BIOTECHNOLOGY TECHNIQUES
Volume 9 No.6 (June 1995) pp.417-422
Received 24th April

SCX SEPARATION OF RECOMBINANT THROMBOLYTIC PROTEIN FROM COMPLEX BIOLOGICAL FEEDS

Young J. Kim*

Research Institute of Engineering and Technology, Department of Environmental Engineering, Chonnam National University, KwangJu 500-757, Korea (South)

The purification of recombinant thrombolytic protein from a crude fermentation broth mixture was examined in strong cation exchange (SCX) chromatographic system. SCX system was shown to exhibit unique selectivity for the recombinant protein broth mixture. While SCX system showed lower total binding capacity for the recombinant protein than Anti-body Exchange system, it showed sharper separation boundaries in both displacement and step gradient module. The research with the SCX indicated that this separating system could be successfully employed in the displacement mode using DEAE-dextran as the displacer.

INTRODUCTION

The purification of therapeutic proteins from the complex mixtures presents one of the most pressing engineering challenges facing the pharmaceutical and biotechnological industry today. The displacement mode of chromatographic operation offers high throughput and product purity associated with the process. The ability to carry out simultaneous concentration and purification in a single displacement step has significant advantages for downstream processing of bioproducts (Horváth, 1985, Cramer *et al.*, 1987, Frenz and Horváth, 1988, Cramer and Subramanian, 1990). These advantages are particularly significant for the isolation of biopolymers from dilute solutions such as those encountered in biotechnology processes.

The recombinant protein which is to be separated from complex biological mixtures is a form of intact proenzyme. There are few cleavage sites within the amino acid sequence which result in active enzyme possessing thrombolytic activity. The molecule is proprietary and its identity may not be disclosed in this paper. The composition of the recombinant protein crude mixture is consisted of albumins, insulin, transferrin, aprotinin, methotrexate, and bovine serum. Antibody exchanger (ABx) from J.T. Baker (Phillipsburg, NJ, USA) has been tested for the separation of the dilute fermentation mixture (Kim, 1994). ABx is a mixed mode chromatographic material with silica gel support and hydrophilic polymeric coverage to increase stability, eliminate non-specific protein binding, and maximize recovery of mass and immunological activity (Nau, 1989,1990). ABx was shown to exhibit unique selectivity for the recombinant protein broth

mixture, high total binding capacities. Even though ABx showed many advantages for the separation, the displacement with DEAE-dextran showed rather long tail in the displacer zone. While surface interaction of ABx consists of weak cation exchange, mild anion exchange, and mild hydrophobic interactions (Nau, 1989,1990), strong cation exchange (SCX) material has a single-mode cationic interaction. Since DEAE-dextran is well known as a cation exchange displacer (Jayaraman *et al.*, 1993, Gadam *et al.*, 1993), we examined SCX system with a single-mode DEAE-dextran separation module. In this paper, the utility of SCX displacement chromatography for the simultaneous concentration and purification of a recombinant thrombolytic protein from a crude fermentation broth mixture was investigated.

EXPERIMENTAL

Materials

Prepacked POROS R/H reversed phase chromatographic columns (100 x 4.6 mm I.D.) were purchased from PerSeptive Biosystems (Cambridge, MA, USA). Bulk strong cation-exchange (sulfopropyl, 8 μm) material was obtained from Millipore (Waters Chromatography Division, Milford, MA, USA). Waters SCX material was slurry packed into various column dimensions.

Acetonitrile and sodium mono-phosphate were purchased from Fisher Scientific (Fairlawn, NJ, USA). MES, sodium chloride, and all the proteins were obtained from Sigma (St. Louis, MO, USA). 20 kDa diethyl-aminoethyl dextran (DEAE-dextran) and all the electrophoresis supplies including SDS-PAGE gels, molecular weight calibration kits, and PhastGel Silver Kit were supplied by Pharmacia-LKB Biotechnology (Uppsala, Sweden). 5 kDa molecular weight cut-off membranes were purchased from Sartorius (Goettingen, Germany). Polyvinylsulfuric acid potassium salt (PVSK) and indicator o-toluidine blue were obtained from Nalco Chemical Company (Naperville, IL, USA). The crude broth mixture was supplied by Abbott Biotech, Inc. (Needham Heights, MA, USA).

Apparatus

Ultrafiltration of polyelectrolytes was carried out using an Amicon 8050 stirred cell (Amicon, Danvers, MA, USA). A fast protein liquid chromatography (FPLC) apparatus (Pharmacia LKB, Piscataway, NJ, USA) was employed for the protein displacement experiments as well as all frontal and elution chromatographic experiments. This system consisted of two Model P-500 pumps connected to the chromatographic column via a Model MV-7 valve. The column effluent was monitored at 280 nm by a Model UV-M detector and a Pharmacia strip-chart recorder. Fractions of the column effluent were collected with a Model Frac-100 fraction collector for further analysis. The system was controlled using a LCC-500-Plus controller. Lyophilization was carried out using a Model Lyph Lock 4.5 Freeze Dry System (Labconco, Kansas City, MO, USA).

Procedures

Purification of 10 kDa DEAE-dextran displacer. The DEAE-dextran was ultrafiltered to remove salts and other low molecular weight impurities. A 5 kDa molecular weight cut off membrane was employed to purify the displacer. After ultrafiltration, the retentate was lyophilized.

Recombinant protein crude mixture preparation. The crude broth mixture was ultrafiltered using a 0.4 μm hydrophilic membrane and adjusted to pH 5.6 just before experiments.

Operation of displacement chromatograph. A schematic of the displacement chromatograph system employed in this work is illustrated elsewhere (Cramer and Horváth, 1988). In all displacement experiments, the columns were initially equilibrated with the carrier and then sequentially perfused with feed, displacer, and regenerant solutions. The carrier consisted of 0.1 M NaCl in 25 mM MES buffer, pH 6.0. Fractions of the column effluent were collected directly from the column outlet to avoid extra column dispersion of the purified components. Fractions collected throughout the displacement runs were assayed by analytical chromatography. At the end of displacement procedures, the column was regenerated by passing five column volumes of 1.5 M NaCl in 25 mM phosphate buffer, pH 10.0.

Frontal chromatography of recombinant protein crude mixture. To measure the column capacity of the SCX column, crude mixture was flowed through the column. Fractions collected throughout the frontal chromatography mode were assayed by analytical chromatography.

High-performance liquid chromatographic (HPLC) analysis using reversed phase column. Fractions collected during the displacement experiments were analyzed by gradient elution reversed phase chromatography. The analyses were carried out using a 100 x 4.6 I.D. POROS reversed phase column on the FPLC chromatographic system. A six minute linear gradient of 10 - 70 % acetonitrile in 50 mM phosphate buffer, pH 2.2 was employed. Displacement fractions were diluted 20 - 100 fold with the first eluent buffer and 25 µl samples were injected. The flow rate was 1.0 ml/min. The column effluents were monitored at 280 nm and column temperature was maintained at 22 ^{o}C. Quantitative analyses were carried out and the data were used to construct displacement chromatograms.

10 kDa DEAE-dextran displacer analysis. All DEAE-dextran concentrations were analyzed using a colloidal titration assay provided by Nalco Chemical Company. The displacer solution containing 2 drops of o-toluidine was titrated against PVSK to produce a colorimetric change. Linear calibrations were obtained with this titration.

RESULTS AND DISCUSSION

To develop an appropriate analytical assay, a linear gradient reversed phase chromatography of the recombinant thrombolytic protein mixture was developed using a POROS reversed phase convective chromatographic system (PerSeptive Biosystems). A gradient of 2 to 80 per cent acetonitrile in 50 mM phosphate buffer, pH 2.2 in 10 minutes, flow rate of 1.0 ml/min was employed for the base line separation of the mixture as shown in Figure 1. Furthermore, electrophoresis using SDS-PAGE was also employed for the analysis of the preparative separations.

Before the separations of the crude fermentation broth mixture in SCX systems were attempted in large scale, the binding capacity of the SCX material for the recombinant thrombolytic protein was established. SCX material from Millipore company has particle size of 8 µm and density of 0.5 g/ml. A frontal chromatographic experiment of crude mixture was carried out as shown in Figure 2. By monitoring the protein profile in the fractions collected at various stages, the emerging volume of the crude mixture and the total binding capacity for the recombinant protein could be determined (Li-Chan *et al.*, 1990). This chromatogram presents only the recombinant protein content of the collected fractions which was determined by RPLC analytical chromatography. This experiment indicated that the total binding capacity of the SCX

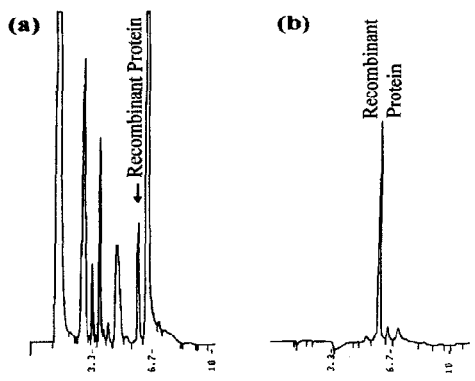

Figure 1. Separation of complex crude mixture (a) and pure recombinant thrombolytic protein (b) by gradient elution chromatography. Column, 100 x 4.6 mm I.D. POROS R/H; feed, 25 µl of crude mixture undiluted (a) and pure recombinant protein 20 times diluted; gradient condition (b), 2 to 80 per cent acetonitrile in 50 mM phosphate buffer in 10 minutes, pH 2.2; flow rate 1.0 ml/min; temperature 22 °C; detector wavelength, 280nm.

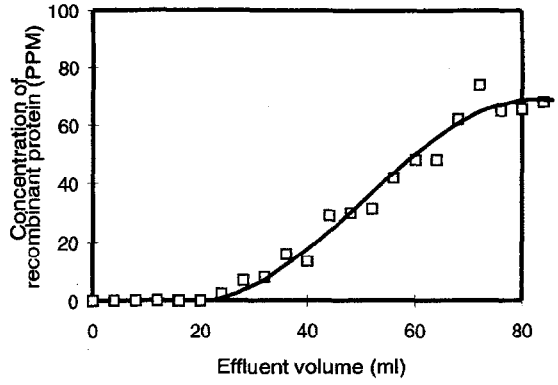

Figure 2. Breakthrough profile for the recombinant protein during application of crude mixture pH 5.6 containing 65 PPM recombinant protein on a 25 x 2 mm I.D. SCX (8 µm); flow rate 0.5 ml/min; temperature 22 °C; fraction volume, 4 ml.

system for the recombinant thrombolytic protein under these conditions was 83 mg protein/g-SCX. An emerging volume of 510 ml of the crude mixture/g-SCX could be introduced into the column before any recombinant protein emerged from the column effluent. The total binding capacity of the SCX system was lower than that of ABx material from J.T. Baker which showed 112 mg protein/g-ABx (Kim, 1994). An emerging volume of SCX was also lower than that of ABx material which showed 1130 ml of the crude mixture/g-ABx. The possible explanation is 1) ABx material consists of harder matrix of silica gel with larger pore size (300 Å) and smaller particle size (5 µm) than SCX material, 2) ABx consists of multi-mode interactions comprising cation exchange, mild anion exchange, and mild hydrophobic interaction, which may contribute stronger affinity than single-mode SCX material (Nau, 1989,1990).

For the effective separation of the recombinant protein, a displacement protocol was examined firstly with 2 ml of purified recombinant protein solution, which was provided as a purified form of 0.381 mg protein/ml. The resulting displacement, shown in Figure 3, indicates that the recombinant thrombolytic protein was well displaced by the DEAE-dextran displacer with relatively sharp tail. The recombinant protein displacement zone appeared to be fully developed and high concentration was achieved.

A scaled-up displacement experiment was then carried out by introducing 327 ml of crude mixture containing approximately 21 mg of the recombinant thrombolytic protein into the 88 x 5.0 mm I.D. analytical SCX column corresponding to 0.9 g-SCX column material. The resulting displacement, shown in Figure 4, indicates that most of the weakly adsorbing impurities emerged by elution chromatography, while the recombinant thrombolytic protein was well displaced by the

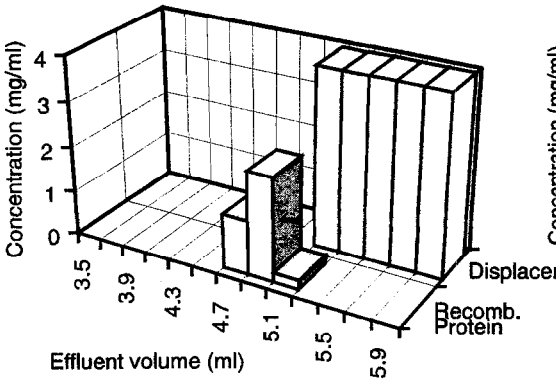

Figure 3. Displacement chromatogram of pure recombinant protein. Column, 88 x 5.0 mm I.D. SCX (8 μm); carrier, 0.1 M NaCl in 25 mM MES buffer, pH 6.0; displacer, 20 mg/ml DEAE-dextran in carrier; flow rate 0.1 ml/min; temperature 22 °C; feed, 0.762 mg recombinant protein in 2 ml carrier; fraction volume, 200 μl.

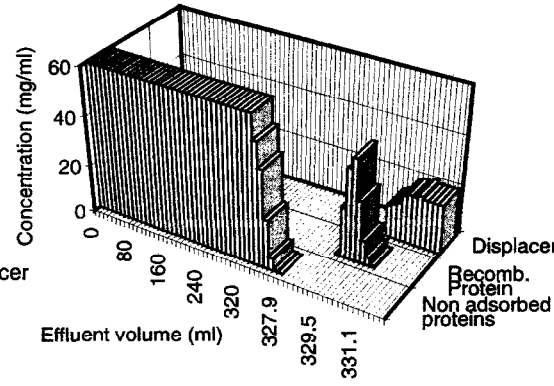

Figure 4. Displacement chromatogram of complex crude mixture. Column, 88 x 5.0 mm I.D. SCX (8 μm); carrier, 0.1M NaCl in 25 mM MES buffer, pH 6.0; displacer, 20 mg/ml DEAE-dextran in carrier; flow rate 0.5 ml/min in feed stage, 0.1 ml/min in wash and displacement stage; temperature 22 °C; feed, 327 ml of complex crude mixture, pH 5.6; fraction volume, 200 μl.

DEAE-dextran displacer. This separation resulted in relatively sharp displacement boundaries and 600 fold concentration of the recombinant protein. It showed sharper boundaries than the ABx displacement, while a little less concentration of the recombinant protein was achieved (Kim, 1994). This experiment demonstrates that DEAE-dextran is a powerful displacer in cation-exchange systems as well as in mainly cationic interaction systems such as ABx. The protein and displacer in the mixed zone can be recycled or separated easily by gel permeation chromatography afterwards. The sharper boundary indicates that the productivity could be higher by recycling less amount of the mixed zone product. Through the RPLC analysis of the fractions collected during the experiment, the weekly adsorbing impurities emerged by elution chromatography contained no recombinant protein in its analytical chromatograms. The purified recombinant protein had no significant amount of other proteins satisfying the purification constraints of higher than 90%, which was confirmed by both RPLC and SDS-PAGE gel electrophoresis.

In order to compare these results to conventional step-gradient operation, the following step gradient experiment was carried out. 50 ml of the crude mixture were loaded onto the column, followed by 5 column volumes of 0.1 M NaCl in 10 mM MES buffer, pH 6.0, to remove weakly retained species. The recombinant thrombolytic protein was then eluted from the column using a step gradient of 0.5 M NaCl in 10 mM MES buffer, pH 6.0. The resulting separation, shown in Figure 5, indicates that this mode of operation was also able to effect adequate separation of the target protein from the impurities. However, under these conditions, the recombinant protein was significantly less concentrated during the separation and the non-linear adsorption resulted in a prolonged tail at the end of the recombinant protein zone. Thus, displacement might

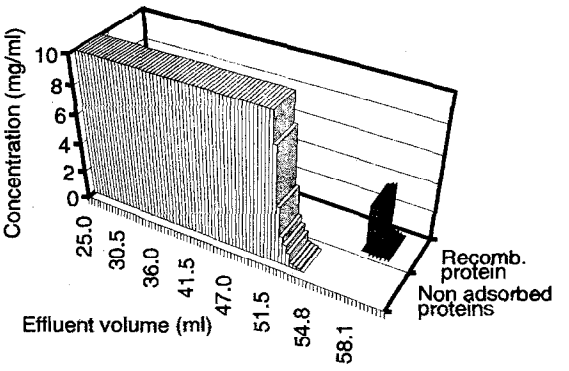

Figure 5. Step-gradient separation of complex crude mixture on 100 x 4.6 mm I.D. SCX (8 μm) column. Chromatographic conditions as in Figure 3 with the displacer replaced by a solution of 500 mM NaCl in 10 mM MES buffer, pH 6.0; feed, 50 ml of complex crude mixture, pH 5.6; fraction volume, 300 μl.

have some advantages with respect to product concentration and recovery.

In conclusion, this research involved the development of appropriate analytical assays and appropriate preparative chromatographic separation schemes including both displacement and step-gradient protocols. While SCX had lower total binding capacity for the recombinant protein than ABx system, the displacement using DEAE-dextran showed sharper boundaries resulting in higher productivity. It is expected that SCX system with DEAE-dextran displacer would have significant advantages for the isolation of biopolymers from dilute solutions such as those encountered in biotechnology processes.

Acknowledgments: Especial thanks to Professor Steven M. Cramer, Department of Chemical Engineering in Rensselaer Polytechnic Institute, for his kind advice and use of research equipment and to Dr. Indu Isaacs in Cambridge Neuro Science Co. for her technical guidance.

REFERENCES

Cramer, S.M., El Rassi, Z., and Horváth, Cs., (1987). *J. Chromatogr.*, **394**, 305-314.

Cramer, S.M., and Horváth, Cs., (1988). *Prep. Chromatogr.*, **1**, 29-49.

Cramer, S.M., and Subramanian, G., (1990). *Sepn. and Purif. Methods*, **19(1)**, 31-91.

Frenz, J., and Horváth, Cs., (1988). High-Performance Displacement Chromatography. In: *High Performance Liquid Chromatography, Advances and Perspectives*, Horváth, Cs., ed. vol. 8, pp. 211-314, Orlando: Academic Press.

Gadam, S.D., Jayaraman, G., and Cramer, S.M., (1993). *J. Chromatogr.*, **630**, 37-52.

Horváth, Cs., (1985). Displacement Chromatography: Yesterday, Today, and Tomorrow. In: *The Science of Chromatography*, Bruner, F., ed. pp. 179-203, Amsterdam: Elsevier.

Jayaraman, G., Gadam, S.A., and Cramer, S.M., (1993). *J. Chromatogr.*, **630**, 53-68.

Kim, Y.J., (1994). *Biotech. Tech.*, **8(7)**, 457-462.

Li-Chan, E., Kwan, L., and Nakai, S., (1990). *J. Dairy Sci.*, **73**, 2075-2086.

Nau, D.R., (1989). *Biochromatogr.*, **4(1)**, 4-18.

Nau, D.R., (1990). Design of Chromatographic Matrices for the Purification and Analysis of Antibodies. In: *High Performance Liquid Chromatography in Biotechnology*, Hancock, W.S., ed. pp. 399-530, New York: John Wiley & Sons.