CHROM. 21 925

DISPLACEMENT CHROMATOGRAPHY OF BIOMOLECULES WITH LARGE PARTICLE DIAMETER SYSTEMS

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SUMMARY

Displacement chromatography was employed for the preparative-scale separation of peptides and proteins using large particle diameter chromatographic systems. Peptide displacements were successfully scaled-up with respect to particle and column diameter with no adverse effects on product recovery. Protein displacements on 30- and 90-μm agarose-based adsorbent systems resulted in well separated displacement zones of pure material. The present work extends the scope of biopolymer displacement chromatography to large particle diameter systems and is expected to further increase the distinct economic advantages associated with preparative-scale displacement chromatography.

INTRODUCTION

Displacement chromatography is rapidly emerging as a powerful preparative bioseparation technique due to the high throughput and purity associated with the process[1–3]. The operation of preparative elution systems at elevated concentrations has been shown to result in significant tailing of the peaks with the concomitant loss of separation efficiency[4]. In contrast, displacement chromatography offers distinct advantages in preparative chromatography as compared to the conventional elution mode[1,2]. The process takes advantage of the non-linearity of the isotherms such that a larger feed can be separated on a given column with the purified components recovered at significantly higher concentrations. Furthermore, the tailing observed in nonlinear elution chromatography is greatly reduced in displacement chromatography due to the self-sharpening boundaries formed in the process. Whereas in elution chromatography the feed components are diluted during the separation, the feed components are often concentrated during displacement chromatography[1,5]. These advantages are particularly significant for the isolation of biopolymers from dilute solutions such as those encountered in biotechnology processes.

Although the physico-chemical basis of the displacement mode of chromatography was established by Tiselius in 1943[6], the potential of this technique for preparative bioseparations was not realized until the recent work on displacement chroma-

tography employing high-performance liquid chromatography (HPLC) sorbents which exhibit rapid kinetics and mass transfer[1,3,5,7-25].

We have demonstrated that displacement chromatography can be successfully employed for the simultaneous concentration and purification of peptides, antibiotics, and proteins[1]. A mathematical model for the simulation of non-ideal displacement chromatography has also been developed to facilitate the optimization of these separations[26]. We have recently extended our work with biopolymer displacement to relatively complex mixtures and examined displacement behavior under elevated flow-rate and crossing isotherm conditions[8]. While the recent advances with displacement chromatography have been carried out with small particle diameter HPLC materials, there is a significant economic driving force for using larger particle diameter supports for preparative and process-scale liquid chromatography. The present work extends the scope of biopolymer displacement chromatography to large particle diameter systems and is expected to further increase the economic advantages associated with the elevated throughput and product purity of displacement chromatographic systems.

EXPERIMENTAL

Materials

μBondapak octadecylsilica (10 μm and 15–20 μm) columns in various column dimensions were gifts from Waters Chromatography Division (Millipore, Milford, MA, U.S.A.). 30-μm Sepharose S and 90-μm Sepharose S Fast-Flow bulk cation-exchange materials and a 50 × 5 mm I.D. column packed with 10-μm Mono-S cation-exchange material were donated by Pharmacia LKB Biotechnology (Piscataway, NJ, U.S.A.). Bulk Zorbax octadecylsilica and strong cation exchanger (SCX) chromatographic materials were gifts from DuPont (Wilmington, DE, U.S.A.). Methanol, 2-(2-butoxyethoxy) ethanol (BEE), sodium monophosphate and ammonium sulfate were purchased from Fisher Scientific (Rochester, NY, U.S.A.). N-Carbobenzoxy-L-alanyl-L-glycyl-L-glycine (Cbz-Ala-Gly-Gly), N-carbobenzoxy-L-alanyl-L-alanine (Cbz-Ala-Ala), N-benzoyl-L-arginine (Bz-Arg), cytochrome *c*, α-chymotrypsinogen and lysozyme were obtained from Sigma (St. Louis, MO, U.S.A.). Water-soluble coagulant, Nalcolyte 7105, was a gift from Nalco (Chicago, IL, U.S.A.).

Apparatus

The chromatograph employed for the peptide displacements consisted of a Model LC 2150 pump (Pharmacia LKB) connected to the chromatographic columns via a Model C10W 10-port valve (Valco, Houston, TX, U.S.A.). The column effluent was monitored by a Model 757 Spectroflow UV detector (Applied Biosystems, Ramsey, NJ, U.S.A.) and a Model L6512 strip chart recorder (Linseis, Princeton, NY, U.S.A.). Fractions of the column effluent were collected with an LKB Model 2212 Helirac fraction collector. The column temperature was controlled using a Model RM20 Lauda recirculating water bath (Brinkman, Westbury, NY, U.S.A.).

An FPLC chromatograph (Pharmacia LKB) was employed for the protein displacement experiments. This system consisted of a Model P-500 pump connected to the chromatographic column via a Model MV-7 valve. The column effluent was

monitored 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. The system was controlled using a LCC-500-Plus controller.

Procedures

Operation of displacement chromatograph. A schematic of the displacement chromatograph system employed in this work is illustrated elsewhere[5]. In all displacement experiments, the columns were sequentially perfused with carrier, feed, displacer, and regenerant solutions. Fractions of the column effluent were collected throughout the displacement runs and were assayed by analytical chromatography.

Displacement of peptides. Feed mixtures containing Bz-Arg, Cbz-Ala-Gly-Gly, and Cbz-Ala-Ala were separated by displacement chromatography on μBondapak octadecylsilica columns of various column dimensions (Waters). The carrier solution was methanol–50 mM phosphate buffer, pH 2.2 (40:60, v/v). The displacer for these separations was 30 mg/ml BEE in the carrier. The column temperature was maintained at 45°C and flow-rates of 0.1, 0.4 and 2.5 ml/min were employed for the displacement experiments with columns of 3.9, 7.8 and 19 mm I.D., respectively.

Displacement chromatography of proteins on Mono-S 10-μm supports. Displacement experiments were carried out using 50 × 5 mm I.D. columns packed with 10-μm Mono-S cation exchange materials. The displacer was 30 mg/ml Nalcolyte 7105 in a carrier of 0.1 M ammonium sulfate in 25 mM phosphate buffer, pH 7.5. The regenerant contained 0.8 M ammonium sulfate in 50 mM phosphate buffer, pH 3.0. The feed mixture was a 0.5-ml solution containing α-chymotrypsinogen, cytochrome c and lysozyme at 1.67 mg/ml each. The protein displacement employed a flow-rate and temperature of 0.1 ml/min and 22°C, respectively.

Purification of proteins on 30-μm Sepharose S particles. Displacement experiments were carried out using 300 × 10 mm I.D. columns packed with 30-μm Sepharose S cation-exchange materials. The carrier contained 0.1 M ammonium sulfate in 25 mM phosphate buffer, pH 7.5. The displacer was 75 mg/ml Nalcolyte 7105 in the carrier. The regenerant solution and temperature employed were the same as described above. The flow-rate was 0.4 ml/min. The feed solution was 6 ml of 1.67 mg/ml each of α-chymotrypsinogen, cytochrome c and lysozyme.

A step-gradient experiment was carried out using the same conditions as described above, with the displacer replaced by a solution of 0.25 M ammonium sulfate in 25 mM phosphate buffer, pH 7.5.

The displacement experiment was repeated using a carrier of 25 mM phosphate buffer, pH 7.5, all other conditions as stated above.

Purification of proteins on 90-μm Sepharose S particles. Displacement experiments were carried out using 300 × 10 mm I.D. columns packed with 90-μm Sepharose S cation-exchange material. The displacer was 75 mg/ml Nalcolyte 7105 in a carrier of 0.1 M ammonium sulfate in 25 mM phosphate buffer, pH 7.5. The feed solution was a 6-ml solution containing 1.67 mg/ml each of α-chymotrypsinogen and lysozyme. All other conditions were the same as described above for the 30-μm displacement.

Preparative elution of the feed mixture was carried out in the absence of the displacer, all other conditions the same.

HPLC analysis. Fractions collected during the chromatographic runs were ana-

lyzed by HPLC. A Model LC 2150 pump (Pharmacia LKB), a Model 7125 sampling valve with a 20-μl sample loop (Rheodyne, Cotati, CA, U.S.A.), a Model 757 spectroflow UV detector (Applied Biosystems), and a Model C-R3A integrator (Shimadzu, Kyoto, Japan) were assembled to carry out HPLC analysis. Peptide analyses were carried out using a 100 × 4.6 mm I.D. Zorbax C_{18} column (DuPont). The eluent consisted of methanol–50 mM phosphate buffer, pH 2.2 (50:50, v/v). Protein analyses were performed with a 100 × 4.6 mm I.D. SCX column (DuPont). The eluent contained 0.15 M ammonium sulfate in 25 mM phosphate buffer, pH 7.5. Displacement fractions were diluted 50–500 fold with the eluent and 20-μl samples were injected. The flow-rate was 1.0 ml/min and column temperature was maintained at 22°C. The column effluents were monitored at 254 and 280 nm for the peptide and protein analyses, respectively. Quantitative analysis was carried out and the data was used to construct displacement chromatograms.

RESULTS AND DISCUSSION

Displacement chromatography has been established as a powerful technique for the simultaneous concentration and purification of biomolecules[1,3,5,7-25]. However, the biopolymer displacements performed to date have employed stationary phase materials with particle diameters of 10 μm or less. Clearly, for displacement chromatography to become a useful preparative separation tool in the biotechnology
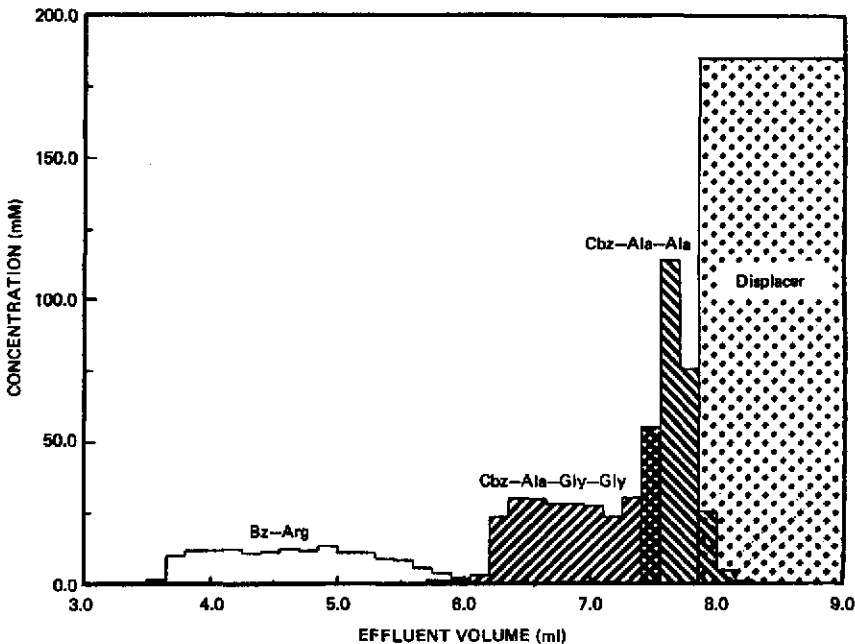

Fig. 1. Displacement chromatogram of a peptide mixture. Column, 300 × 3.9 mm I.D. μBondapak ODS (10 μm); carrier, methanol–50 mM phosphate buffer, pH 2.2 (40:60, v/v); displacer, 30 mg/ml BEE; feed, 7.2 mg Bz-Arg, 16.4 mg Cbz-Ala-Gly-Gly and 18.2 mg Cbz-Ala-Ala in 2 ml of carrier; flow-rate, 0.1 ml/min; temperature, 45°C; fraction volume, 150 μl.

industry, the economics of the process must be compelling. Since the cost of carrying out preparative chromatography decreases dramatically with increasing particle diameter, it is important to investigate the efficacy of displacement chromatography of biomolecules with large particle diameter systems. Accordingly, the present work examines the displacement chromatographic purification of peptides and proteins in such systems.

Scale-up of peptide displacement

In a previous report[1] we described the displacement purification of the peptides Bz-Arg, Cbz-Ala-Gly-Gly, and Cbz-Ala-Ala using BEE as the displacer on a reversed-phase analytical HPLC system. This model displacement was employed in this study to investigate the scale-up of displacement chromatography of peptides with respect to particle and column diameter.

The model displacement was first carried out on a 300 × 3.9 mm I.D. analytical column packed with 10-μm octadecylsilica material. The resulting displacement chromatogram is shown in Fig. 1. Under these conditions, Bz-Arg eluted ahead of the displacement train and Cbz-Ala-Gly-Gly and Cbz-Ala-Ala were well displaced as expected from the previously reported results.

The displacement separation was then scaled-up with respect to column diameter using two 150 × 19 mm I.D. columns in series packed with the same 10-μm stationary phase material. In this experiment, the feed volume and flow-rate were linearly scaled-up with respect to the cross-sectional area. The resulting displacement chromatogram, shown in Fig. 2, demonstrates that the same degree of separation was
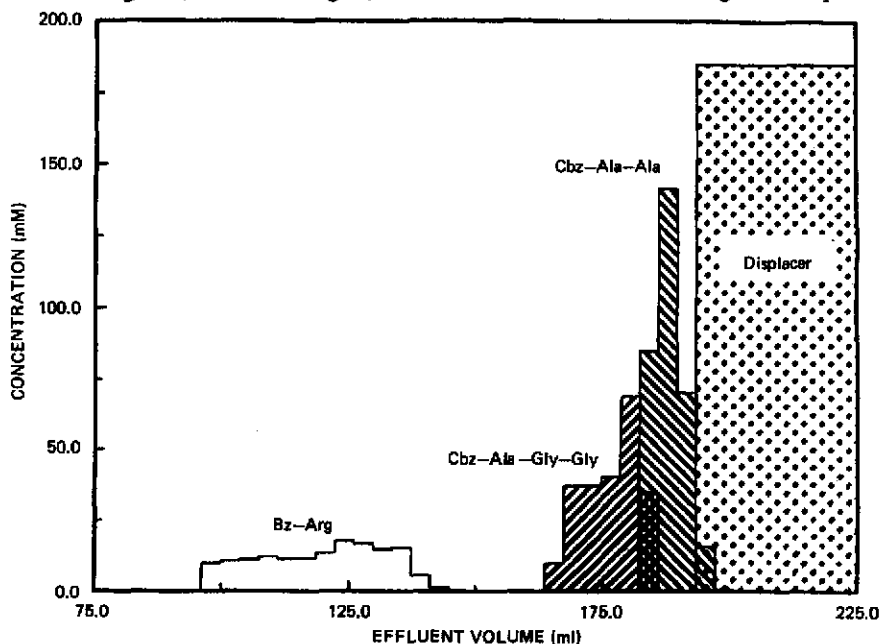

Fig. 2. Preparative-scale displacement of a peptide mixture. Chromatographic conditions as in Fig. 1 with the exception of: column, two 150 × 19 mm I.D. μBondapak ODS columns (10 μm) in series; feed, 161.9 mg Bz-Arg, 303.1 mg Cbz-Ala-Gly-Gly and 412.5 mg Cbz-Ala-Ala in 45.2 ml of carrier; flow-rate, 2.5 ml/min; fraction volume, 3.75 ml.

achieved as with the analytical column. In fact, the breakthrough time of the displacer in both experiments was identical, confirming the linear scale-up of the process. This separation corresponds to the purification of approximately one gram of peptide per displacement experiment. This result indicates that preparative HPLC columns can be readily employed for the displacement purification of peptides.

The displacement separation was also scaled-up with respect to particle diameter. Fig. 3 shows the displacement chromatogram of the model separation using a 300 × 7.8 mm I.D. column packed with 15–20-μm reversed-phase material. Under these conditions, the same degree of separation was obtained as with the 10-μm stationary phase.

We are presently extending this work to process-scale column systems and employing our model of non-ideal displacement chromatography[26] for the optimization of such systems. This work will be the subject of a future report.

Displacement chromatography of proteins

We have previously demonstrated that displacement chromatography can be successfully employed for the simultaneous purification and concentration of protein mixtures using silica-based stationary phase materials[1,8]. In this report, we extend this work to larger particle diameter polymer-based systems.

The separation of the proteins α-chymotrypsinogen, cytochrome *c* and lysozyme by displacement chromatography was first investigated using a 50 × 5 mm I.D. column packed with 10-μm Mono-S cation-exchange material. The resulting dis-
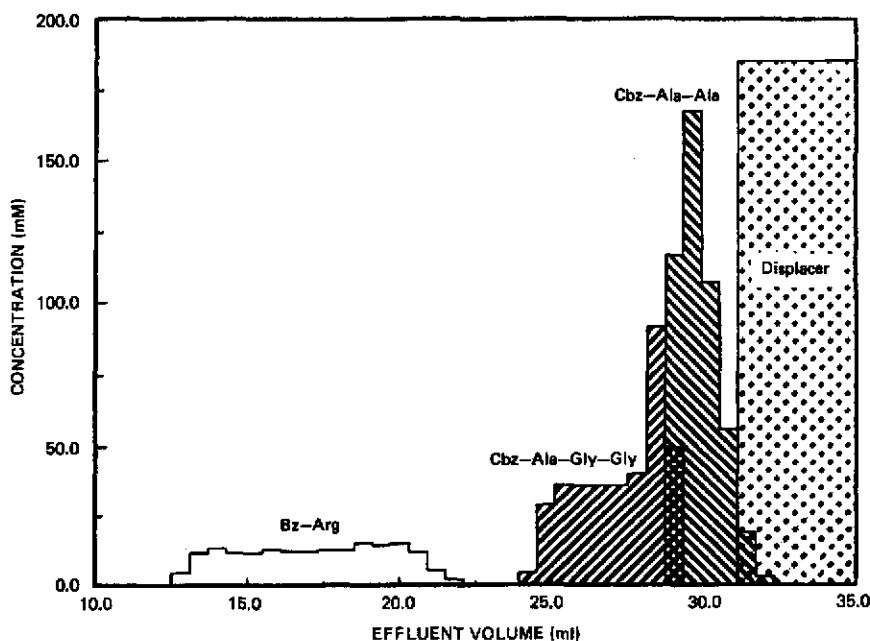

Fig. 3. Displacement chromatogram of a peptide mixture on a 20-μm particle diameter system. Chromatographic conditions as in Fig. 1 with the exception of: column, 300 × 7.8 mm I.D. μBondapak ODS (15–20 μm); feed, 27.8 mg Bz-Arg, 66.6 mg Cbz-Ala-Gly-Gly and 74.9 mg Cbz-Ala-Ala in 8 ml carrier; flow-rate, 0.4 ml/min; fraction volume, 600 μl.
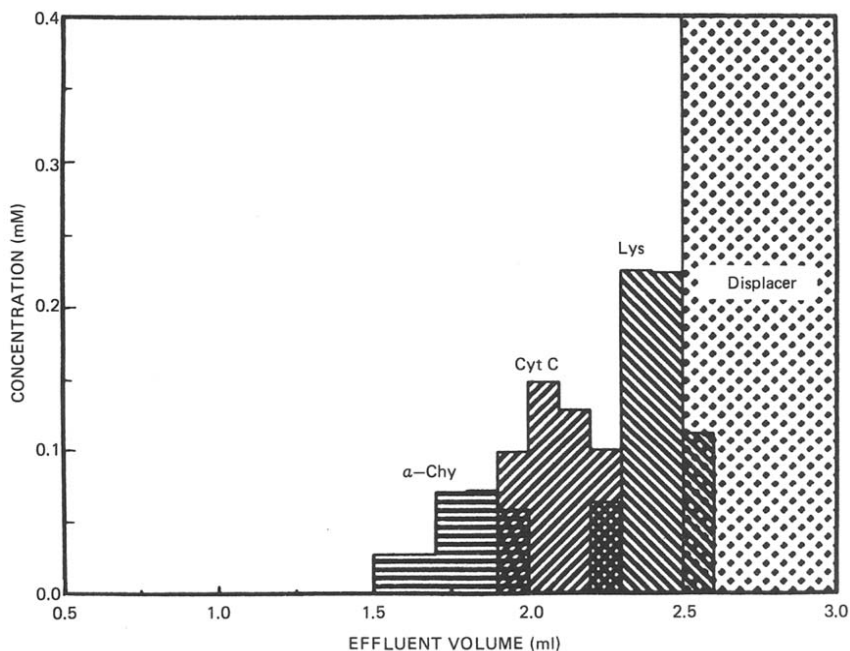
Fig. 4. Displacement chromatogram of a three-component protein mixture. Column, 50 × 5 mm I.D. Mono-S cation exchanger (10 μm); carrier, 0.1 M ammonium sulfate in 25 mM phosphate buffer, pH 7.5; displacer, 30 mg/ml Nalcolyte 7105 in carrier; flow-rate, 0.1 ml/min; temperature, 22°C; feed, 500 μl of 1.67 mg/ml each of α-chymotrypsinogen (α-Chy), cytochrome c (Cyt C) and lysozyme (Lys); fraction volume, 100 μl.

placement chromatogram, shown in Fig. 4, demonstrates that the proteins were well-separated during the displacement process. While the Nalcolyte displacer was readily removed from the column using standard regeneration techniques, these polymer-based materials have the distinct advantage of being able to withstand extremes of pH. In fact, column regeneration was also easily achieved by the perfusion of 6 ml of 2 M NaOH.

Scale-up of protein displacement

The purification of proteins by displacement chromatography was investigated with larger particle diameter materials. Fig. 5 shows the displacement chromatogram of the proteins α-chymotrypsinogen, cytochrome c and lysozyme using 75 mg/ml Nalcolyte 7105 to displace the proteins from a chromatographic column packed with 30-μm Sepharose S cation-exchange material. Under these conditions, the proteins were well separated and concentrated during the displacement process. These results are indeed dramatic in that they demonstrate that displacement chromatography of proteins is not limited to small particle diameter systems.

However, in ion-exchange displacement chromatography, the adsorption of the displacer not only results in the displacement of the feed components but can also result in desorption of the salt molecules in the system. This in turn can result in an effective "salt-gradient" moving down the column ahead of the displacer front. Thus,
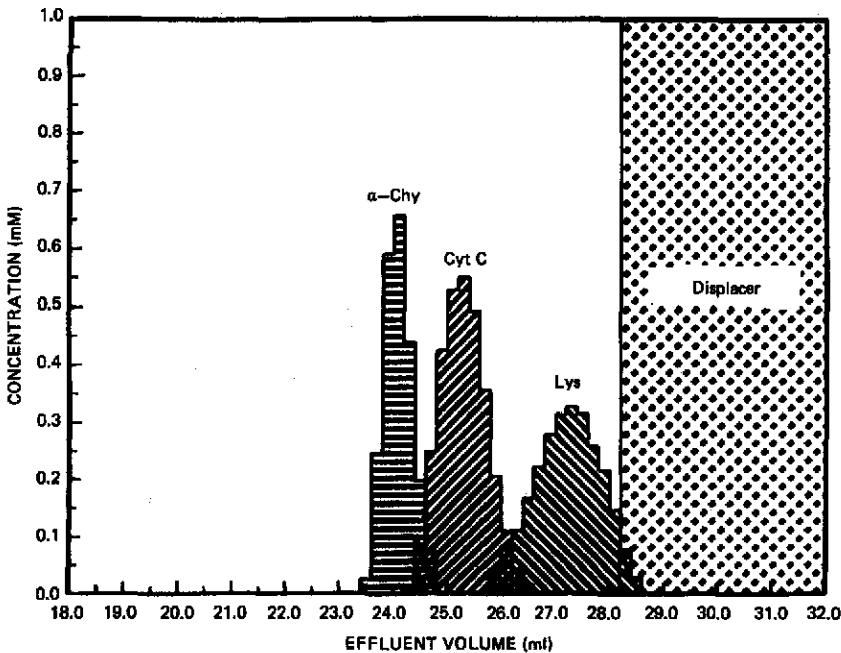
Fig. 5. Displacement chromatogram of a three-component protein mixture on a 30-μm particle diameter system. Column, 300 × 10 mm I.D. Sepharose S cation exchanger (30 μm); carrier, 0.1 M ammonium sulfate in 25 mM phosphate buffer, pH 7.5; displacer, 75 mg/ml Nalcolyte 7105 in carrier; flow-rate, 0.4 ml/min; temperature, 22°C; feed 6 ml of 1.67 mg/ml each of α-chymotrypsinogen, cytochrome c and lysozyme; fraction volume, 200 μl.

it is critical that the effect of the desorbed salt molecules on the feed components downstream of the displacer front be investigated.

In the above displacement purification, a carrier containing 0.1 M ammonium sulfate in 25 mM phosphate buffer, pH 7.5, was employed. In order to examine whether this separation was due primarily to the action of an effective "salt-gradient" induced by the displacer, the following idealized control experiment was carried out. After the introduction of the feed mixture, a step change in the salt concentration from 0.1 to 0.25 M ammonium sulfate was carried out at the column inlet. This step-gradient was selected such that the breakthrough times of the proteins in the two experiments were comparable. The profile obtained in this control experiment is shown in Fig. 6. While the proteins had comparable effluent concentrations and retention times under these conditions, there was more mixing and significant tailing of the feed components in the step gradient experiment. Although this does not represent an optimal step gradient for these components, it illustrates that the displacement separation is not solely due to the action of the induced salt gradient.

In order to examine the displacement behavior of this system in the absence of the ammonium sulfate salt, the displacement experiment was repeated with no salt present in the carrier. The resulting profile from this control experiment is shown in Fig. 7. Again, the three proteins were well separated and concentrated during the
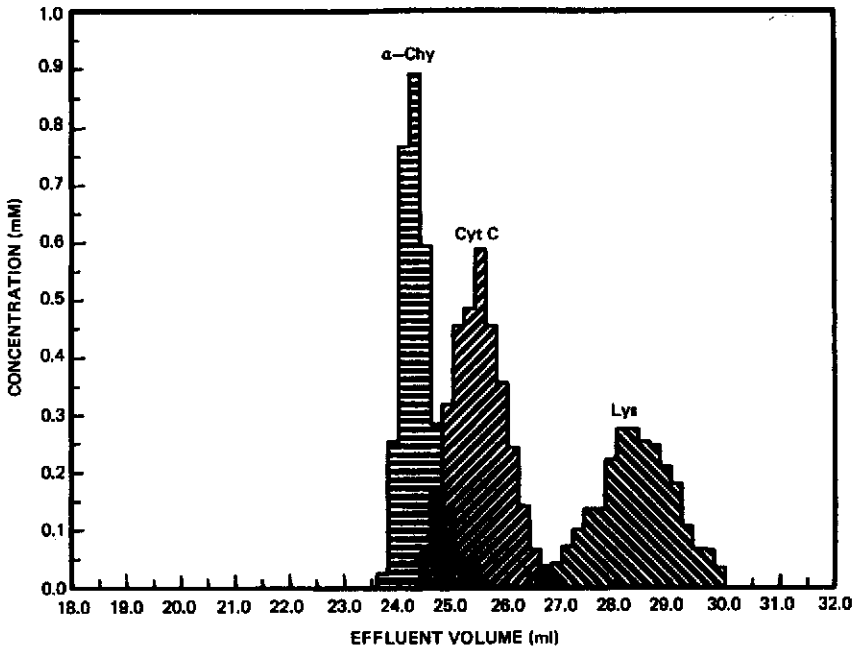
Fig. 6. Step-gradient separation of a three-component protein mixture on a 30-μm particle diameter system. Chromatographic conditions as in Fig. 5 with the displacer replaced by a solution of 0.25 M ammonium sulfate in 25 mM phosphate buffer, pH 7.5.
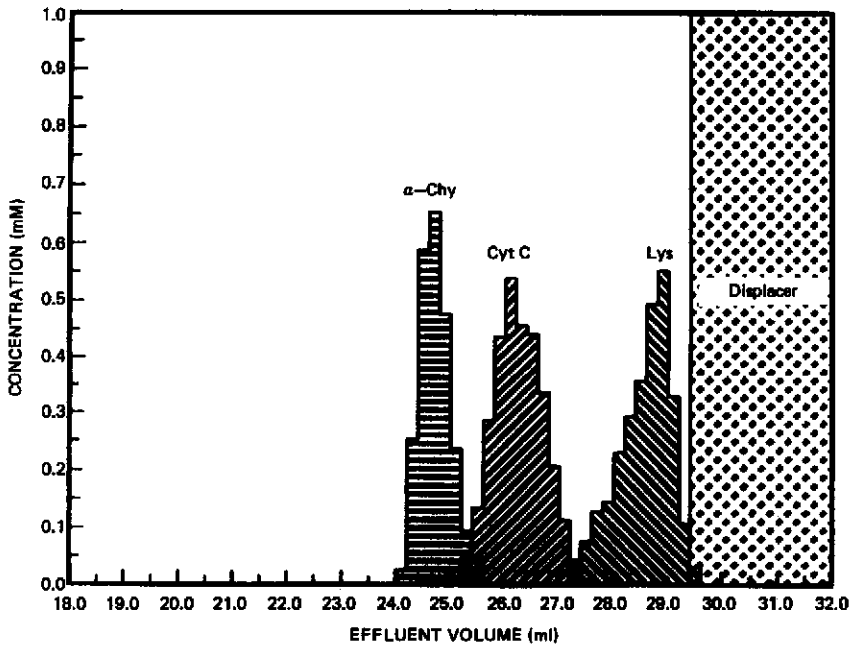
Fig. 7. Displacement chromatogram of a three-component protein mixture on a 30-μm diameter system. Chromatographic conditions as in Fig. 5 with the exception of: carrier, 25 mM phosphate buffer, pH 7.5.
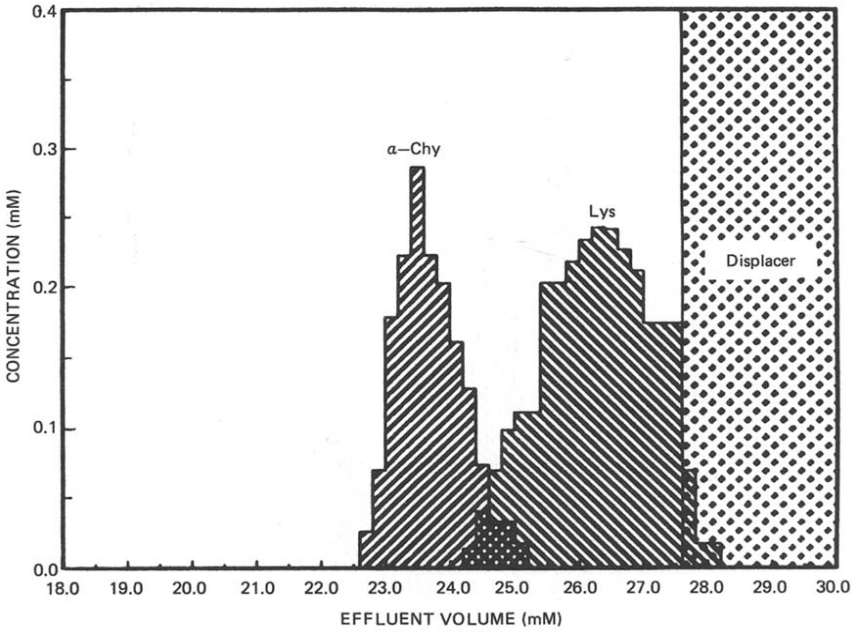
Fig. 8. Displacement chromatogram of a two-component protein mixture on a 90-μm particle diameter system. Chromatographic conditions as in Fig. 5 with the exception of: column, 300 × 10 mm I.D. Sepharose S cation exchanger (90 μm); feed, 6 ml of 1.67 mg/ml each of α-chymotrypsinogen and lysozyme.
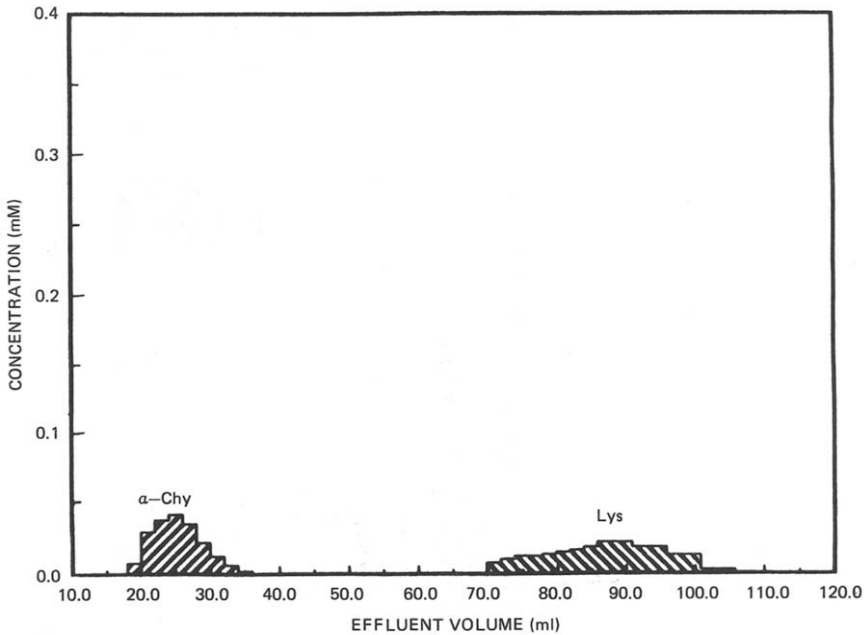

Fig. 9. Preparative elution chromatogram of a two-component protein mixture on a 90-μm particle diameter system. Column, 300 × 10 mm I.D. Sepharose S cation exchanger (90 μm). Chromatographic conditions as in Fig. 8 with the absence of the displacer.

displacement process. Thus, displacement chromatography of proteins can be readily carried out with or without the presence of salt in the carrier.

Displacement chromatography of proteins was further scaled-up to 90-μm agarose-based materials. The simultaneous concentration and purification of the proteins α-chymotrypsinogen and lysozyme on these materials is shown in Fig. 8. The displacement zones exhibited relatively sharp boundaries, which is quite remarkable for such large particle diameter systems. The separation was repeated in the absence of the displacer. Preparative non-linear elution chromatography under the same carrier conditions resulted in extremely long elution times with significant dilution of the feed proteins as shown in Fig. 9. While this preparative elution experiment was not optimized, it serves to dramatize the action of the displacer in these systems.

CONCLUSIONS

In this report we have demonstrated that the displacement purification of biomolecules can be readily scaled-up to larger particle and column diameter systems. Indeed, the ability to simultaneously concentrate and purify biomolecules by displacement chromatography using preparative columns packed with large particle diameter materials may have a significant impact on the economics of preparative chromatography.

ACKNOWLEDGEMENTS

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