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Rapid ion-exchange displacement chromatography of proteins on perfusive chromatographic supports

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Abstract

Displacement chromatography is an extremely powerful separation technique. However, it has not been widely accepted as a preparative technique due to the creeping flow-rates at which displacement separations have been demonstrated. In this work, the enhanced mass transport of perfusive chromatographic particles enabled displacement separations to be dramatically scaled-up with respect to flow-rate. At a flow-rate of 4 ml/min (1440 cm/h) on an analytical-scale column, crude β-lactoglobulin was displaced in only 90 s to yield ca. 18 mg each of pure β-lactoglobulin A and B. This represents an order of magnitude decrease in the time required for displacement separations. Thus, the operation of perfusive columns in the displacement mode offers an ultra-high throughput separation technique with outstanding resolving power even under heavily overloaded conditions.

1. Introduction

The last decade in liquid chromatography has seen the emergence of two potentially powerful advances in preparative chromatography: perfusive chromatographic supports and displacement chromatography. Perfusion chromatographic supports (POROS, PerSeptive Biosystems, Cambridge, MA, USA) attempt to minimize mass transfer resistances associated with conventional diffusive chromatographic supports by incorporating large diameter (6 000–8 000 Å) "through" pores into the sorbent particles [1,2]. These through pores provide for convective fluid flow into the particle that greatly enhances mass transfer to the sorbent phase enabling perfusion supports to operate at high flow-rates while retaining high efficiency [3]. Lloyd and Wagner [4] separated a six-protein mixture in approximately 1 min on a perfusion column at a flow-rate of 4.0 ml/min while the identical separation required 20 min at 1 ml/min on a conventional column. Using 20-μm reversed-phase particles, Fulton et al. [5] performed an analytical separation of a five-protein feed mixture in under 15 s. Fulton et al. [6] have also successfully employed perfusive supports for preparative separations by purifying 95 mg of a vasoactive intestinal polypeptide in a single 12-min run. The immobilization of antibodies onto perfusive chromatographic supports has enabled immunoassays to be performed in seconds which greatly facilities real-time monitoring of bioprocesses [7,8]. Gordon and Adams [9] have reported the

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distinct advantages that preparative-scale perfusive particles possess over diffusive supports with regard to process development, scale-up and validation.

Displacement chromatography is an extremely powerful bioseparation tool due to the high throughput and product purity associated with the process [10–12]. This technique offers several advantages in preparative chromatography as compared to traditional preparative elution and gradient modes. The displacement process takes advantage of the non-linear adsorptive properties of biomolecules 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 preparative isocratic elution is greatly reduced in displacement chromatography due to the self-sharpening boundaries developed during the process. In contrast to preparative elution where the feed components are often diluted during the separation, the feed components can be significantly concentrated during the displacement procedure.

Preparative gradient separations also typically exhibit reduced peak tailing and significant concentration of feed components. However, if the feed components are readily separated employing step or linear gradients, then displacement may not offer significant advantages over these traditional techniques. Displacement chromatography should be considered primarily for separations possessing low separation factors. For these difficult separation problems, the desired level of purity is obtainable, if at all, only through shallow gradients (or isocratic conditions) where peak tailing typically becomes a concern. The ability of gradient separations to concentrate the product is not without its pitfalls. Since many proteins exhibit concentration-dependent aggregation affects, the high product concentrations reached in gradient separations can lead to aggregation and loss of bioactive product. Product concentration can be reduced in gradient separations by altering the "steepness" of the gradient, however, this changes the elution conditions and may lower product purity and recovery. Through appropriate manipulation of the displacer concentration in displacement separations, product concentration is readily controlled without affecting the overall separation. These advantages combine to make displacement chromatography an extremely attractive preparative technique for the isolation of biomolecules from the dilute solutions often encountered in biotechnology processes.

Displacement chromatography has been employed for the purification of a variety of protein mixtures using both anion- and cation-exchange supports. Peterson and co-workers [13–20] have extensively investigated the utility of carboxymethyldextrans (CM-D) to act as protein displacers in anion-exchange systems. Ghose and Mattiasson [21] have used carboxymethylstarch to displace lactate dehydrogenase from beef heart homogenate. Chondroitin sulfate, a constituent of connective tissue, has been used by Horvath and co-workers [22–24] for the displacement of protein mixtures. Cramer and co-workers [25,26] have explored the potential of cation-exchange displacement chromatography using Nalcolyte 7105, a water soluble coagulant, as a displacer. Gerstner and Cramer [27,28] have utilized protamine and heparin as displacers in cation- and anion-exchange systems, respectively. Jayaraman et al. [29] examined the effect of displacer molecular mass on separation efficiency by using dextran-based polyelectrolytes of various molecular masses as displacers in both cation- and anion-exchange systems. Jen and Pinto [30,31] have employed relatively low molecular mass displacers in anion-exchange systems.

These separations demonstrated the resolving power of displacement, but they were typically performed at volumetric flow-rates of less than 0.2 ml/min (ca. 60–75 cm/h) which in some instances lead to displacement run times exceeding 1 h. While linear flow-rates of 50–100 cm/h are typically employed for large diameter (>50 μm) preparative particles, these displacements were performed using analytical-scale particles (ca. 10 μm) where much higher linear velocities (300–700 cm/h) are the operating norm. At these higher linear velocities, displacement run times as short as 10 min should have been possible. Operating analytical columns in the

displacement mode at linear velocities well below those typically utilized for traditional techniques (i.e. gradient elution) has clouded the issue as to whether displacement is a more productive technique (in terms of mass of product/unit time) as compared to traditional modes. Subramanian and Cramer [32] explored the efficacy of displacement chromatography at a higher flow-rate (1 ml/min–360 cm/h) in 1989, however, displacements continue to be reported at reduced flow-rates.

In this work, we will couple the advantages of displacement chromatography with the high flow-rate efficiency of perfusive chromatographic supports to transform displacement chromatography into an extremely high throughput separation technique. The work also serves to demonstrate the dramatic effect that POROS perfusive supports can have on significantly reducing total chromatographic processing time for all modes of chromatography.

2. Experimental

2.1. Materials and apparatus

Analysis and perfusive displacement experiments and were performed using POROS 10 HQ (100 mm × 4.6 mm I.D., 10-μm particles) and POROS HQ/M (100 mm × 4.6 mm I.D., 20-μm particles) columns (PerSeptive Biosystems, Cambridge, MA, USA). A SynChropak Q300 column (100 mm × 4.6 mm I.D.) packed with 6.5-μm (300 Å pores) particles was employed for diffusive displacement experiments (SynChrom, Lafayette, IN, USA). Sodium chloride, sodium hydroxide, Tris–HCl, β-lactoglobulin A, β-lactoglobulin B, β-lactoglobulin (crude mixture of A and B) and heparin ammonium were purchased from Sigma (St. Louis, MO, USA). Polydiallyl dimethyl ammonium chloride (poly-DADMAC) and *o*-toluidine blue indicator were obtained from Nalco Chemical Company (Naperville, IL, USA). The HPLC system used in this study was a BioCAD 20 (PerSeptive Biosystems) which included an Advantec Model SF-2120 fraction collector (Advantec Toyo Kaisha, Japan).

2.2. Procedures

Displacement chromatography —perfusive

A POROS 10 HQ or a POROS HQ/M column was sequentially perfused with carrier, feed, displacer and regenerant solutions. The feed and displacer solutions were prepared in the carrier which was 100 m*M* Tris–HCl, pH 7.0. The feed consisted of 60 mg β-lactoglobulin (crude mixture of A and B) in 1.7 ml of carrier and the displacer was 7 ml of 12 mg heparin/ml. The fraction size was set at 200 μl. The regenerant was 1.0 *M* sodium chloride in 0.1 *M* sodium hydroxide. Flow-rates employed for the displacement experiments were varied from 1 to 4 ml/min (360–1440 cm/h). The feed was loaded at the same flow-rate employed for the displacement portion of the experiment.

Displacement chromatography —diffusive

A SynChropak Q300 column was employed for diffusive displacements. The feed and displacer solutions were prepared in the carrier which was 75 m*M* NaCl in 25 m*M* Tris–HCl, pH 6.5. The feed consisted of 50 mg β-lactoglobulin (crude mixture of A and B) in 2.0 ml of carrier and the displacer was 7 ml of 14.1 mg heparin/ml. The fraction size was set at 200 μl. The regenerant was 3.0 *M* sodium chloride. Flow-rates employed for the displacement experiments were varied from 0.2 to 1 ml/min (72–360 cm/h). The feed was loaded at the same flow-rate employed for the displacement portion of the experiment.

HPLC analysis

Fractions collected during displacement experiments were diluted 10 × with water and were analyzed by HPLC using linear gradient elution. Analysis for the β-lactoglobulins was performed using a POROS 10 HQ column with a linear gradient from 50 to 500 m*M* NaCl (constant buffer concentration of 10 m*M* Tris–HCl, pH 5.5) in 16.6 ml (10 column volumes) at a flow-rate of 2.0 ml/min (720 cm/h).

Titration assay for heparin analysis

Heparin concentration was measured using a colloidal titration assay provided by Nalco Chemical Company. For analysis of heparin, two drops of *o*-toluidine indicator were added to 100 ml of distilled water, the subsequent addition of heparin produced a colorimetric change. Titrating against polyDADMAC produced another colorimetric change. A linear calibration plot which was generated using known amounts of heparin, was used to quantify the unknown concentrations of heparin in the displacement fractions.

3. Results and discussion

The milk proteins β-lactoglobulin A and B form a good model system to demonstrate the resolving power of displacement chromatography since they differ in structure by only two amino acids [33]. Figs. 1–3 show the displacement separation of β-lactoglobulin A and B on a 10 HQ column (10-μm particles) at flow-rates from 1 to 4 ml/min (360–1440 cm/h). These separations demonstrate both the high flow-rate efficiency of POROS supports and the resolving power of displacement chromatography. The profiles at 1 (360 cm/h) and 2 ml/min (720 cm/h) (Figs. 1 and 2) are essentially identical as there are only two fractions of overlap between the zones of pure β-lactoglobulin B and β-lactoglobulin A. These displacement profiles also compare very favorably to those previously reported [15,22,23,28,29,31] which is remarkable since the current displacements were performed at flow-rates 10 to 20 times faster. As the flow-rate was increased to 4 ml/min (1440 cm/h) (Fig. 3), an additional fraction of overlap developed, but the overall profile still indicates an efficient displacement. As the flow-rate was increased from 1 to 4 ml/min, the protein displacement zones change from classic squares waves to roughly gaussian peaks. These changes in profile indicate that non-ideal effects (mass transfer and adsorption/desorption kinetics) are also increasing in magnitude. However, the result to focus attention on is the amount of overlap between the zones of pure β-lactoglobulin B and pure β-lactoglobulin A which does not substantially increase at the elevated flow-rates.

The histograms presented above do not always convey the true nature of the compositional changes that occur in the column effluent during a displacement. Fig. 4 presents the analytical chromatograms of the 200-μl fractions collected during the displacement from which the histogram presented in Fig. 2 was constructed. From

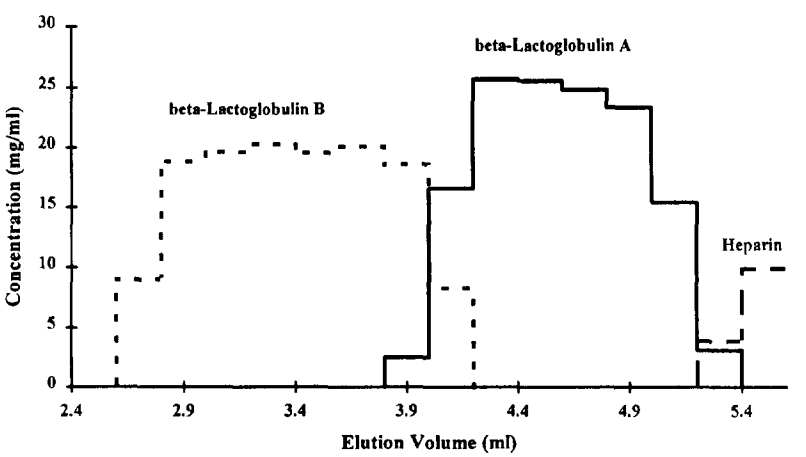

Fig. 1. Displacement chromatography of β-lactoglobulins at 1 ml/min. Column, 100 mm × 4.6 mm I.D. POROS 10 HQ (10-μm particles); carrier, 100 m*M* Tris–HCl, pH 7.0, displacer, 12 mg/ml ammonium heparin in carrier; feed, 60 mg β-lactoglobulin crude mixture in 1.7 ml carrier; fraction volume, 200 μl.

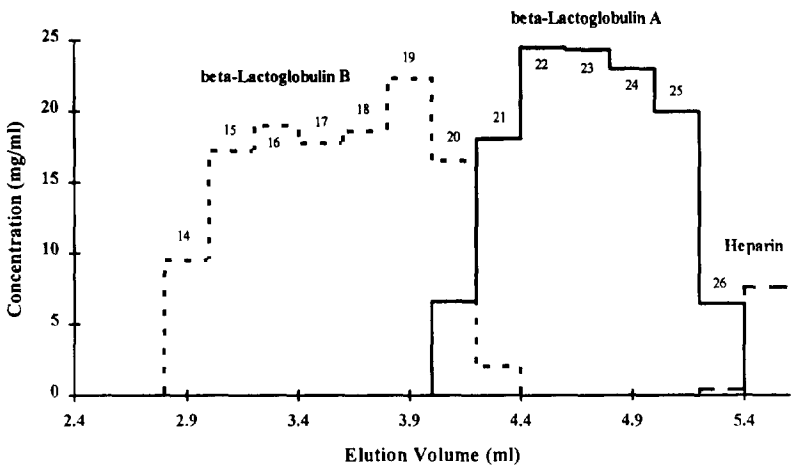

Fig. 2. Displacement chromatography of β-lactoglobulins at 2 ml/min. Chromatographic conditions as stated in Fig. 1 with the exception of flow-rate. Fraction numbers indicated on histogram.

fraction 13 to 14, the column effluent changed from no detectable protein to ca. 9 mg/ml β-lactoglobulin B. The next 5 fractions (15–19) contain the plateau region of the β-lactoglobulin B zone. Fractions 20 and 21 are the change-over region from pure β-lactoglobulin B to pure β-lactoglobulin A and the remaining fractions contain pure β-lactoglobulin A. The end of the displacement train is abrupt as the column outlet goes from ca. 5 mg/ml β-lactoglobulin A to no detectable protein in only one fraction (26 to 27). Closer examination of the cross-over fractions provides more evidence concerning the efficiency of the perfusion particles employed for this separation. From fractions 19–22, it can be seen that the effluent of the column during the displacement separation goes from pure β-lactoglobulin B (ca. 20 mg/ml) to pure β-lactoglobulin A (ca. 24 mg/ml) with only 400 μl of a mixed zone in-between. Clearly even at these elevated flow-rates, the experimental displacement profiles approach the square wave zones that are predicted by theory under ideal conditions.

While displacement of these proteins is impre-

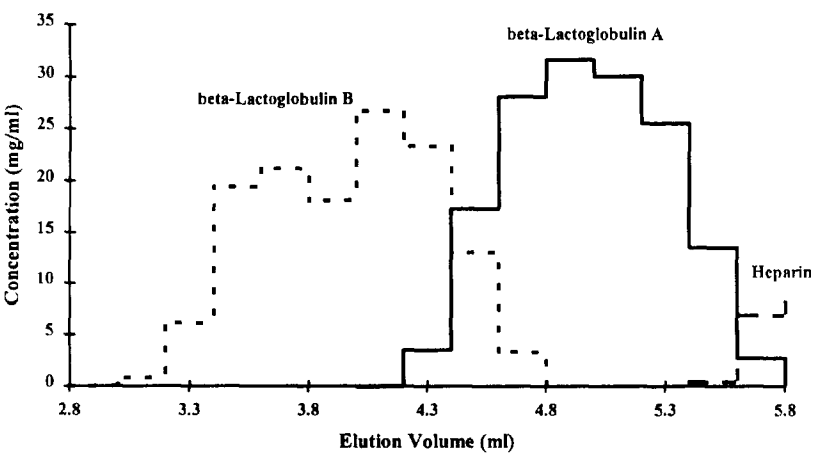

Fig. 3. Displacement chromatography of β-lactoglobulins at 4 ml/min. Chromatographic conditions as stated in Fig. 1 with the exception of flow-rate.

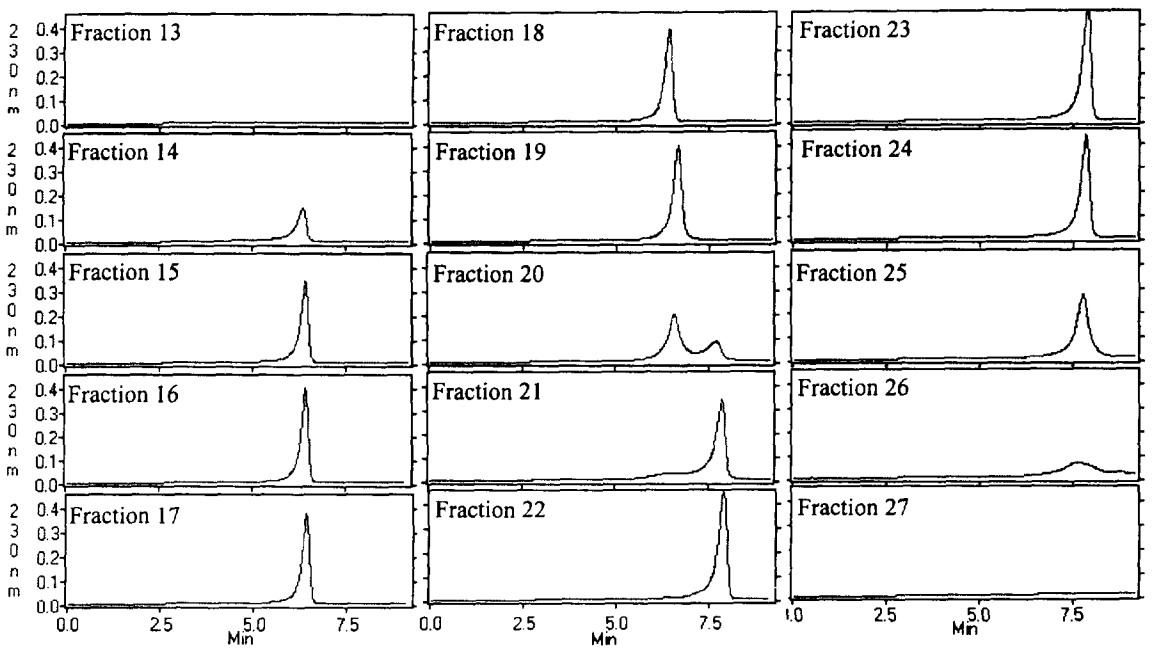

Fig. 4. Analytical chromatograms of fractions collected during the displacement separation performed at 2 ml/min on POROS 10 HQ column. *y*-Axes represent absorbance units.

ssive on the 10-μm system employed for these experiments, in order for displacement to be accepted as a preparative technique it must be performed on larger preparative-scale particles. Therefore, the displacement was scaled-up to a 20-μm particle system at a flow-rate of 2 ml/min (720 cm/h) with a minor loss in separation efficiency (Fig. 5). This elevated flow-rate should be compared to the 100 cm/h typically used on conventional soft gel chromatographic media.

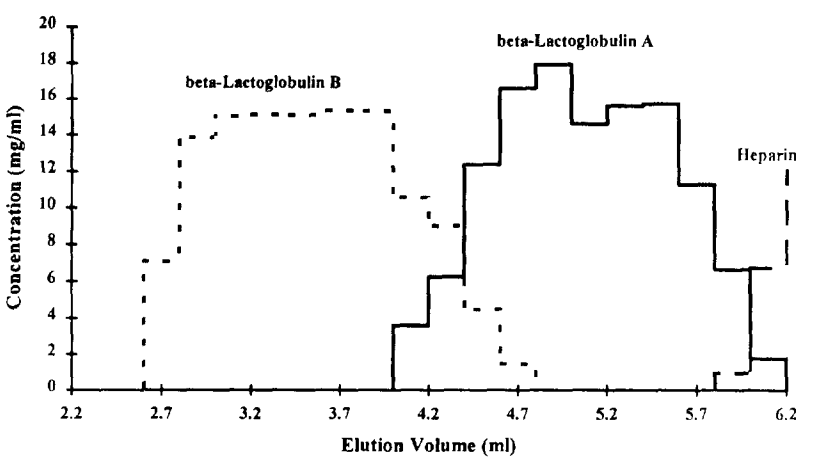

Fig. 5. Displacement chromatography of β-lactoglobulins at 2 ml/min using preparative POROS 20-μm particles. Chromatographic conditions as stated in Fig. 2 with the exception of the column: 100 mm × 4.6 mm I.D. HQ/M (20-μm particles).

Thus, the perfusive, high-efficiency nature of POROS particles enables high flow-rate displacements even on preparative-scale particles.

The high flow-rate efficiency of the POROS particles can be confirmed by comparing these perfusive displacements to displacements performed on a traditional diffusive particle. For this comparison, a particle of 6.5 μm diameter with 300-Å pores was chosen. The small pore diameter should assure that mass transport into the particle will primarily occur by diffusion. Displacements were performed at 0.2 ml/min (72 cm/h) and 1.0 ml/min (360 cm/h) to test the efficiency of a diffusive system at elevated linear flow-rates (Fig. 6). The initial displacement at a flow-rate of 0.2 ml/min is not as efficient as would be expected (Fig. 6A). There is a considerable amount of overlap between β-lactoglobulin A and the displacer. However, the small overlap (only 200 μl) between the protein zones is indicative of an efficient system. A plausible explanation is that the displacer experienced a higher degree of hindered diffusion as compared to the proteins. When the flow-rate was in-

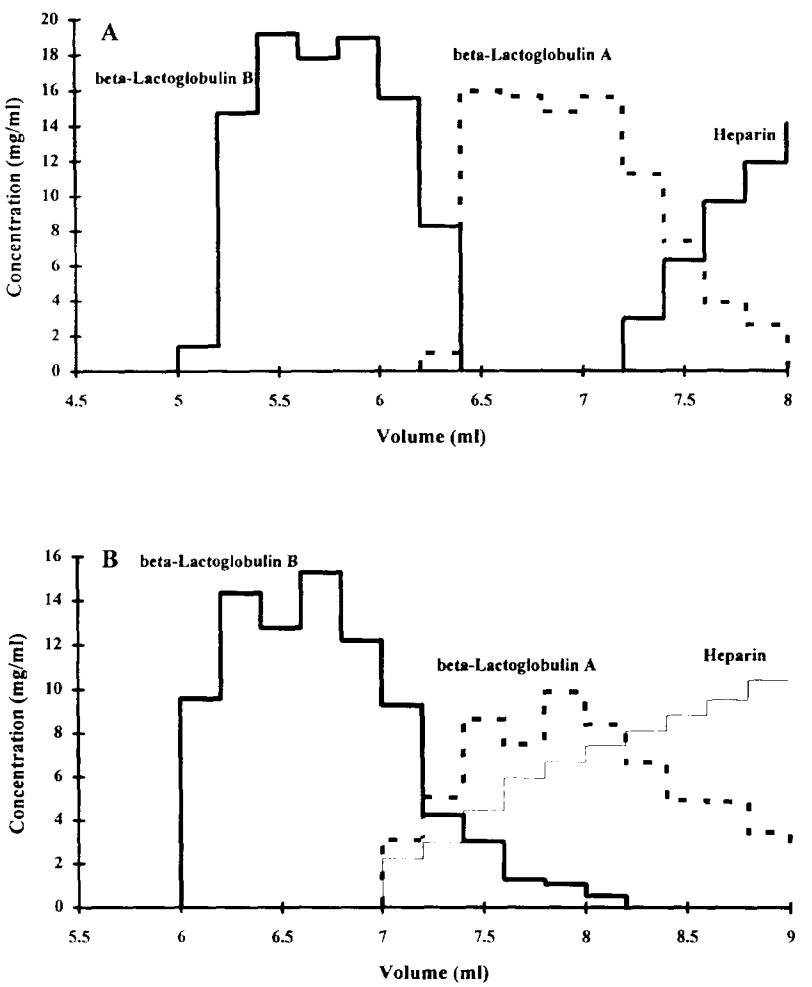

Fig. 6. Displacement chromatography of β-lactoglobulins on diffusive particles. Column, 100 mm × 4.6 mm I.D. Synchropak Q300 (6.5-μm particles); carrier, 75 m*M* NaCl in 25 m*M* Tris–HCl, pH 6.5, displacer, 14.1 mg/ml ammonium heparin in carrier; feed, 50 mg β-lactoglobulin crude mixture in 2.0 ml carrier; fraction volume, 200 μl; flow-rate, A (0.2 ml/min), B (1.0 ml/min).

creased to 1.0 ml/min, the displacement was a complete failure. The β-lactoglobulin A zone was completely engulfed by the displacer front which was now relatively diffuse. The zone of β-lactoglobulin A was also contaminated with a tail of β-lactoglobulin B. These results differ with those reported by Subramanian and Cramer [32] who showed efficient protein displacements at 1.0 ml/min (360 cm/h) on 5-μm, 300-Å pore particles.

The purity of β-lactoglobulin A and B recovered from displacement experiments is chromatographically compared to pure β-lactoglobulin A and B purchased from Sigma (Figs. 7 and 8). From these chromatograms, the β-lactoglobulins recovered from the displacement experiments appear as pure as the proteins obtained from Sigma. Figs. 7 and 8 show chromatograms from the middle of the respective displacement zone (either β-lactoglobulin A or B). These mid-

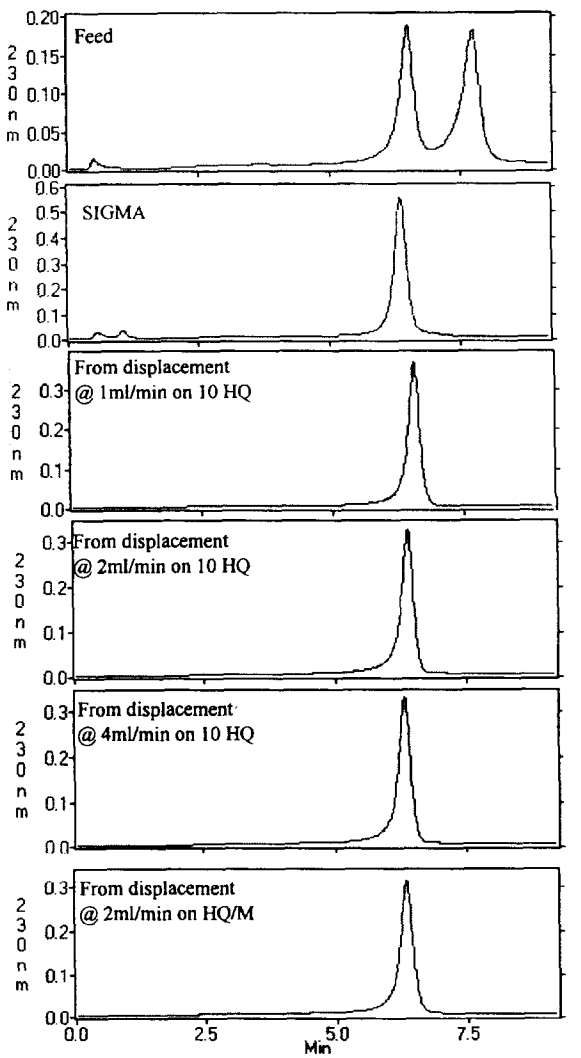

Fig. 7. Analytical chromatograms of representative fractions of β-lactoglobulin B recovered from displacement experiments compared to β-lactoglobulin B obtained from Sigma. y-Axes represent absorbance units.

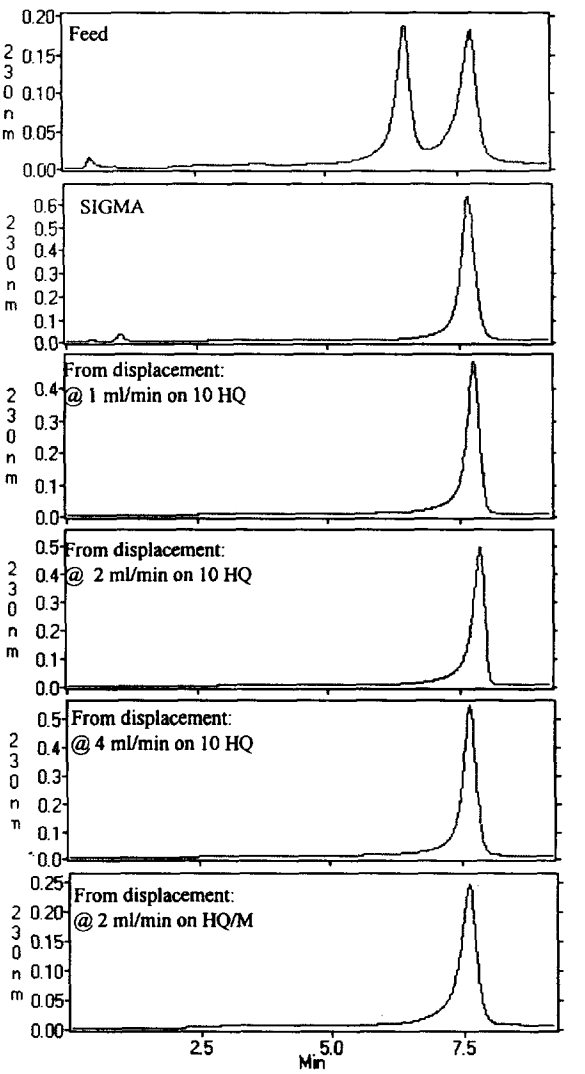

Fig. 8. Analytical chromatograms of representative fractions of β-lactoglobulin A recovered from displacement experiments compared to β-lactoglobulin A obtained from Sigma. y-Axes represent absorbance units.

zone fractions are representative of the pure β-lactoglobulin contained in that zone. (This observation is evident from Fig. 4 which shows analytical chromatograms of all fractions collected during a displacement.)

The productivity (defined as mg purified product/ml of column volume/h) of displacement chromatography performed on perfusive chromatographic supports is compared to that obtained using a traditional diffusive support (Table 1). The cycle time for a displacement run, which is composed of equilibration, feed, displacement and regeneration time, is an order of magnitude faster on the perfusive supports. The cycle time for the 4 ml/min displacement would be only 12 min as compared to approximately 2 h for a similar displacement performed on a diffusive support. The actual displacement portion of the 4 ml/min run was only 90 s in which time 18 mg of each β-lactoglobulin was recovered, pure. This order of magnitude increase in the productivity of this displacement chromatographic separation means that ca. 85 mg of pure β-lactoglobulin B *and* 85 mg of pure β-lactoglobulin A could be recovered every hour using only an analytical-scale column (100 mm × 4.6 mm I.D.). This is compared to only 5 mg each of β-lactoglobulin A and B that would be recovered using a diffusive matrix [28].

The purification of these closely related proteins is not a trivial task using traditional chromatographic techniques. Considerable effort was required to obtain the analytical gradient separation presented in the manuscript. The preparative separation of these proteins must be extremely difficult with the pure β-lactoglobulins recovered at low yields. The difficulty of this preparative separation is anecdotally confirmed by the pricing structure of β-lactoglobulins found in the Sigma catalog. The crude β-lactoglobulin mixture is valued at $25 (US) per gram whereas pure β-lactoglobulin B and β-lactoglobulin A are $496 (US) and $456 (US) per gram, respectively. Clearly, displacement chromatography offers several advantages for the preparative purification of these proteins as well as for more complex mixtures.

4. Conclusions

In this work we have demonstrated the ability of perfusion chromatographic supports to greatly enhance the throughput of displacement chromatography by enabling efficient displacements at elevated flow-rates. In only 90 s at 4 ml/min, we were able to purify by displacement chromatography 18 mg each of β-lactoglobulin A and B. We were also able to scale the displacement separation up to preparative scale (20-μm) particles and still obtain efficient displacements at 2 ml/min. In future work, we will explore displacement separations using even larger perfusive particles (50 μm) and will examine the resolving power of displacement using more complex mixtures.

Table 1

Displacement flow-rate (ml/min)	Recovery of β-lactoglobulin B (mg)[b]	Recovery of β-lactoglobulin A (mg)[b]	Cycle time[a] (min)	Productivity of β-lactoglobulin B ($mg/ml^{-1}h^{-1}$)	Productivity of β-lactoglobulin A ($mg/ml^{-1}h^{-1}$)
0.1[c]	13.2	11.6	133.9	3.0	2.7
1.0	21.5	23.0	17.7	44.0	47.1
2.0	20.9	18.4	13.8	54.6	48.1
4.0	18.5	17.4	11.9	56.2	53.1

[a] Cycle time = (feed volume + displacer breakthrough volume)/(displacement flow-rate) + (10 column volumes equilibration + 20 column volumes regeneration)/operating flow-rate. POROS 10 HQ operating flow-rate = 5 ml/min, for Ref. [28] operating flow-rate = 1 ml/min.

[b] Recovery of chromatographically pure protein.

[c] Data from Ref. [28], total β-lactoglobulin load = 40 mg.

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