resin and required high amounts of salt to cause it to elute (Protein A location is indicated by the shaded region of each chromatogram). At progressively higher pH values less salt was required for elution until finally at pH 6.5 it no longer bound to the column at all. Protein A location in each chromatogram was automatically determined by employing On-Board Analysis using the ID assay.

were performed using automated software features. Gradient durations of 15, 20, 25 and 30 CVs were entered and the system automatically generated and executed the four different methods immediately followed by fraction analysis after each run. It was not possible to increase product-pool purity higher than 75 to 85% while maintaining yield above 75% for gradient durations longer than 20 CVs. On the other hand, decreasing the gradient duration (increasing the slope) to 15 CVs significantly diminished protein A resolution from adjacent contaminants. This caused product yield to drop below 75% when purity was maintained at 70% or greater. To achieve the overall design goals, it was necessary to maintain a minimum yield of 75% and 70% purity in the first step. Therefore, to keep processing times as short as possible and maintain adequate recovery, the 20-CV gradient duration of the original method was chosen.

3.4.2. Loading study

With the other critical parameters determined, the next step was to determine resin dynamic capacity by performing small-scale loading studies. Using the previous method as the base, sample volumes of 0.5, 1, 3, 5 ml were entered and the system automatically executed the methods and performed On-Board Analysis for fraction analysis. Because no product was observed in the flow-through fractions for any of the four loads, the study was extended to include a 10-ml load. Fig. 4 shows the chromatogram and selected assays for this larger sample load experiment. A small amount (approximately 5% breakthrough) of protein A was observed in the flow-through fractions indicating that roughly 8.5 to 9 ml of crude *E. coli* lysate (9 to 9.5 mg total protein per ml of resin) could safely be loaded onto the column.

3.5. Scale-up

Based on the development work completed above, the optimized method consisted of (1) equilibrating a 100×4.6-mm POROS 20 HS column at 3000 cm/h (8.5 ml/min) with 50 m*M* buffer (pH 5.0), (2) applying maximally 9.5 mg total protein per ml of

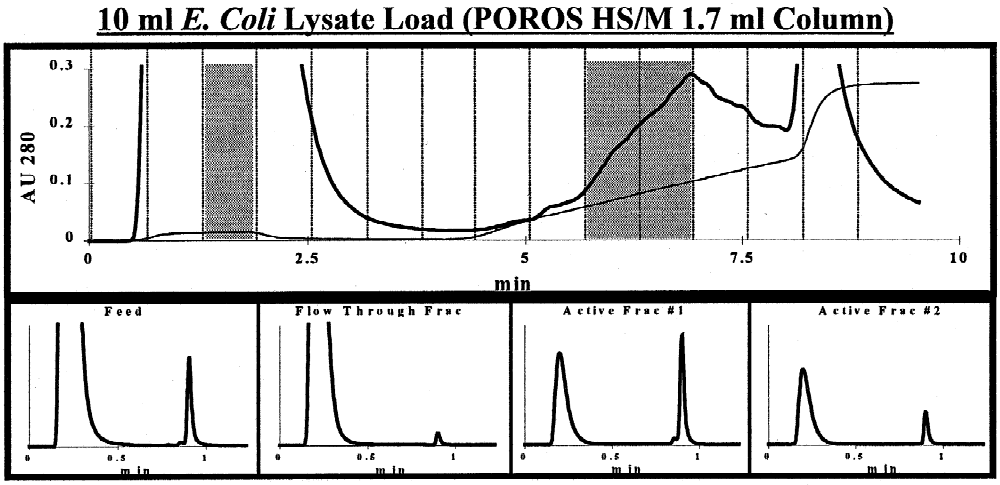

Fig. 4. Chromatograms and selected ID assays for the 10 ml *E. coli* lysate sample load on a POROS HS/M 100×4.6-mm column. The first assay is of the crude sample for reference. Notice no significant protein A in the flow-through fraction (second assay from left in bottom panel) indicating that the column is not saturated with this molecule.

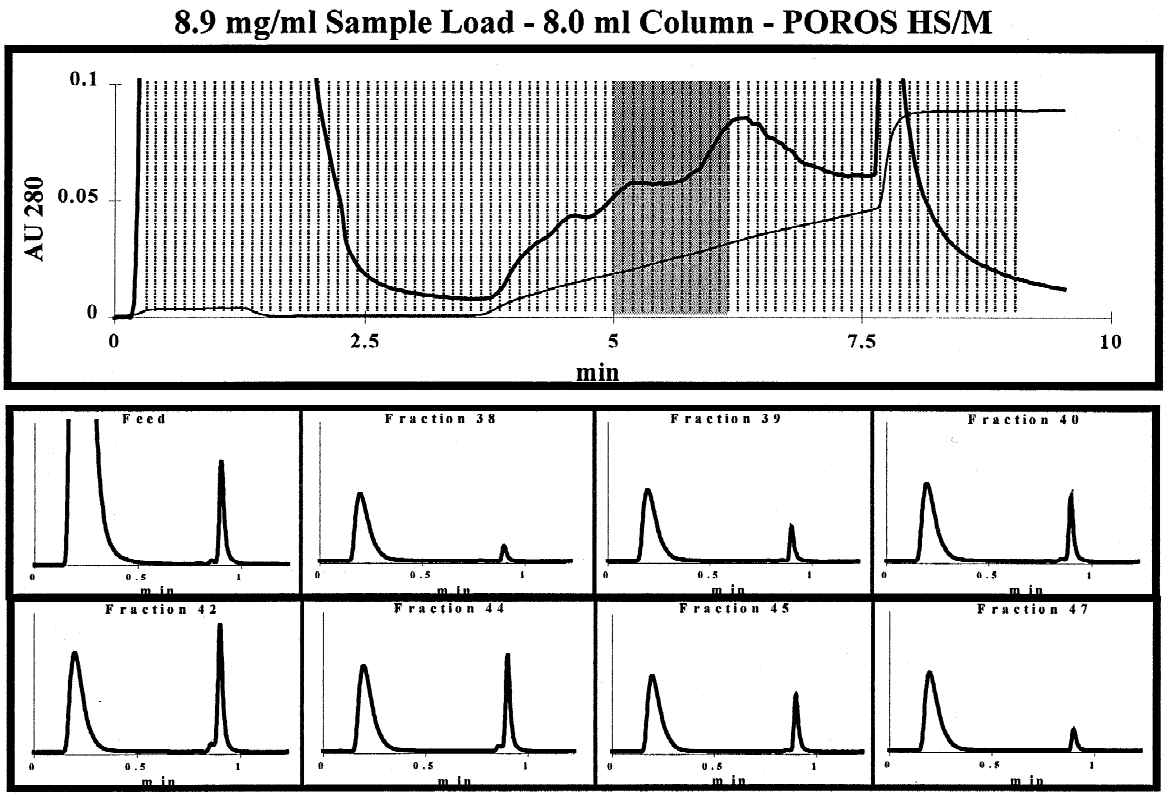

Fig. 5. Scale-up of the cation-exchange step on a 100×10-mm (8 ml) column. On-Board Analysis confirmed that no protein A was present in the flow-through and that its retention time was similar to that observed in small-scale runs (shaded area) indicating that scale-up was performed correctly.

resin of crude *E. coli* lysate, (3) washing contaminants off the column, and (4) eluting the bound protein with a 0–500 m*M* NaCl gradient over 20 CVs. Results of several runs using this procedure indicated 80–85% product recovery and 75–85% purity in the pooled fractions, depending on which fractions were combined. Although the recovery goal was satisfied, the product purity goal was not. Therefore, a second purification step was required. To produce enough partially purified material to develop a second step, the cation-exchange purification was scaled up five-fold. Fig. 5 gives an example of one of the scale-up runs with some selected On-Board Analysis assays. In this case the preparative run was more heavily fractionated to provide higher resolution analysis and more optimal pooling than in the development runs. No product was observed in the flow-through fractions.

3.6. Development of the second purification step

Development and optimization of the second step

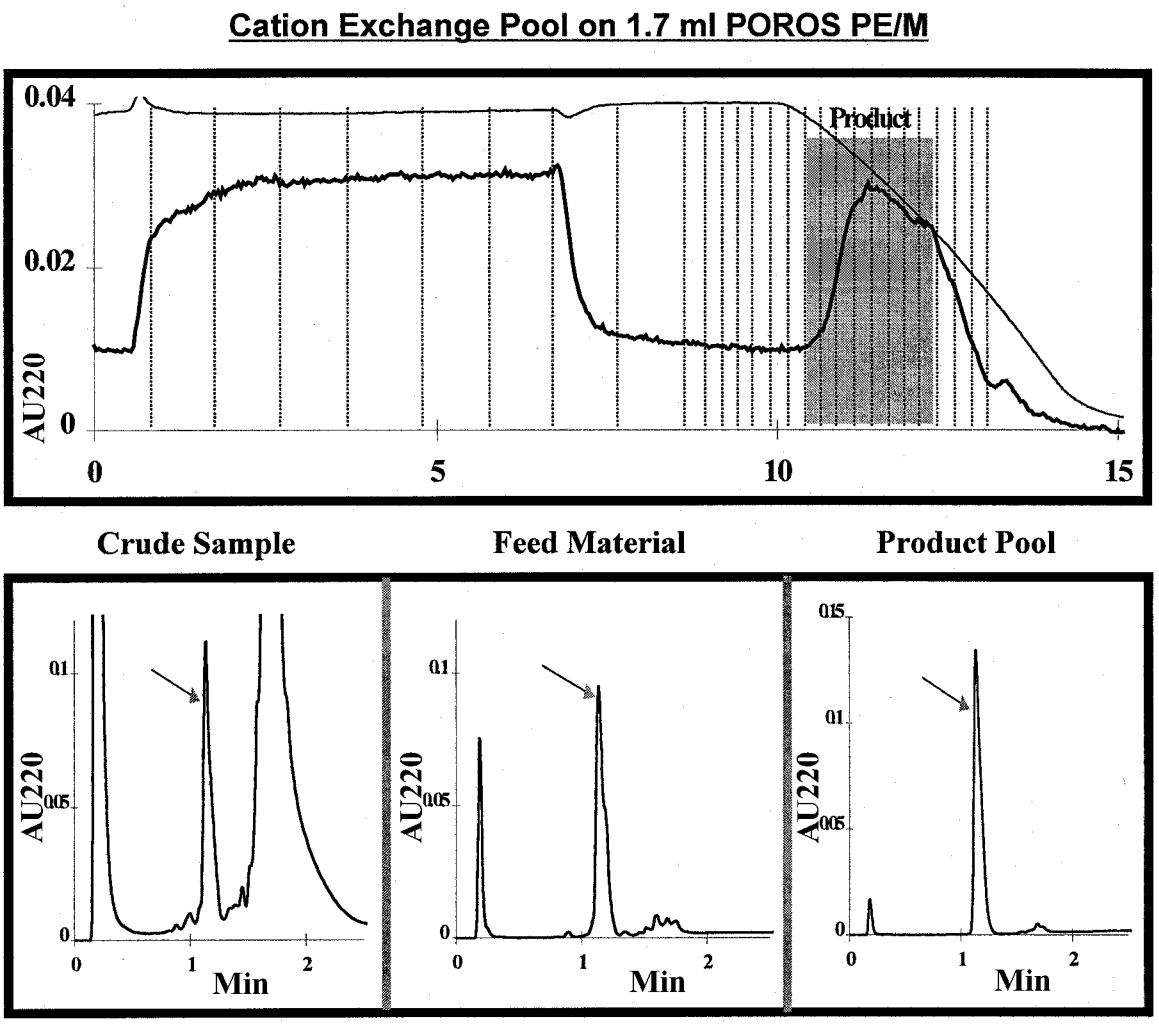

Fig. 6. Chromatogram of a representative Poros PE/M polishing step of partially purified material. The shaded region indicates fractions that were pooled to produce final product. The bottom panel shows reversed-phase assays, from left to right, of the crude sample, partially purified pool from the HS cation-exchange step, and final product pool (shaded fractions) from this step. Product purity went from 12.3% to 85% to 96% during processing.

Table 1
Mass balance of overall process

Material	Yield (%)	Purity (%)	Total protein (mg)	Protein A (mg)
Crude	100	12.3	63	8.3
HS pool	82.4	84.3	8.1	6.8
PE pool	82.3	96.1	5.8	5.6
Overall	67.8	96.1	5.8	5.6

followed a similar course to the first step. Systematic examination of the effectiveness of hydrophobic interaction and anion-exchange chemistries for enhanced purification of partially purified material was undertaken. Better selectivity was achieved with hydrophobic interaction chromatography; hence, POROS 20 PE was chosen for the second step of the purification. The following separation conditions were used:

Column:	100×4.6 mm (1.7 ml) POROS PE/M
Sample:	pH 5.0, partially purified on POROS HS/M, diluted 1:2 on line with 3 *M* $(NH_4)_2SO_4$ to make it 1.5 *M* in $(NH_4)_2SO_4$
Running buffer:	1.5 *M* $(NH_4)_2SO_4$, pH 5
Elution:	1.5–0 *M* $(NH_4)_2SO_4$ in 20 CVs
Linear velocity:	2880 cm/h (8.5 ml/min)
Load:	70 ml

Fig. 6 shows a chromatogram of the second step and reversed-phase assay results illustrating product purity throughout the entire process. The shaded region indicates the pooled fractions. Overall yield for the two steps was 68% with protein A purity of greater than 95%.

4. Conclusions

Table 1 summarizes mass balance results for the two purification steps as well as for the overall process. All of the purification development goals were met or exceeded including demonstration of accelerated development whereby this work was completed in two weeks. It should also be noted that by using high-throughput POROS media and the Vision Workstation, total purification time from crude *E. coli* lysate to purified protein A was 2 h. This included analysis of fractions generated in both purification steps (ID assay for fraction pooling decisions and reversed-phase analysis of all pools for mass balance calculations).

Processing time should not change as the production scale increases provided equipment to supply the required volumetric flow-rates is available. However, modifications to this procedure must be made as the processing scale becomes large. For example, at large-scale it is impractical to fractionate runs to the same extent as at small-scale. Normally only a few fractions can be collected and they are most often based on time or volume into elution. An advantage of accelerating development and automating fraction analysis such as illustrated in this paper is that it is convenient to perform several development runs. Information gathered during those runs affords improved statistical confidence in large-scale cutting decisions.

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