*Journal of Chromatography*, 596 (1992) 173–180
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 921

# Membrane chromatographic systems for high-throughput protein separations

Joseph A. Gerstner

*Bioseparations Research Center, Howard P. Isermann Department of Chemical Engineering, Rensselaer Polytechnic Institute, Troy, NY 12180-3590 (USA)*

Richard Hamilton

*Millipore Corporation, Bioseparations R, D, & E, Bedford, MA 01730 (USA)*

Steven M. Cramer*

*Bioseparations Research Center, Howard P. Isermann Department of Chemical Engineering, Rensselaer Polytechnic Institute, Troy, NY 12180-3590 (USA)*

(First received September 17th, 1991; revised manuscript received November 8th, 1991)

ABSTRACT

This paper explores the utility of a membrane chromatographic system (MemSep) for analytical and preparative separations of biomolecules. These column systems consist of stacked disks of macroporous cross-linked regenerated cellulose membranes functionalized with ion-exchange moieties. Fluid flow through the macropores of these membranes results in rapid mass transport to and from the adsorbent surface. Elution and frontal experiments demonstrated that these systems were relatively insensitive to flow-rate. Linear gradient experiments under analytical conditions indicated that rapid separations could be readily carried out. Preparative-scale separations of proteins on ion-exchange MemSep systems were scaled-up with respect to flow-rate and mass loading with minimal adverse effect on bioproduct purity. A cation-exchange CM MemSep 1010 device was able to concentrate and purify 30 mg and 15 mg of proteins in 3 min when operated in the step and linear gradient modes, respectively. The design of these membrane chromatographic systems enables efficient gradient elution of proteins under elevated flow-rate and mass loading conditions.

## INTRODUCTION

The field of preparative chromatography has seen a period of rapid growth in the last decade [1–3]. The development of novel stationary phases with increased selectivities has enabled the separation of complex mixtures of biomolecules. In addition, the use of preparative chromatographic columns in alternative modes of operation have greatly increased the throughputs attainable with these systems [4–9]. There is currently great interest in redesigning the morphology of stationary phase materials to enable rapid capture steps in the early stages of downstream processing of biopharmaceuticals. Several hollow-fiber-based membrane systems have been reported for affinity, ion-exchange and reversed-phase separations of proteins [10–12]. While these systems are potentially quite useful for bind–release separations, their use for conventional chromatographic operations have met with minimal success to date. A recent development in the chromatographic engineering field has been the emergence of perfusion chromatography [13–15]. These systems have been reported to exhibit convective fluid flow in the macropores of the support resulting in flow-rate-insensitive separations under linear

0021-9673/92/$05.00 

gradient conditions. Another flow-rate-insensitive chromatographic technology is non-porous supports which have shown great utility for analytical biotechnology applications [16–23].

Although membrane systems have been used for adsorption–desorption operations for quite some time it is only recently that membrane chromatographic systems have been developed for high resolution gradient operations. Tennikova *et al.* [24] have demonstrated the utility of methacrylate copolymeric membranes for hydrophobic interaction chromatographic gradient separations of proteins. A low pressure (0–100 p.s.i.) bioseparations system consisting of stacked disks of macroporous, cross-linked regenerated cellulose membranes in a column configuration has been employed for analytical gradient chromatography [25,26]. Mass transport to and from the adsorbent surfaces in these membrane chromatographic systems is facilitated by convective transport in the macropores (12 000 Å). In this paper, we will investigate the ability of these stacked membrane chromatographic systems to carry out efficient gradient elution of proteins under elevated flow-rate and mass loading conditions.

## EXPERIMENTAL

### *Materials*

Carboxymethyl (CM) MemSep and diethylaminoethyl (DEAE) MemSep 1010 (1.0 × 2.8 cm I.D.) and 1000 (0.5 × 1.9 cm I.D.) ion-exchange membrane separation systems were supplied by Millipore (Bedford, MA, USA). Sodium acetate, sodium phosphate dibasic, sodium chloride, Tris–HCl, α-chymotrypsinogen A, cytochrome *c*, bovine serum albumin (BSA), human transferrin, human serum albumin (HSA), conalbumin, α-lactoalbumin, ovalbumin, trypsin inhibitor and lysozyme were purchased from Sigma (St. Louis, MO, USA).

### *Apparatus*

Two chromatographic systems were employed in this work. Experimental system 1 consisted of a fast protein liquid chromatography (FPLC) system (Pharmacia-LKB Biotechnology, Uppsala, Sweden) which included a Model LCC-500-Plus controller, two Model P-500 pumps, a 0.6-ml mixer, and an MV-7 injection valve. The MemSep effluent was monitored at 280 nm by a Model UV-M detector and a Pharmacia strip-chart recorder. Fractions of the effluent were collected with a Model Frac-100 fraction collector. System 1 was used for all experiments unless otherwise noted.

Experimental system 2 consisted of a Waters 650 advanced protein purification system (Waters Chromatography Division, Millipore, Milford, MA, USA) which included a Model 600E controller, a Model 650 four buffer solvent system and a Model 484 variable-wavelength UV detector.

### *Procedures*

*Height equivalent to a theoretical plate (HETP) for MemSep.* The HETP values for the DEAE and CM MemSep 1010 systems were evaluated using human transferrin and cytochrome *c*, respectively. These experiments were carried out under unretained conditions to evaluate the dispersive effects due to non-uniform fluid flow in these systems. Mobile phase conditions for the DEAE and CM MemSep analyses were 25 m*M* sodium phosphate, pH 7.5, containing 500 m*M* NaCl and 400 m*M* sodium acetate, pH 5.5, respectively.

*Dynamic binding capacities of DEAE and CM MemSeps.* The effect of flow-rate on the dynamic binding capacity of the DEAE MemSep 1000 was evaluated using sequential perfusions of: 44.8 ml of 1 mg/ml of HSA in 20 m*M* Tris, pH 8.0; 11.2 ml of 20 m*M* Tris, pH 8.0; and 11.2 ml of 20 m*M* Tris, pH 8.0, containing 1 *M* NaCl. Flow-rates of 1.4, 2.8, 5.6, 11.2 and 22.4 ml/min were employed. The shape of the resulting breakthrough curve as well as the total protein bound at each flow-rate was evaluated to investigate the effect of flow-rate on "dynamic" protein binding (Experimental system 2).

The binding capacities of the DEAE MemSep 1010 and the CM MemSep 1010 for BSA and lysozyme, respectively, were also determined by frontal chromatography under strongly retained conditions. 5.0 mg/ml BSA in 25 m*M* Tris, pH 8.5, and 5.0 mg/ml lysozyme in 25 m*M* sodium acetate, pH 5.5, were used for the DEAE and CM MemSeps, respectively.

### *Linear gradient*

*Anion exchange.* A 5-mg amount of a protein mixture containing conalbumin, human transferrin, α-lactoalbumin, ovalbumin and trypsin inhibitor was separated using a linear gradient of 0 to 0.2 *M*

NaCl in 20 m*M* Tris, pH 8.1 on a DEAE MemSep 1010. Gradient times of 30, 20 and 10 min were employed at a volumetric flow-rate of 10 ml/min (Experimental system 2).

*Effect of flow-rate.* A 25-μl feed solution consisting of 3.0 mg/ml α-chymotrypsinogen A, 4.0 mg/ml cytochrome *c* and 3.0 mg/ml lysozyme in 25 m*M* sodium acetate, pH 5.5, was separated using a linear gradient of 0 to 400 m*M* sodium acetate, pH 5.5, on the CM MemSep 1010. Volumetric flow-rates of 2, 5 and 10 ml/min were employed to evaluate the efficacy of the CM MemSep in linear gradient chromatography under various flow-rate conditions. Gradient volume was held constant at 30 ml at each flow-rate by appropriate adjustment of the gradient time.

*Effect of mass loading.* A feed solution containing 5 mg/ml each of α-chymotrypsinogen A, cytochrome *c* and lysozyme in 25 m*M* sodium acetate, pH 5.5, was employed to evaluate the effect of mass loading on the performance of the CM MemSep 1010 operated in the linear gradient mode. Injection volumes ranging from 25 μl to 1 ml were used at flow-rates ranging from 2–10 ml/min. A linear gradient of 0 to 400 m*M* sodium acetate, pH 5.5, with a gradient volume of 30 ml was used to elute the proteins.

*Linear elution studies.* Linear elution chromatography of α-chymotrypsinogen A, cytochrome *c* and lysozyme was carried out at various concentrations of sodium acetate, pH 5.5, to examine the effect of salt on protein retention time. From these experiments, a plot of log capacity factor (*k'*) *vs.* log [$Na^+$] was generated in order to predict appropriate "windows" of salt concentration for the step gradient experiments.

*Multiple step gradients.* Feed solutions of 1 ml containing 6, 15 and 30 mg total protein of α-chymotrypsinogen A, cytochrome *c* and lysozyme in 25 m*M* sodium acetate, pH 5.5, were separated by step gradient chromatography on the CM MemSep 1010 module. The CM MemSep was initially equilibrated with distilled water. The buffered protein feed solutions were introduced into the CM MemSep 1010 after which the first step change in salt concentration was performed. The subsequent step changes in salt concentration were executed when the eluting peak obtained a maximum value. All experiments were performed at a flow-rate of 5.0 ml/min. The 30-mg experiment was also carried out at a flow-rate of 10 ml/min. The following step gradients were carried out: 6 mg injection: 100, 175 and 400 m*M* sodium acetate; 15 mg injection: 90, 165 and 400 m*M* sodium acetate; 30 mg injection: 60, 150 and 400 m*M* sodium acetate.

## RESULTS AND DISCUSSION

### *HETP for DEAE and CM MemSep*

In order to evaluate the inherent efficiency of these stacked membrane chromatographic columns a traditional HETP evaluation was carried out. While the exact meaning of "plate height" in such a membrane chromatographic column is unclear, it nonetheless served as a comparative measure of efficiency in these systems. Since these modules are typically employed for gradient chromatography where adsorption–desorption kinetics have minimal effects on system efficiency, we examined the dispersion due solely to the fluid flow characteristics of the system. The resulting HETP plots for unretained human transferrin and cytochrome *c* on the DEAE MemSep and CM MemSep modules, respectively, are presented in Fig. 1. Under these conditions, the plate heights were on the order of 20 μm and were relatively insensitive to flow-rate. Thus, there appears to be minimal dispersive contributions from fluid flow irregularities in these systems under typical operating conditions of 1–10 ml/min.

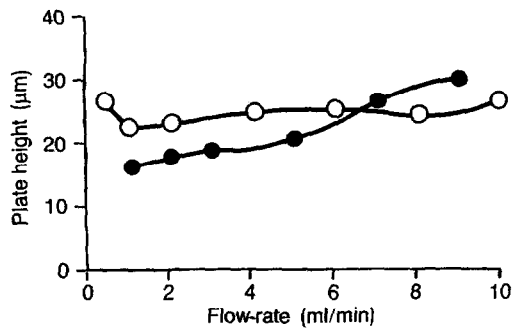


Fig. 1. Height equivalent to a theoretical plate (HETP) *vs.* flow-rate for DEAE and CM MemSeps 1010 for unretained proteins. DEAE MemSep: human transferrin (●) in 25 m*M* sodium phosphate, pH 7.5, containing 500 m*M* sodium chloride; CM MemSep: cytochrome *c* (○) in 400 m*M* sodium acetate, pH 5.5.

*Binding capacity of DEAE and CM MemSeps*

The effect of flow-rate on the dynamic binding capacity of the DEAE MemSep 1000 was evaluated as described in the Experimental section. The resulting chromatographic profiles are shown in Fig. 2. As seen in the figure, the breakthrough fronts were relatively sharp at flow-rates of 1.4 and 11.2 ml/min with a slightly more diffuse front occurring at 22.4 ml/min. The dynamic binding capacities of these systems remained essentially constant at approximately 35 mg as shown in Table I. Thus, these membrane chromatographic systems can be readily scaled-up with respect to flow-rate when operated in the adsorption–desorption mode.

The binding capacities of the DEAE MemSep 1010 for BSA and the CM MemSep 1010 for lysozyme were also measured by frontal chromatography as described in the experimental section. Under these strongly retained conditions, the binding capacity of the DEAE MemSep 1010 was 34.2 mg BSA. For the CM MemSep 1010, the binding capacity was 67.2 mg lysozyme. While these represent lower binding capacities than generally obtained with conventional chromatographic supports, the ability to operate at elevated flow-rates makes these systems attractive for high-throughput capture operations in downstream bioprocessing.

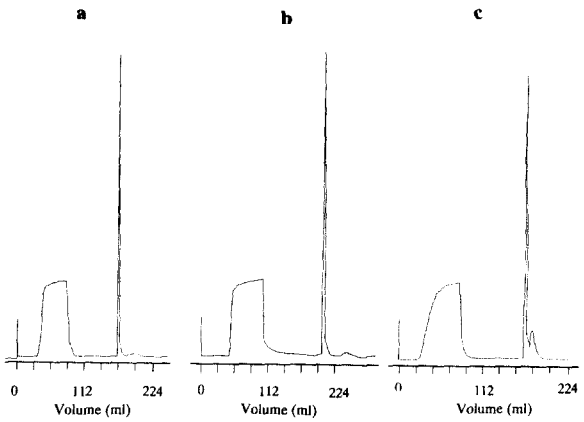


Fig. 2. Effect of flow-rate on dynamic binding capacity of the DEAE MemSep 1000. Feed: 44.8 ml of 1 mg HSA/ml in 20 m*M* Tris, pH 8.0; wash: 11.2 ml of 20 m*M* Tris, pH 8.0; eluent: 11.2 ml of 20 m*M* Tris, pH 8.0, containing 1 *M* NaCl; flow-rates: (a) 1.4, (b) 11.2 and (c) 22.4 ml/min.

TABLE I

DYNAMIC BINDING CAPACITY OF DEAE MemSep 1010 FOR HUMAN SERUM ALBUMIN

| Flow-rate (ml/min) | Binding capacity (mg) |
|---|---|
| 1.4 | 37.8 |
| 2.8 | 33.6 |
| 5.6 | 34.7 |
| 11.2 | 35.8 |
| 22.4 | 34.8 |

*Linear gradient*

The DEAE MemSep 1010 system was employed in the linear gradient mode for the analytical separation of a five-component protein mixture under various flow-rate conditions. The resulting separations for gradient runs ranging from 30 to 10 min are shown in Fig. 3. These results demon-

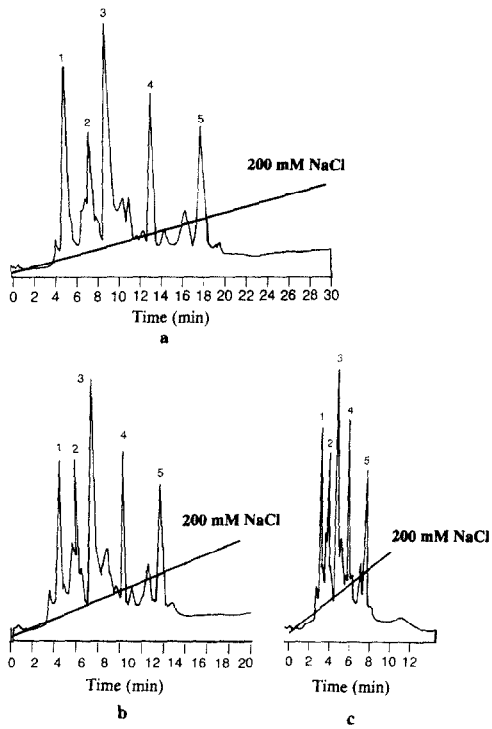


Fig. 3. Linear gradient separation of a five-component protein mixture on DEAE MemSep 1010. Feed: 1 = conalbumin; 2 = human transferrin; 3 = α-lactoalbumin; 4 = ovalbumin; 5 = trypsin inhibitor (5 mg total protein); flow-rate: 10 ml/min; gradient conditions: 0 to 200 m*M* NaCl in 20 m*M* Tris, pH 8.1, in 30 min (a), 20 min (b) or 10 min (c).

strate that these systems can indeed be employed for linear gradient separations of complex protein mixtures.

The CM MemSep 1010 column was employed in a series of linear gradient experiments to examine the effects of flow-rate and mass loading on the performance of the system. These experiments were carried out under conditions where baseline resolution of the proteins was maintained. Linear gradient conditions were established using analytical-scale protein loading (0.25 mg total protein). The resulting chromatogram, depicted in Fig. 4a, demonstrates that these cation-exchange MemSep systems can also be readily employed for linear gradient protein separations. In order to examine the effect of flow-rate in these systems, the experiment was repeated at volumetric flow-rates of 5 and 10 ml/min. The resulting chromatograms, shown in Fig. 4b and c, indicate that these analytical linear gradient separations can be readily scaled-up with respect to flow-rate with minimal adverse effect on the separation efficiency. Clearly, these ion-exchange membrane systems have significant potential for rapid analytical chromatographic applications. Furthermore, the ability to carry out rapid analytical experiments will significantly shorten method development time with these membrane chromatographic systems.

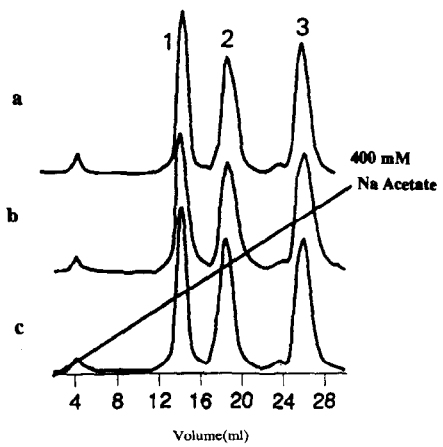


Fig. 4. Linear gradient separation of a three-component protein mixture on CM MemSep 1010. Feed: 1 = α-chymotrypsinogen A; 2 = cytochrome *c*; 3 = lysozyme (0.25 mg total protein). Gradient conditions: 0–400 m*M* sodium acetate, pH 5.5; in (a) 15 min at a flow-rate of 2.0 ml/min, (b) 6 min at a flow-rate of 5.0 ml/min or (c) 3 min at a flow-rate of 10.0 ml/min.

While these results indicate that the MemSep systems can be a powerful tool for analytical chromatography, in order for it to have utility for preparative separations, it must be capable of high-throughput bioseparations. Accordingly, the linear gradient experiment on the CM MemSep 1010 was repeated at mass loadings in the range of 0.75–15 mg total protein. As seen in Fig. 5, this preparative separation was readily scaled-up to 15 mg total protein with minimal adverse effect on the separation efficiency.

In an effort to further increase the throughput in this linear gradient system, the 15-mg separation was repeated at 10 ml/min resulting in the chromatogram shown in Fig. 6. The gradient time for this separation of 15 mg of proteins was 3 min. These results demonstrate that these membrane chromatographic systems can be readily employed in the linear gradient mode at both elevated mass loadings and volumetric flow-rates for high-throughput bioseparations.

*Multiple step gradients*

In order to establish appropriate conditions for multiple step gradient protein separations, linear

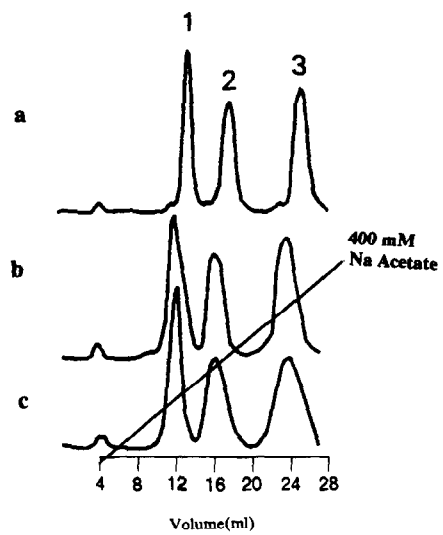


Fig. 5. Linear gradient separation of a three-component protein mixture on CM MemSep 1010 at elevated protein loadings. Feed: 1 = α-chymotrypsinogen A; 2 = cytochrome *c*; 3 = lysozyme. Gradient conditions: 0–400 m*M* sodium acetate, pH 5.5 in 15 min, flow-rate: 2.0 ml/min. Total protein loadings of 0.75 mg (a), 7.5 mg (b) and 15.0 mg (c).

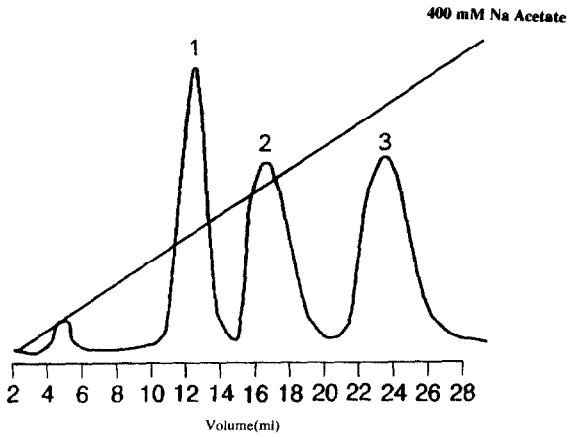


Fig. 6. Linear gradient separation of a three-component protein mixture on CM MemSep 1010 at both elevated flow-rate and protein loading. Feed: 1 = α-chymotrypsinogen A; 2 = cytochrome *c*; 3 = lysozyme (15 mg total protein). Gradient condition: 0–400 m*M* sodium acetate, pH 5.5 in 3 min, flow-rate: 10.0 ml/min.

elution studies were carried out. Various concentrations of sodium acetate were used to examine the effect of salt on the retention of α-chymotrypsinogen A, cytochrome *c* and lysozyme in the CM MemSep 1010. The resulting plot of log *k'* *vs.* log [$Na^+$] is shown in Fig. 7. As seen in the figure, concentrations of 100 m*M* and 175 m*M* sodium acetate result in negligible *k'* values for α-chymotrypsinogen A and cytochrome *c*, respectively. For lysozyme, any concentration above 300 m*M* sodium acetate resulted in rapid elution of the protein. Accordingly, these "windows" of salt concentration were employed in

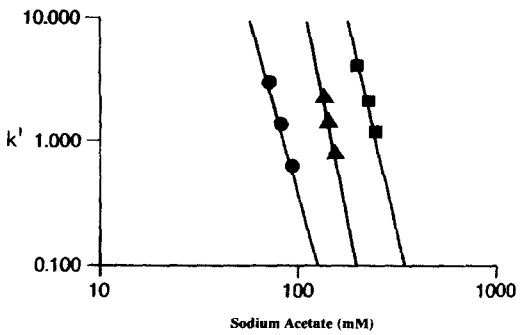


Fig. 7. Effect of salt on protein capacity factors on CM MemSep 1010. ● = α-Chymotrypsinogen A; ▲ = cytochrome *c*; ■ = lysozyme.

the multi-step gradient separation of 6 mg total protein as shown in Fig. 8a. Under these conditions, the multi-step gradient procedure resulted in complete separation of the three proteins in approximately 5 min.

The separation was then scaled-up with respect to mass loading to investigate the capacity of these systems for multiple step-gradient operation. As expected, at higher mass loadings the non-linearity of the protein isotherms resulted in elution of the proteins at lower salt concentrations. Accordingly, the step gradient conditions were appropriately modified, as the mass loading was increased, to maintain baseline separation. For a total protein loading of 15 mg, the "windows" employed for the multi-step gradient were changed to 90, 165 and 400 m*M* sodium acetate. As seen in Fig. 8b, under this mass loading, baseline separation of the proteins was achieved with a separation time of approximately 5 min.

At a protein loading of 30 mg, a significant reduction in the initial step change was required to eliminate contamination of the α-chymotrypsinogen A by the cytochrome *c*. For this mass loading, the "windows" employed for the multi-step gradient were changed to 60, 150 and 400 m*M* sodium

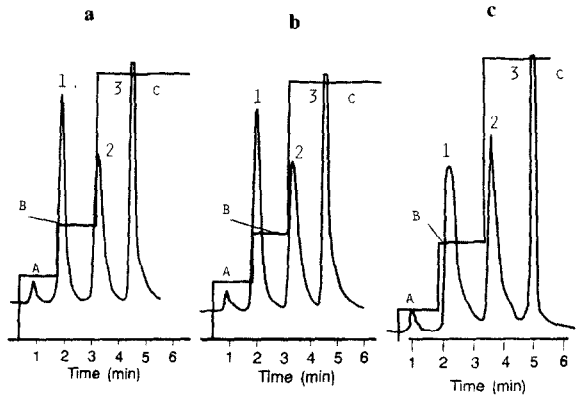


Fig. 8. Step gradient separation of a three-component protein mixture on CM MemSep 1010. Feed: 1 = α-chymotrypsinogen A; 2 = cytochrome *c*; 3 = lysozyme. Flow-rate: 5.0 ml/min. (a) Total protein: 6 mg. Gradient conditions: sequential step changes of 100 (A), 175 (B) and 400 (C) m*M* sodium acetate, pH 5.5. (b) Total protein: 15 mg. Gradient conditions: sequential step changes of 90 (A), 165 (B) and 400 (C) m*M* sodium acetate, pH 5.5. (c) Total protein: 30 mg. Gradient conditions: sequential step changes of 60 (A), 150 (B) and 400 (C) m*M* sodium acetate, pH 5.5. Flow-rate: 5.0 ml/min.

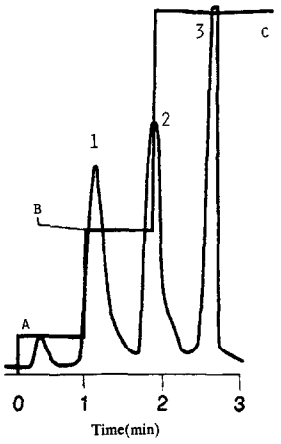


Fig. 9. Step gradient separation at elevated flow-rate and mass loading of a three-component protein mixture on CM MemSep 1010. Conditions as in Fig. 8c with the exception of flow-rate: 10.0 ml/min.

acetate. While, the reduced initial step gradient resulted in a more diffuse α-chymotrypsinogen A peak than in the previous experiments, baseline separation of the proteins was achieved with a separation time of approximately 6 min (Fig. 8c).

To further increase the throughput of these systems, the 30-mg step gradient separation was repeated at a volumetric flow-rate of 10 ml/min, resulting in the chromatogram shown in Fig. 9. Again, baseline separation of the proteins was achieved, now with a separation time of about 3 min. In fact, the bioproduct throughput could be readily increased by further optimization of the step gradient conditions as well as relaxation of the baseline resolution constraint. In addition, recovery of the feed proteins was found to be greater than 95% in all gradient experiments. If one assumes a re-equilibration time of 2 min (corresponding to 20 ml or 5 column volumes), this system would have a throughput of 360 mg purified protein/h. Thus, the CM MemSep 1010 module can be employed for high-throughput protein purification when operated in the multiple step gradient mode.

## CONCLUSIONS

In this report we have demonstrated that chromatographic systems consisting of stacked adsorptive membranes can be employed for high-throughput protein purification. Linear and step gradient chromatographic separations of proteins on ion-exchange membrane systems were successfully scaled up with respect to flow-rate and mass loading with minimal adverse effect on resolution or bioproduct purity. The ability to operate these systems at elevated flow-rates enables rapid analytical chromatography as well as significantly shortened methods development time. In addition, the ability of these efficient, low-pressure systems to operate at high mass loadings in both the linear and step gradient modes of operation make these systems particularly attractive for preparative chromatographic applications.

## ACKNOWLEDGEMENTS

This work was supported by Grant No. CTS-8957746 from the National Science Foundation and by Millipore Corporation.

## REFERENCES

1 S. M. Cramer and G. Subramanian, in G. Keller and R. Yang (Editors), *New Directions in Sorption Technology*, Butterworth, Boston, MA, 1988, pp. 187–226.
2 G. Guiochon and A. Katti, *Chromatographia*, 24 (1987) 165.
3 E. Grushka (Editor), *Preparative-Scale Chromatography (Chromatographic Science Series*, Vol. 46), Marcel Dekker, New York, 1989.
4 S. M. Cramer and G. Subramanian, *Sep. Purif. Methods*, 19 (1990) 31.
5 J. Frenz and Cs. Horváth, in Cs. Horváth (Editor), *High-Performance Liquid Chromatography —Advances and Perspectives*, Vol. 5, Academic Press, Orlando, FL, 1988, pp. 211–314.
6 Cs. Horváth, in F. Bruner (Editor), *The Science of Chromatography (Journal of Chromatography Library*, Vol. 32), Elsevier, Amsterdam, 1985, pp. 179–207.
7 K. Veeraragavan, A. Bernier and E. Bracndli, *J. Chromatogr.*, 54 (1991) 207.
8 J. Newburger and G. Guiochon, *J. Chromatogr.*, 523 (1990) 63.
9 R. S. Hodges, T. W. L. Burke and C. T. Mant, *J. Chromatogr.*, 444 (1988) 349.
10 S. Brandt, R. A. Goffe, S. B. Kessler, J. L. O'Connor and S. E. Zale, *Biotechnology*, 6 (1989) 779.
11 H. Ding, M. C. Yang, D. Schisla and E. L. Cussler, *AIChE J.*, 35 (1989) 814.
12 H. Ding and E. L. Cussler, *Biotechnol. Prog.*, 6 (1990) 472.
13 N. B. Afeyan, S. P. Fulton, N. F. Gordon, I. Mazsaroff and L. Varady, *Biotechnology*, 8 (1990) 203.
14 L. L. Lloyd and F. P. Warner, *J. Chromatogr.*, 512 (1990) 365.
15 N. B. Afeyan, N. F. Gordon, I. Mazsaroff, L. Varady, S. P.

Fulton, Y. B. Yang and F. E. Regnier, *J. Chromatogr.*, 519 (1990) 1.
16 D. J. Burke, J. K. Duncan, L. C. Dunn, L. Cummings, C. J. Siebert and G. S. Ott, *J. Chromatogr.*, 353 (1986) 425.
17 K. K. Unger, G. Jilge, J. N. Kinkel and M. T. Hearn, *J. Chromatogr.*, 359 (1986) 61.
18 Y. Kato, T. Kitamura, A. Mitsui and T. Hashimoto, *J. Chromatogr.*, 398 (1997) 327.
19 L. Varady, K. Kalghatgi and Cs. Horváth, *J. Chromatogr.*, 458 (1988) 207.
20 K. Kalghatgi, *J. Chromatogr.*, 499 (1990) 267.
21 S. Hjertén and J.-L. Liao, *J. Chromatogr.*, 457 (1988) 165.
22 J.-L. Liao and S. Hjertén, *J. Chromatogr.*, 457 (1988) 175.
23 S. Hjertén, J.-L. Liao and R. Zhang, *J. Chromatogr.*, 473 (1989) 273.
24 T. B. Tennikova, B. G. Belenkii and F. Svec, *J. Liq. Chromatogr.*, 13 (1990) 63.
25 I. M. Bird, I. H. Sadler, B. C. Williams and S. W. Walker, *Mol. Cell. Endocrinol.*, 66 (1989) 215.
26 I. M. Bird, I. Meikle, B. C. Williams and S. W. Walker, *Mol. Cell. Endocrinol.*, 64 (1989) 45.