Journal of Chromatography, 519 (1990) 87–98
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 680

Use of the sodium salt of poly(vinylsulfonic acid) as a low-molecular-weight displacer for protein separations by ion-exchange displacement chromatography

S.-C. DAVID JEN and NEVILLE G. PINTO*
Department of Chemical Engineering, University of Cincinnati, Cincinnati, OH 45221-0171 (U.S.A.)
(First received February 26th, 1990; revised manuscript received July 5th, 1990)

ABSTRACT

The sodium salt of poly(vinylsulfonic acid) (PVSNa), molecular weight 2000, a low-molecular-weight polyelectrolyte, has been identified as a suitable displacer for the concentration and purification of protein mixtures. This displacer has been tested on the separation of ovalbumin from conalbumin, and the fractionation of heterogeneous ovalbumin. The displacement characteristics of the polyelectrolyte were a strong function of the carrier pH, and a pH for good displacement development of heterogeneous ovalbumin has been identified. The displacer can be efficiently removed from the exchanger with a mild regeneration protocol. In this regard, the low-molecular-weight polyelectrolyte appears to have a significant advantage over high-molecular-weight ion-exchange displacers used in the past. Solvent requirements for regeneration and re-equilibration are significantly lower with PVSNa, suggesting the use of molecular weight to tailer ion-exchange displacers with desirable characteristics with respect to *both* column development and regeneration.

INTRODUCTION

In recent years, liquid chromatography has been the focus of much attention as a large-scale process for the purification of proteins. Much of this effort has focused on the scale-up of analytical elution chromatography [1–4]. The impetus for this approach has been extensive accumulated experience from analytical applications, commercial availability of suitable stationary phases and chromatographic hardware, and the potential for attainment of high separation efficiencies. However, elution chromatography has a number of shortcomings in process-scale applications. These include poor column utilization, loss of separation efficiency due to tailing at elevated sample loadings, large solvent requirements for chromatographic development, and product dilution during the elution process.

Recently, Frenz and Horváth [5] have shown that preparative-scale separations can be accomplished by high-performance liquid chromatography (HPLC) in the displacement mode. This mode of column development has some significant beneficial characteristics for process-scale chromatography. First, and probably most significantly, is the potential for achieving product concentration and separation in a single step. Secondly, since the displacement process operates in the non-linear region of the equilibrium isotherm, high column loadings, and, hence, good column uti-

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lization are possible. Thirdly, column development, *per se*, requires less solvent than a comparable elution process. And, finally, the operating procedure, involving only step changes, is simple, in comparison to a gradient elution process.

However, displacement chromatography has a number of problems that must be overcome before it can find wider acceptance. Mixing between adjacent bands in the displacement train, due to mass transfer resistances and mass dispersion effects, generally lowers the separation efficiency. Also, in order to minimize band spreading due to mass transfer resistances, low carrier fluid velocities must be used. Both these problems may have a solution in the novel, macroporous resins that are currently being developed [6]. The most severe problem with displacement chromatography, however, relates to the regeneration and re-equilibration steps [7]. Since the displacement process uses a high affinity compound as the displacer, the time for regeneration and/or re-equilibration is long compared to elution chromatography. Furthermore, large amounts of solvent are generally required during regeneration/re-equilibration, generally negating any advantage with respect to solvent consumption that may be gained during column development. For example, Liao *et al.* [8] required over 100 column volumes of solvent to regenerate and re-equilibrate a DEAE column used in the ion-exchange displacement chromatography of proteins. Also, Subramanian *et al.* [9] have reported using 50 column volumes of solvent for regenerating a cation-exchange column used in protein separations.

A common characteristic of ion-exchange displacers used in protein separations in the past is their high molecular weights; Liao *et al.* used chondroitin sulfates with molecular weights of 50 000, while Subramanian *et al.* used Nalcolyte 7105 which has a molecular weight of 20 000. High-molecular-weight displacers have probably been favored to ensure higher binding coefficients than the high-molecular-weight proteins they are to displace. However, it has been shown [10] that protein retention on ion exchangers may result from charge asymmetry on the protein surface, and only a fraction of the surface interacts with the stationary phase. Therefore, it may not be necessary to use high molecular weight displacers, which bind extremely strongly, when less strongly bound low-molecular-weight compounds may suffice. This will facilitate column regeneration and re-equilibration. It must, however, be emphasized that the low molecular weight displacers should have affinities greater than those of the proteins to be displaced.

In this communication, results from a study to evaluate poly(vinylsulfonic acid) (PVSNa) as an ion-exchange displacer for protein purifications have been reported. This low-molecular-weight polyelectrolyte has been used for the separation and purification of conalbumin and ovalbumin, and the fractionation of heterogeneous ovalbumin. Both displacement and regeneration characteristics of the new displacer have been studied.

EXPERIMENTAL

Materials

Matrex PAE-300, a polyethyleneimine-coated, silica-based, weak anion exchanger (10 μm diameter, 350–500 Å pore size), was purchased from Amicon (Danvers, MA, U.S.A.). The resin was slurry packed into 25 cm × 0.46 cm I.D. and 10 cm × 0.21 cm I.D. columns at 6000 p.s.i. The sodium salt of PVSNa was used as the

TABLE I

PROPERTIES OF THE DISPLACER AND PROTEINS

Displacer	Sodium salt of poly(vinylsulfonic acid); mol.wt. 2000 Structure: $-(CH_2-CH)_n-$ $SO_3^-Na^+$
Proteins	(1) Conalbumin: p*I* 5.9; mol.wt. 77 000 (2) Ovalbumin: p*I* 4.7; mol.wt. 43 500

displacer, and was purchased from Polysciences (Warrington, PA, U.S.A.). Tris(hydroxymethyl)aminomethane, imidazole, histidine, sodium citrate and sodium bicarbonate were obtained from Aldrich (Milwaukee, WI, U.S.A.). Conalbumin and ovalbumin were obtained from Sigma (St. Louis, MO, U.S.A.). Table I lists some properties of the displacer and proteins.

Apparatus

A Haskel air-driven liquid pump was used for slurry packing the columns, and was purchased from Alltech (Deerfield, IL, U.S.A.). The displacement apparatus (Fig. 1) consisted of a SP8800 ternary gradient HPLC pump (Spectra-Physics, San Jose, CA, U.S.A.) connected to a 25 cm × 0.46 cm I.D. chromatographic column via

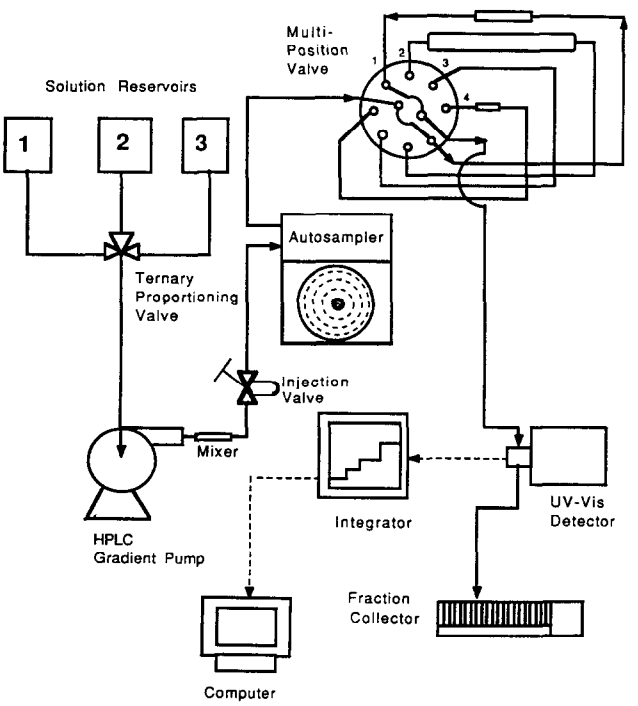

Fig. 1. Schematic diagram of experimental set-up for elution and displacement chromatography.

a Model CST4UW multiposition valve (Valco, Houston, TX, U.S.A.). The column effluent was monitored with a Model 2550 UV–VIS detector (Varian Instruments, Sunnyvale, CA, U.S.A.) and recorded on a HP 3396A integrator (Hewlett-Packard, Palo Alto, CA, U.S.A.). Fractions collected from displacement runs were analyzed on a 10 cm × 0.21 cm I.D. column. Samples to be analyzed were injected automatically by a Model 655A-40 autosampler (Hitachi, Danbury, CT, U.S.A.). A 100-μl polypropylene sample vial with a PTFE-faced silicone septum (Wheaton, Milville, NJ, U.S.A.) was used for holding the fraction collected. A manual ten-port sampling valve (Supelco, Bellefonte, PA, U.S.A.) was used for calibration, as well as for the isocratic elution experiments.

Procedures

Displacement chromatography. The displacement experiments were performed on the 25 cm × 0.46 cm I.D. PAE-300 column connected to the No. 2 position of the multiposition valve (MPV). A by-pass line was plumbed to the No. 3 position of the MPV. The displacement column was first equilibrated with a buffer solution of a known pH. The MPV was then switched to the No. 3 position, and the connecting line upstream of the MPV was primed with the feed protein mixture. Subsequently, the MPV was switched to the No. 2 position, and the feed was loaded onto the column. Upon loading the feed, the MPV was switched to the No. 3 position, and the connecting line upstream of the MPV was primed with the displacer solution. The MPV was then switched to the No. 2 position, and the displacement development was initiated. The effluent was collected and subsequently analyzed by gradient elution chromatography. During the displacement, effluent from the column did not pass through the detector, to prevent distortion of the displacement train by mass dispersion in the flow cell.

On completion of the displacement development, the column was regeneratered with 0.1 *M* sodium citrate in 0.3 *M* sodium bicarbonate solution at pH 9. A flow-rate of 0.5 ml/min was used. The column was re-equilibrated to the desired pH using 0.3 *M* tris(hydroxymethyl)aminomethane at pH 8, 0.3 *M* imidazole at pH 7, and 0.3 *M* histidine at pH 6. These buffers were chosen for their good buffering capacity, and inability to form anions in solution [11]. A flow-rate of 0.5 ml/min was used for the re-equilibration. Table II summarizes the conditions used in the various stages of the displacement cycle.

Isocratic elution chromatography. Isocratic elution chromatography was evaluated as a tool for identifying a suitable pH for the displacement of heterogeneous ovalbumin. These elution experiments were performed on a 10 cm × 0.21 cm I.D. PAE-300 column with a carrier flow-rate of 1 ml/min. The carriers used were 0.3 *M* tris(hydroxymethyl)aminomethane at pH 8 and 0.3 *M* histidine at pH 6. 10 μl samples of the protein mixtures were used, and the UV–VIS detector was set at 280 nm.

Analytical procedure. As was stated earlier, fractions from the displacement runs were analyzed by gradient elution chromatography. The analyses were performed on a 10 cm × 0.21 cm I.D. PAE-300 column. The carrier was maintained at pH 8 with 0.01 *M* Tris buffer, and a carrier flow-rate of 0.7 ml/min was used. A linear salt gradient of 0.0 to 0.25 *M* sodium chloride in 15 min was used. Samples (5 μl) of the displacement effluent were injected automatically by the autosampler. The column effluent was monitored at 280 nm.

TABLE II

OPERATING CONDITIONS USED FOR DISPLACEMENT CHROMATOGRAPHY

Carrier	(1) pH 8, 0.3 M Tris; (2) pH 7, 0.3 *M* imidazole; (3) pH 6, histidine. Flow-rate 0.5 ml/min
Feed	(1) 3 ml of 1 mg/ml each of ovalbumin and conalbumin. Flow-rate 0.1 ml/min (2) 3 ml of 1 mg/ml ovalbumin. Flow-rate 0.1 ml/min
Displacer	PVSNa in carrier.
Regenerant	0.1 *M* sodium citrate in 0.3 sodium bicarbonate, pH 9. Flow-rate 0.5 ml/min

RESULTS AND DISCUSSION

In order to establish the utility of PVSNa as a displacer, two separations were attempted: the separation of ovalbumin from conalbumin, and the fraction of heterogeneous ovalbumin. For ovalbumin–conalbumin mixtures, the effects of displacer concentration and carrier flow-rate were investigated. The primary objectives of these experiments were to determine if the separation of conalbumin from ovalbumin was achievable, and, if so, to establish that the mechanism was indeed displacement. The separation of heterogeneous ovalbumin was used to probe the resolution achievable with PVSNa. In this case, the effects of pH on the separation were also studied.

Fig. 2 represents a typical chromatogram obtained for the displacement of an ovalbumin–conalbumin mixture. The feed mixture consisted of 3 ml of 1 mg/ml each

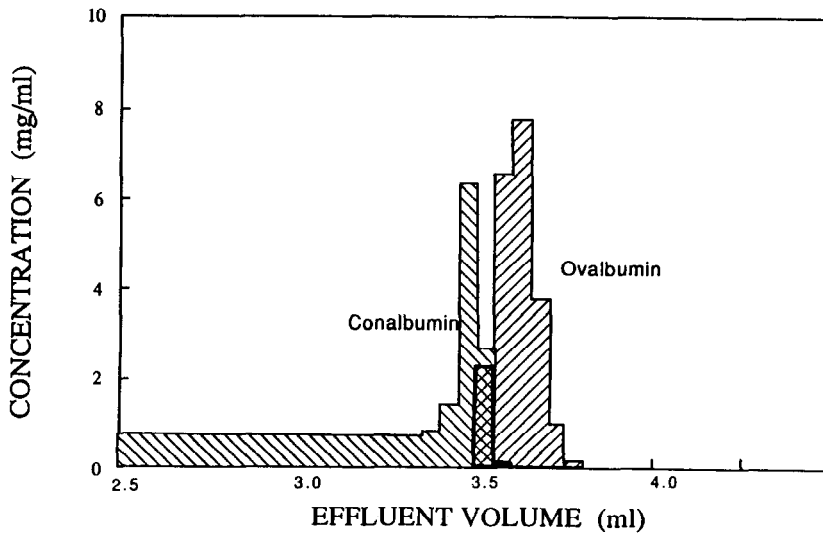

Fig. 2. Displacement chromatogram of ovalbumin and conalbumin. Carrier, 0.3 *M* Tris buffer, pH 8; displacer, 25 mg/ml of PVSNa in carrier; flow-rate, 0.1 ml/min; feed, 3 ml of 1 mg/ml each of conalbumin and ovalbumin in carrier; temperature, 20°C; fraction volume, 50 μl.

of conalbumin and ovalbumin, fed at a flow-rate of 0.1 ml/min. The carrier was maintained at pH 8 with Tris buffer, and a displacer concentration of 25 mg/ml was used. The chromatogram indicates that a zone of pure conalbumin is followed by a protein mixture zone, which is followed by a pure ovalbumin zone. Behind the ovalbumin is a mixture region containing both the displacer and the ovalbumin, and this is followed by the pure displacer. The concentrations of the proteins in this chromatogram peak above their respective feed concentrations; this response is characteristic of displacement chromatography. However, the separation has not been achieved entirely by displacement. It is apparent from the column response, that the affinity of the stationary phase for conalbumin is much weaker than that for ovalbumin. Pure conalbumin is quickly obtained in the column effluent at a concentration only slightly above its feed concentration. This is typical of frontal chromatography. Thus, Fig. 2 represents a displacement chromatogram for which the isotachic condition has not been attained. A longer column length would ensure completion of wave interferences in the column, and the characteristic displacement pattern.

The chromatogram in Fig. 2 clearly shows that mass transfer resistances contribute to lowered separation efficiency in the column. Overlap of zones in displacement chromatography, prior to the achievement of the final pattern, can be due to interferences between waves in the column, and to mass transfer resistances [5,12]. In Fig. 2, the overlap between the two protein zones could be due to both these factors. However, the rear boundary of the ovalbumin band would be sharp in the absence of mass transfer resistances, since wave interferences in this region are instantaneously resolved. The most likely mass transfer resistance is pore diffusion; PAE-300 has pore sizes in the range 350–500 Å. The use of PAE-1000, which has pore sizes of about 1000 Å, is likely to significantly reduce the overlap between zones.

The displacement chromatogram in Fig. 2, and all subsequent chromatograms, do not show the displacer front. The reason for this is that the absorbance of PVSNa in the UV–VIS region is so low that its effluent composition history cannot be determined accurately by the gradient elution method. Thus, an indirect procedure was used to check the displacer front. A separate frontal experiment was performed with only PVSNa, at a concentration of 25 mg/ml. The effluent composition history was obtained by monitoring the column effluent directly, and this history was superimposed on the displacement chromatograph in Fig. 2. The frontal boundary was approximately at the location where the protein band ended, consistent with theoretical expectations for displacement chromatography.

In order to study the effects of displacer concentration on the separation, an experiment identical to that of Fig. 2 was performed, but with a displacer concentration of 75 mg/ml. The chromatogram obtained in this case is shown in Fig. 3. The higher displacer concentration leads to a higher peak concentration of proteins, and a higher displacement velocity. This is consistent with previously reported results [5] and with theoretical expectations [12], and confirms the separation to be a displacement process. The higher displacer concentration also resulted in a sharper rear for the ovalbumin band, once again indicating that mass transfer resistances do play a role. However, the overlap region between the two protein bands increased in width. This probably occurred because wave interferences are primarily responsible for the overlap in this region.

One of the disadvantages of displacement chromatography is that it is usually

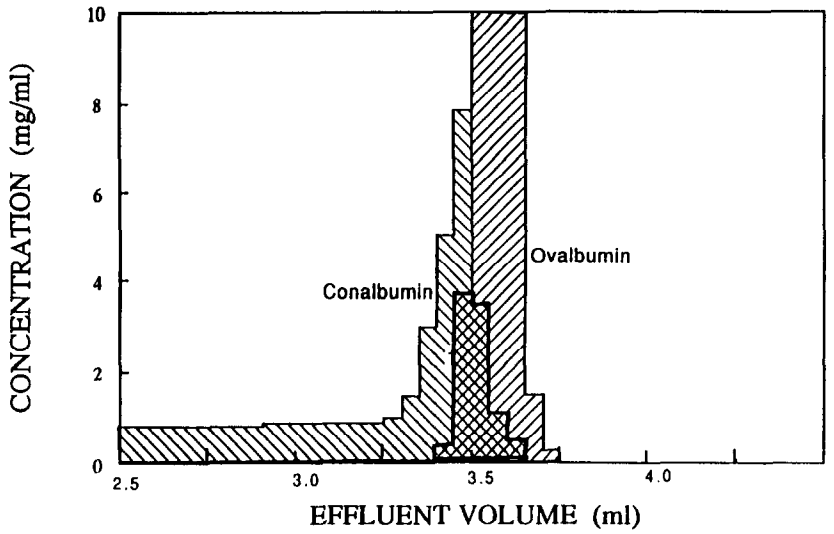

Fig. 3. Displacement chromatogram of ovalbumin and conalbumin. Conditions as in Fig. 2, except 75 mg/ml was the displacer concentration.

performed at low carrier flow-rates. This results in a low throughput. Thus, it is important to investigate the possibility of increasing the throughput by increasing the carrier flow-rate. To this end, an experiment was performed at a flow-rate of 0.4 ml/min, keeping all other conditions identical to the conditions used to obtain the chromatogram in Fig. 2. The results from this experiment are shown in Fig. 4. As

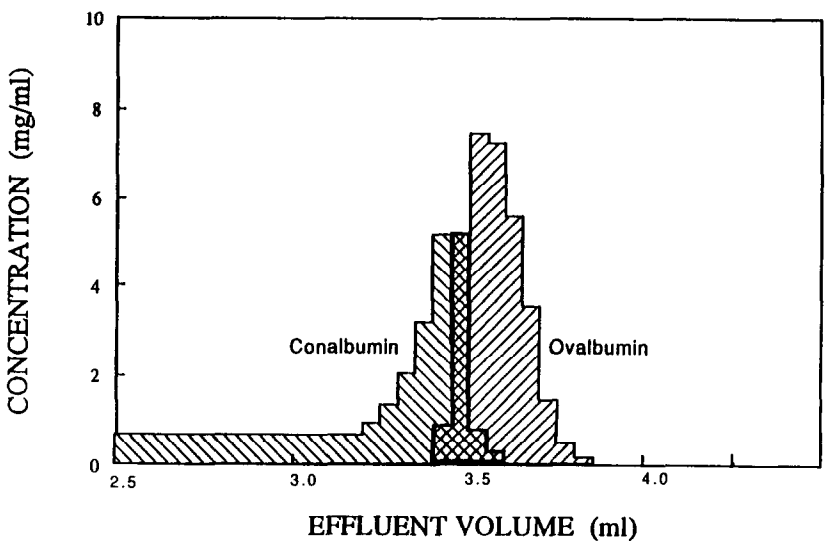

Fig. 4. Displacement chromatogram of ovalbumin and conalbumin. Conditions as in Fig. 2, except 0.4 ml/min was the flow-rate.

expected, mass transfer effects are more significant at the higher flow-rate. This is seen clearly from the rear of the ovalbumin band, which increases in width at the higher flow-rate. In fact, 0.4 ml/min does not appear to be a suitable carrier flow-rate at the sample loading used, since the pure protein regions are essentially swamped by the mixture zones. It must however be noted that at higher sample loadings the width of the pure bands will increase, since the plateau concentration of the bands is established by the displacer concentration. Thus, at higher loadings higher carrier fluid velocities will be feasible. Cramer and co-workers [9,13] have addressed in detail the problem of raising carrier fluid velocities without adversely affecting product purities.

From the data presented so far, it is apparent that PVSNa has potential as a displacer for the separation of conalbumin from ovalbumin, and, if desired, conditions can be optimized to attain an isotachic condition in the bed. However, the separation of conalbumin from ovalbumin is not a challenging separation, as is evident from the large difference in the affinities of the two proteins for the stationary phase. Thus, a more stringent test for the displacement process was sought.

It has been reported in the literature that pure ovalbumin has some degree of heterogeneity, due to variations in the number of phosphate groups [14] as well as the carbohydrate structure [15]. This molecular dispersity results in minor variations in the ion-exchange affinity of ovalbumin, which can be exploited to provide an excellent test of the resolving power of ion-exchange-based chromatographic systems [16,17].

Shown in Fig. 5 is a displacement chromatogram obtained for ovalbumin with PVSNa as the displacer. The feed mixture consisted of 3 ml of 1 mg/ml of ovalbumin fed at a flow-rate of 0.1 ml/min. The column pH was maintained at 6, with 0.3 *M* histidine as the buffer. It is clear from this chromatogram that six forms of oval-

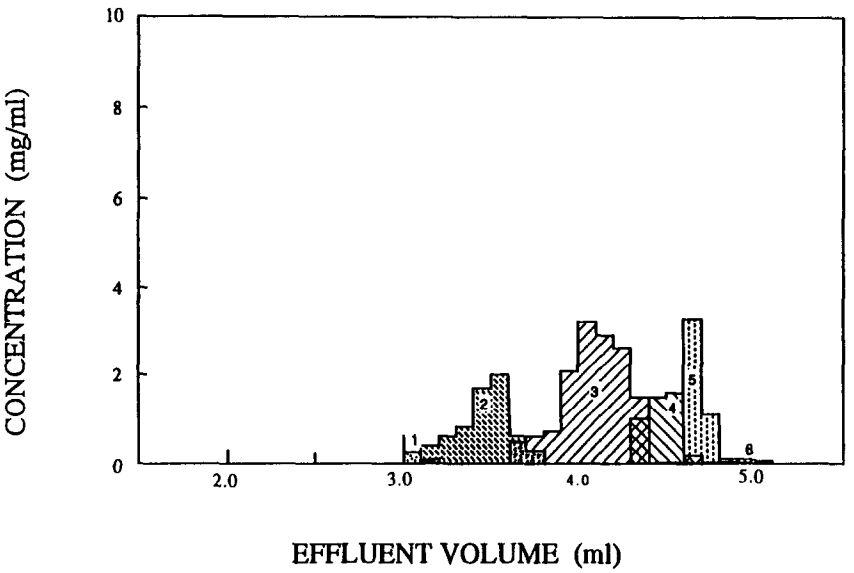

Fig. 5. Displacement chromatogram of ovalbumin. Conditions as in Fig. 2, except 0.3 *M* histidine, pH 6, as the carrier, 3 ml of 1 mg/ml of ovalbumin as the feed, and 100 μl as the fraction volume. The six isoproteins of ovalbumin are numbered 1–6.

bumin, one corresponding to each band in the chromatogram, were obtained. Furthermore, though there is some overlap between bands due to mass transfer limitations, pure zones of each fraction are clearly evident. Shown in Fig. 6A is a gradient elution chromatogram of a fraction of the pure ovalbumin 3 band of Fig. 5. Shown in Fig. 6B is the gradient elution chromatogram for the feed mixture. It is clear from a comparison of these chromatograms, that the displacement process has produced ovalbumin 3 of very high purity. Zones of equal degree of purity were obtained with the other fractions, including ovalbumin 1 and 6 which are present at very low concentrations. In fact, the low concentrations of ovalbumin 1 and 6 make it difficult to identify elution peaks for these fractions in a gradient elution chromatogram of the feed (Fig. 6B). However, for either ovalbumin 1 or 6, for a sample taken from the pure zones in Fig. 5, the elution peaks are clearly seen, as is shown in Fig. 6C for ovalbumin 1. In all three chromatograms in Fig. 6, the peak at approximately 6 min is the solvent peak.

The separation of heterogeneous ovalbumin was found to be strongly dependent on solution pH. Shown in Fig. 7 is the displacement chromatogram obtained at

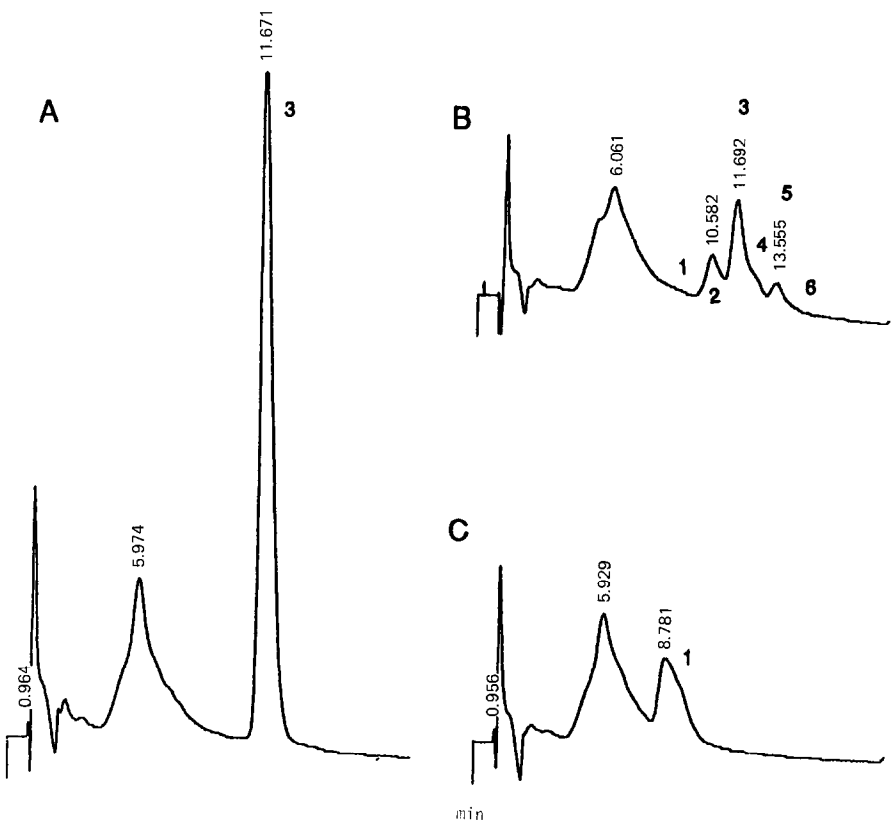

Fig. 6. Analytical chromatograms of (A) one fraction of ovalbumin 3 from pure zone in Fig. 5, (B) 1 mg/ml of ovalbumin sample and (C) one fraction of ovalbumin 1 from pure zone in Fig. 5; the peak with retention time of about 6 min is a solvent peak.

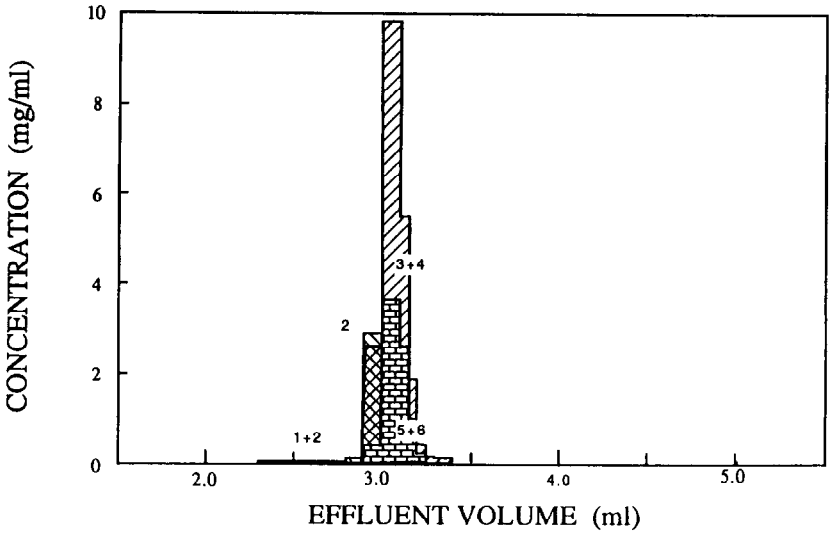

Fig. 7. Displacement chromatogram of ovalbumin. Conditions as in Fig. 5, except 0.3 *M* Tris, pH 8, was the carrier.

pH 8. Clearly, no significant separation of the ovalbumin fractions is obtained at the higher pH. An increase in solution pH results in an increase in the charge density of ovalbumin, but simultaneously decreases the ion-exchange capacity of the stationary phase. The net effect is a decrease in the resolution obtained. This trend was verified with a displacement performed at pH 7, which produced a separation intermediate between pH 6 and pH 8.

Since the separation of heterogeneous ovalbumin is sensitive to solution pH, it is useful to have a simple method for the *a priori* identification of a suitable pH for the displacement process. It was established that isocratic elution could be used as an effective tool for this purpose. Using the same carriers and support that were used in the displacement development, retention data were obtained for ovalbumin at pH 6 and pH 8. At pH 8 a single peak was observed, with a retention time of 1.6 min while at pH 6 a total of three peaks were obtained, with retention times of 2.0, 5.0 and 22.0 min. This shows that separation factors are enhanced at the lower pH, indicating that a better displacement separation can be expected at this pH. At pH values lower than 6, low capacity factors, unfavorable to displacement development, were observed.

The fractionation of heterogeneous ovalbumin with carrier displacement chromatography has been attempted previously by Peterson and Torres [16]. In that study, a mixture of carboxymethyl dextrans was used for displacement and spacing of components. Consistent with this study, the heterogeneous ovalbumin was fractionated into six components.

As was mentioned earlier, ease of regeneration of the column is critical to determining the overall utility of the displacement process [7]. In this study, 0.1 *M* sodium citrate in 0.3 *M* sodium bicarbonate at pH 9 was used as the regenerant. For all the separations performed, no more than three column volumes were required to regenerate the column. Furthermore, re-equilibration requirements for the column

TABLE III

COMPARISON OF REGENERATION PROTOCOLS

Displacer (source)	PVSNa (this study)	Nalcolyte 7105 [9]	Chondroitin sulfates [8]
Molecular weight	2000	20 000	50 000
Column	250 × 4.6 mm, weak base	250 × 4.6 mm, weak acid	75 × 7.5 mm, weak base
Column volume (ml)	4.15	4.15	3.3
Regenerant	0.1 *M* sodium citrate in 0.3 *M* sodium bicarbonate, pH 9	0.8 *M* ammonium sulfate in 0.05 *M* sodium acetate, pH 3.2	50 ml 0.5 *M* sodium phosphate pH 7, 100 ml 0.9 *M* NaCl in 0.01 *M* Tris pH 9.8, and 7.5 ml 0.16 *M* NaOH
Regenerant volume (column volumes)	3	50	78
Re-equilibration volume (column volumes)	10	Not reported	30

are also favorable, with only ten column volumes of solvent necessary. Table III summarizes these results, and also shows regeneration and re-equilibration requirements reported in two other ion-exchange displacement studies [8,9]. Though the separations attempted in each case are different, the much lower solvent requirement with PVSNa indicates the strong potential of low-molecular-weight displacers for future use in ion-exchange displacement chromatography of macromolecules. It is postulated that the key to desirable behavior with respect to *both* displacement development and column regeneration is optimization of the displacer's affinity. Since protein affinity is established by ionic interactions between localized regions of the protein surface and the stationary phase, it is sufficient to select a displacer that has an adequate affinity to overcome these local interactions, simultaneously allowing good column development and easy regeneration. PVSNa appears to fulfill these requirements for the separations studied; it has an affinity strong enough to provide good displacement development of heterogeneous ovalbumin, yet not strong enough to make regeneration of the column difficult. These results suggest the possible use of molecular weight to tailor displacers that will overcome the problem of column regeneration, the most often cited shortcoming of displacement chromatography. The use of low-molecular-weight displacers will also often provide a secondary advantage. In many downstream processing operations, size exclusion chromatography is often used as a final product polishing step. A large difference in molecular weight between the displacer and the biomolecules will facilitate this separation.

CONCLUSION

PVSNa, a low-molecular-weight polyelectrolyte, has been shown to be a suitable displacer for protein separations by ion-exchange displacement chromatography. Experimental evidence obtained for the effects of displacer concentration and mobile phase velocity on the separation of ovalbumin from conalbumin indicate that separation is indeed achieved by displacement. Using PVSNa as the displacer, a displacement protocol has been developed for the fractionation of heterogeneous ovalbumin. Also, it was found that column regeneration and re-equilibration after displacement with PVSNa requires low amounts of solvent, when compared to high-molecular-weight displacers used on similar separations in the past.

ACKNOWLEDGEMENT

This work was supported in part by Grant No. CTS-8909742 from the National Science Foundation. This support is gratefully acknowledged.

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