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Preparative high-performance liquid chromatography on a unique high-speed macroporous resin

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ABSTRACT

The use of high-performance liquid chromatography (HPLC) for the analysis and purification of biological macromolecules has increased with the commercial availability of packing materials specifically designed for large-molecule analysis. However, the speed of analysis is limited as the efficiency of the HPLC columns for macromolecules has been significantly lower that that acheived for small molecule separations. One possible solution to the speed/capacity/resolution triangle experienced with large biological molecules is to increase the pore size of the HPLC matrix. A rigid macroporous polymer with a 4000 Å pore size has, therefore, been developed for evaluation as a high performance matrix for macromolecule HPLC separations. The mechanical stability of this polymer enables the material to be packed in conventional columns, 4.6 mm I.D., and operated under HPLC conditions of flow rate and pressure.

The high speed, fast flow matrices evaluated are for reversed-phase HPLC, PLRP-S 4000 Å, and for anion-exchange HPLC, PL-SAX 4000 Å. The matrices/columns have been assessed for stability, permeability, efficiency, selectivity and capacity with macromolecules and their suitability for analytical and preparative applications evaluated.

INTRODUCTION

As research workers continually strive for increased speed of analysis in the separation/fractionation of biological macromolecules much effort has been put into the optimization and design of chromatographic supports for high-performacne liquid chromatography (HPLC). This is reflected in the fact than HPLC is now one of the fastest growing techniques with a proliferation of column packings and instrumentation.

A suitable adsorbent for HPLC should have a controlled uniform pore geometry, mechanical stability, chemical resistance, clean homogeneous surface and narrow particle size distribution. In the 1970s silica became the base matrix of choice being commercially available in a range of pore and particle sizes and being easily derivatized to produce different functionalities, reversed-phase[1], ion-exchange[2], affin-

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ity[3] and for gel filtration chromatography[4]. However the silica-based packings have restricted chemical stability.

Although liquid chromatography of biopolymers, proteins, nucleic acids etc., was originally performed using polymer gels[5–7] these were microporous semi-rigid gels where the pore size is dependent upon the degree of swelling, hydration of the polymer chains. Unless operated at low pressure with low flow-rates these gels collapse, restricting the flow and so making them unsuitable for high speed separations. It was not until 1964[8] that rigid macroporous polymers were produced for liquid chromatography and in 1985 used for the HPLC analysis of proteins[9].

Even with these rigid macroporous, microparticulate HPLC packing materials the speed of macromolecule analysis is limited due to the reduced efficiency/resolution of large-molecule separations compared to those achieved with small molecule HPLC under similar conditions. This is attributed to both kinetic and thermodynamic factors. The diffusion coefficient of a solute decreases as its molecular size in solution increases so limiting its ability to rapidly diffuse in and out of the matrix pore structure. Large-molecule diffusion coefficients have been measured at 10^{-10} and 10^{-11} m^2/s (ref. 10) but these are further reduced by pore diffusion (solute diffusion into and out of the porous structure of the packing material). It has been shown that when the ratio of the solute diameter to pore diameter exceeds 0.2 the pore diffusion is restricted[11]. This leads to a reduction in efficiency/resolution as band-spreading increases at flow-rates normally used for small-molecule HPLC. In order to reduce band-spreading and improve efficiency the linear velocity must be reduced but this increases the sample residence time in the columns and hence contact time with potentially denaturating conditions. This is obviously undesirable for the analysis of biologically active molecules.

In order to improve intraparticle stagnant mobile phase mass transfer for macromolecules the pore size must be increased. However, the configuration of the molecule in solution and in contact with the stationary phase surface will determine the size of the pore required. One solution to the problem would be to have a non-porous matrix where stagnant mobile phase mass transfer would be eliminated. Unger and co-workers used 1.5-μm non-porous silica as a matrix for affinity chromatography in 1984[12] and subsequently for reversed-phase[13] and hydrophobic-interaction chromatography[14] non-porous polymers have also been used to produce high-speed matrices[15,16]. However, these materials, even when used in small particle form, have reduced capacity due to the loss of internal pore surface area compared with porous matrices. Therefore it is felt that a more general solution to the speed/capacity/resolution triangle is needed for high-load analytical separations and preparative fractionations.

This paper reports on the chromatographic characteristics of a unique highly porous polymer matrix which can be used for reversed-phase separations or in a derivatized form for ion exchange and which when operated at high flow-rates with rapid gradient development times maintains the resolution and capacity of the separation/fractionation.

EXPERIMENTAL

HPLC system

All chromatography and dynamic capacity measurements were carried out using a modular Knauer HPLC system (Knauer, Berlin, F.R.G.) equipped with two reciprocating high-pressure pumps, Model 64, a gradient former, Model 50B, static mixing chamber with a 20-μl internal volume, variable-wavelength UV detector, Model 87, fitted with a standard analytical cell of 10 mm pathlength/10 μl volume and twin-pen chart recorder. To improve mixing at high flow-rates whilst maintaining the minimum volume of mixing for the high speed separations a Lee Viso-Jet® micromixer with a 10 μl internal volume was inserted between the pumps and the static mixing chamber. (Lee Products, Gerrards Cross, U.K.). Sample introduction was achieved with a Rheodyne 7125 injection valve fitted with a 200-μl loop (supplied by HPLC Technology, Macclesfield, U. K.). A Trilab 2000 computer equipped with a liquid chromatography/gas chromatography data handling programme was used for peak detection, integration and data manipulation (Trivector, Sandy, U.K.).

Mobile phases and chemicals

Water used for sample and eluent preparation was purified using an Elgastat UHP system (Elga, High Wycombe, U.K.) and the buffer salts were of analytical or HPLC grade (FSA Laboratory Supplies, Loughborough, U.K.). Proteins used as chromatographic test probes were of high purity (Sigma, Poole, U.K.). Lyophilized bovine serum albumin (BSA), purified by chromatography to $> 99\%$ was used for the determination of dynamic loading capacity (Advanced Protein Products, Brierley Hill, U.K.).

Dynamic load capacity

Frontal analysis was used to determine the binding capacity of the 4000 Å high-speed macroporous resin[17]. The dynamic protein capacity for a medium-molecular-weight globular protein, BSA, was obtained by pumping a dilute solution of the protein in binding buffer through a 50 × 4.6 mm I.D. polyether ether ketone (PEEK) column at 1.0 ml/min (2.5 mg BSA/ml in 0.01 *M* Tris · HCl, pH 8.0). The column was first eluted with a cleaning buffer, 0.01 *M* Tris · HCl + 1.0 *M* NaCl, pH 8.0, followed by conditioning with the binding buffer, 0.01 *M* Tris · HCl, pH 8.0. The protein breakthrough curve was established by monitoring the column eluent at 256 nm and the volume of protein solution required to saturate the column was determined by dropping a perpendicular line from the front breakthrough boundary at 20% of the maximum UV adsorbance.

Chromatographic methods

Matrix permeability and mechanical rigidity was determined using the high-performance 150 × 4.6 mm I.D. stainless-steel hardware. Methanol–water (70:30, v/v) was pumped through the column at constant pressure using a Haskel (Sunderland, U.K.) air-driven pump Model MCP-71 and the flow-rate monitored using an analytical flowmeter (supplied by HPLC Technology).

Reversed-phase analysis of proteins under denaturing conditions was carried out using PLRP-S 300 Å 5 μm and PLRP-S 4000 Å 8 μm material packed in 50 × 4.6

mm I.D. stainless-steel hardware. The test proteins were dissolved in water containing 0.1% trifluoroacetic acid and eluted with a gradient of 0.1% trifluoroacetic acid in acetonitrile.

The resolution of a protein test mixture under non-denaturing conditions by anion exchange, was determined using PL-SAX 4000 Å 8 μm and PL-SAX 1000 Å 8 μm material packed in biocompatible PEEK hardware, 50 × 4.6 mm I.D. The proteins were dissolved in 0.01 *M* Tris · HCl, pH 8.0 and eluted with a gradient of increasing NaCl concentration.

The influence of flow-rate on the band spreading of non-retained solutes was investigated using a PL-SAX 4000 Å 8 μm 250 × 4.6 mm I.D. stainless-steel column. A 0.01 *M* Tris · HCl + 0.5 *M* NaCl, pH 8.0 eluent was used.

The preparative fractionations were carried out using the PL-SAX 4000 Å 8 μm material packed in the biocompatible PEEK hardware 50 × 4.6 mm I.D. The samples were diluted using 0.01 *M* Tris · HCl, pH 8.0 and chromatoghraphed using an increasing salt gradient, 0.01 *M* Tris · HCl + 0.5 *M* NaCl, pH 8.0.

RESULTS AND DISCUSSION

Column stability

The conventional wide pore poly(styrene–divinylbenzene) reversed-phase material, PLRP-S 1000 Å, and the high speed, fast flow poly(styrene–divinylbenzene) matrix, PLRP-S 4000 Å, were compared for permeability and stability. A plot of column pressure *vs.* flow-rate was carried out for each material using the same column dimensions, 150 × 4.6 mm I.D. and eluent, methanol–water (70:30, v/v). The nominal particle size for both materials was 8 μm although there were slight differences in the maxima of the number-average particle size distributions. This is reflected in the off-set of the curves and flow-rates at 2000 p.s.i. The PLRP-S 40000 Å being slightly larger with a flow-rate of 2.07 ml/min compared with the PLRP-S 1000 Å at 1.75 ml/min.

A linear relationship for both materials is achieved up to 3000 p.s.i., the column packing pressure, after which the bed is repacked resulting in deviation from linearity (Fig. 1). Comparing the mechanical rigidity and flow characteristics, permeability, of these two materials it is clear that the fast flow matrix, PLRP-S 4000 Å, is capable of operating at the high flow-rates and pressures necessary for HPLC without particle compression or fragmentation.

By changing the eluent polarity [*i.e.* so that it is more polar than the neutral non-polar poly(styrene–divinylbenzene) surface] it is possible to use this polymeric matrix for reversed-phase separations. Fig. 2 compares the resolution achieved for a representative selection of globular proteins on a conventional reversed-phase material, PLRP-S 300 Å 5 μm operated at 1.0 ml/min with a 20-min gradient development time and the fast flow matrix, PLRP-S 4000 Å 8 μm operated at 4.0 ml/min with a 1-min gradient development time. The separations were both carried out at ambient temperature. No deterioration in separation efficiency was observed for the fast flow matrix, PLRP-S 4000 Å when operated repeatedly with short gradient development times and high flow-rates. Rapid re-equilibration was achieved, 30 s, between the end of the first gradient run and the start of a second.

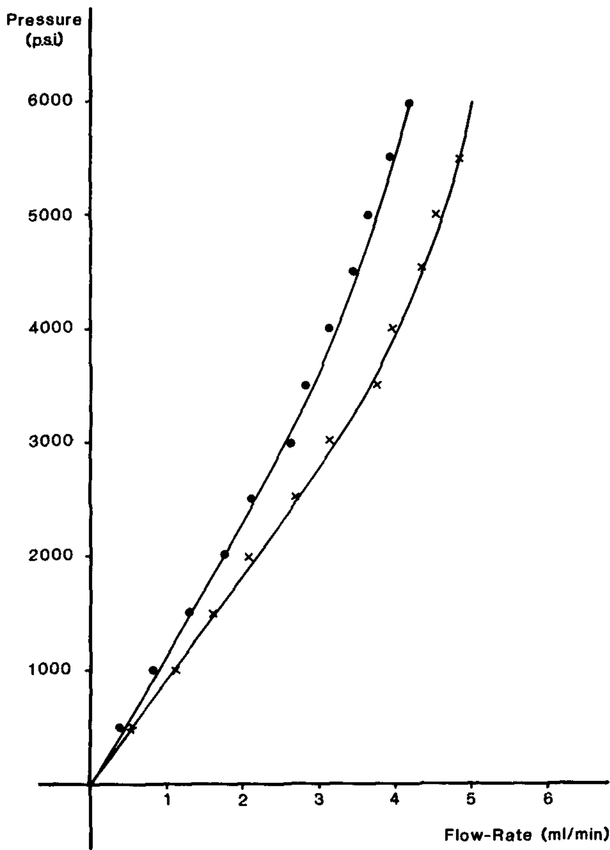

Fig. 1. Column pressure *vs.* flow-rate. Eluent, methanol–water (70:30, v/v); temperature, ambient; column 150 × 4.6 mm I.D.; particle size 8 μm. ● = PLRP-S 1000 Å; × = PLRP-S 4000 Å.

Adsorbent capacity

In order to evalutate the fast flow matrix in non-denaturing eluents for its suitability as a rapid purification system for biologically active molecules a strong anion exchanger was produced. The PLRP-S 1000 Å and 4000 Å materials were derivatized, coated with poly(ethyleneimine) and quaternized following the method published by Rounds *et al.*[18] to produce the strong anion exchangers PL-SAX 1000 Å and PL-SAX 4000 Å.

The influence of pore size on the chromatographic loading was determined by reference to the separation of the proteins ovalbumin and soybean trypsin inhibitor. The resolution factor, R_s as calculated from the difference in retention volume divided by the mean peak (base) volume was determined at various protein loadings.

$$R_s = \frac{t_{R(A)} - t_{R(B)}}{\frac{1}{2}(W_A + W_B)} \quad \text{(ref. 19)}$$

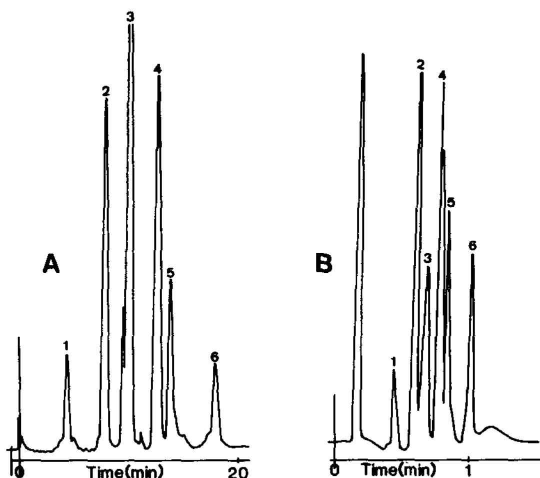

Fig. 2. Comparison of the resolution achieved for 6 globular proteins run under reversed-phase conditions. Proteins: 1 = ribonuclease A; 2 = cytochrome *c*; 3 = lysozyme; 4 = bovine serum albumin; 5 = myoglobin; 6 = ovalbumin. Detection, UV at 280 nm; temperature, ambient; eluent A, 0.1% trifluoroacetic acid in acetonitrile–water (5:95, v/v); eluent B, 0.1 % trifluoroacetic acid in acetonitrile–water (95:5, v/v). (A) column, PLRP-S 300 Å 5 μm 50 × 4.6 mm I.D.; gradient, linear 15–55% B in 20 min at a flow-rate of 1.0 ml/min. (B) Column, PLRP-S 4000 Å 8 μm 50 × 4.6 mm I.D.; gradient, linear 18–60% B in 1 min at a flow-rate of 4.0 ml/min.

where $t_{R(A)}$ and $t_{R(B)}$ are the retention volumes of soybean trypsin inhibitor and ovalbumin respectively and W_A and W_B are the respective peak volumes at the base.

Fig. 3, the plot of resolution factor R_s *vs.* protein load shows that at low loadings comparable resolution is achieved but that the resolution obtained with the

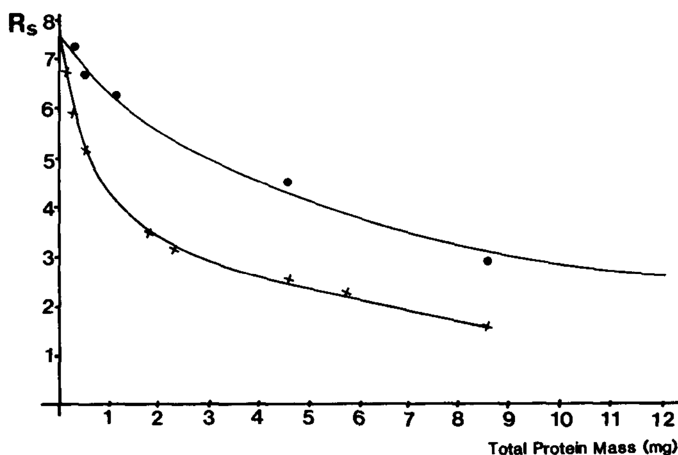

Fig. 3. Resolution factor, R_s, *vs.* total protein load for the anion-exchange separation of ovalbumin and soybean trypsin inhibitor. Eluent A, 0.01 *M* Tris · HCl, pH 8.0; eluent B, A + 0.35 *M* NaCl, pH 8.0; gradient, linear 0–100% B in 20 min; flow-rate, 1.0 ml/min; detection, UV at 280 nm. ● = PL-SAX 1000 Å 8 μm 50 × 4.6 mm I.D. PEEK hardware; × = PL-SAX 4000 Å 8 μm 50 × 4.6 mm I.D. PEEK hardware.

PL-SAX 4000 Å material is more dependent upon column load. Therefore, for high-resolution analytical separations of medium-molecular-weight globular proteins using the PL-SAX 4000 Å 50 × 4.6 mm I.D. column of 0.83 ml volume a typical protein load would be 100 μg. However, 8 mg of total protein can be loaded before the resolution factor drops to 2, the normal criteria for an excellent separation where for gaussian peaks there are 2 base widths between the peak maxima and one base width of near baseline[19].

This would be predicted due to a decrease in surface area with increasing pore size. The total surface area of the poly(styrene–divinylbenzene) 1000 Å and 4000 Å matrices being 267 m^2/g and 139 m^2/g respectively as determined using multipoint nitrogen adsorption, (Digisorb 2600, Coulter Electronics, Luton, U.K.). These values are higher than expected when compared with a silica-based material due to the lower density, 0.3 g/ml, and the presence of microporous structure within the polymer matrix which is accessible to nitrogen but not to an HPLC solute[20]. From the multipoint nitrogen adsorption measurements it is estimated that approximately 1/3 of the surface area is located in pores with an average diameter of less than 20Å. In order to compare available surface area for protein fractionation the dynamic loading capacity was determined by frontal analysis as detailed in the Experimental section. Values obtained were 93 mg BSA/ml of column volume for the PL-SAX 1000 Å and 34 mg/ml for the 4000 Å.

High-speed resolution

As both the PL-SAX 1000 Å and 4000 Å materials are rigid macroporous polymers and are, therefore, capable of operating at high flow-rates and pressures and have exceptionally rapid equilibration rates it is predicted that the packed columns would be stable for high speed operation. As would be expected with porous HPLC matrices a decrease in gradient development time, with a constant flow-rate leads to a decrease in resolution. However, when the flow-rate is increased with a short gradient development time, 2 min, a significant increase in the resolution factor is obtained for the fast flow-rate matrix, PL-SAX 4000 Å, from 4.80 to 7.30. This does not occur with the wide-pore material PL-SAX 1000 Å where the resolution factor increases from 3.25 to 4.00. This suggests that there is a difference in the intraparticle mass transfer of these two materials for large molecules at high flow as the protein peak width (band spreading) decreases for the 4000 Å matrix but not the 1000 Å.

Using an isocratic system and a high salt buffer to give a solute capacity factor (k') of 0 and peak symmetry factors of 1.3 (anion exchange, non-interactive conditions) plate count (efficiency) measurements were carried out for three solutes with increasing linear velocity. The probes used were adenosine 5′-monophosphate and two proteins of increasing molecular weight, myoglobin with a molecular weight of 16 950 daltons and ferritin of 470 000 daltons. As the molecular size in solution of a solute increases so the diffusion coefficient decreases. This significantly increases the band broadening and hence reduces the efficiency of macromolecule separation compared with small molecules at a given flow-rate. As seen in Fig. 4, the *H vs. U* plot, for the PL-SAX 4000 Å material there is a decrease in efficiency with increased molecular size. However, at a linear velocity of approximately 3.5 mm/s the decrease in efficiency for the three probes is halted. It would appear that with the high-speed/fast flow matrix above a critical linear velocity irrespective of solute size the efficiency

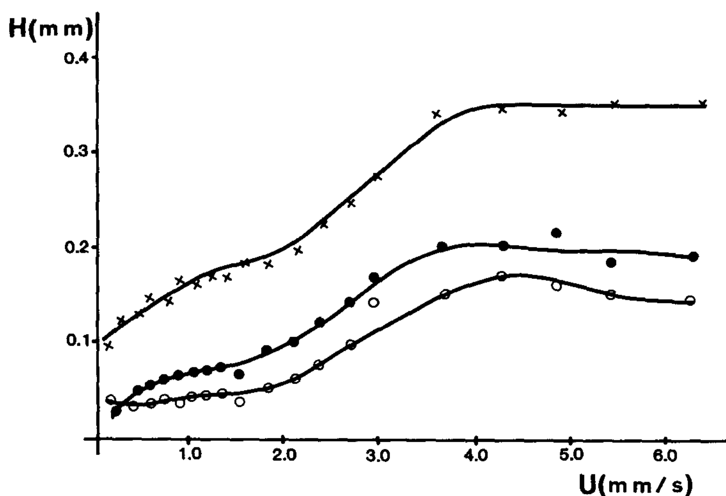

Fig. 4. Plate height (H) *vs.* linear velocity (U) for the PL-SAX 4000 Å 8 μm material run in a non-interactive mode. Eluent, 0.01 M Tris·HCl, 0.5 M NaCl, pH 8.0; detection, UV at 280 nm; column, 250 × 4.6 mm I.D. stainless-steel hardware. ○ = Adenosine 5′-monophosphate; ● = myoglobin; × = ferritin.

starts to improve suggesting an apparent increase in the diffusion coefficient. This type of curve is not typical for porous materials although it has been observed with some non-porous packings.

High-speed capacity

If at high linear velocities the increase in efficiency is due to the matrix performing as a non-porous support then a decrease in dynamic protein capacity and an increased dependence of resolution on sample load would be observed as there would be a decrease in available suraface area.

The plot of resolution factor, R_s, *vs.* total protein load for the analytical separation of ovalbumin and soybean trypsin inhibitor demonstrated that there is no greater dependence of resolution on load at 4.0 ml/min than 1.0 ml/min. However, as would be predicted from the H *vs.* U plot there is an increase in resolution with increased flow-rate at similar protein loads. The dynamic loading capacity as determined by frontal loading was determined as 34 mg BSA/ml of column volume at 1.0 ml/min and 32 mg at 4.0 ml/min. The difference in shape of the two frontal loading curves (Fig. 5) is due to the differences in the flow path through the column. However, at flow-rates of 4.0 ml/min it would be possible to utilize over 80% of the column volume before valuable product is detected in the eluent system. It would, therefore, appear that although the efficiency of separation increases at high linear velocity suggesting a change in the intraparticle diffusion coefficients the high-speed/fast flow material is not performing as a non-porous matrix. There is no decrease in sample load at the increased linear velocities as would be observed if the internal pore surface area was no longer accessible. The surface area of non-porous packings being approximately two orders of magnitude lower than that of porous packings of the same particle size, 0.55 m^2/g for a 5-μm particle[21].

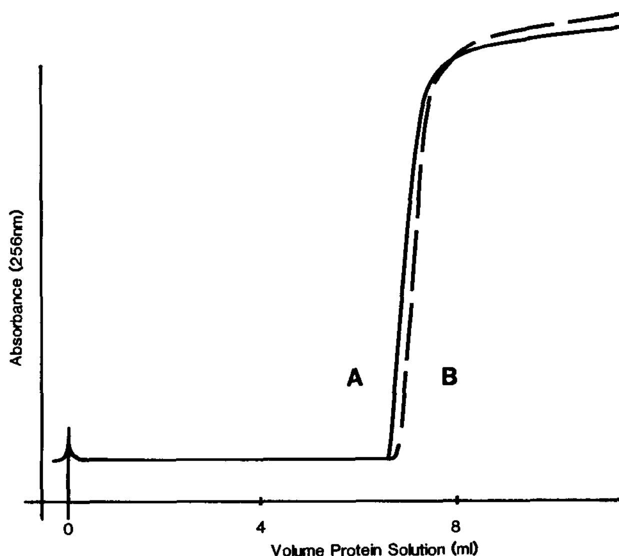

Fig. 5. BSA frontal loading curves. Eluent, 2.5 mg BSA/ml 0.01 *M* Tris-HCl, pH 8.0; detection, UV at 256 nm; column, PL-SAX 4000 Å 8 μm, 50 × 4.6 mm I.D.; PEEK hardware. (A) Flow-rate, 4.0 ml/min; (B) flow-rate, 1.0 ml/min.

Applications

The isolation of antibodies from complex biological matrices necessitates the use of several chromatographic stages if high purity is required. For the isolation of poly/monoclonal antibodies from serum where the composition is relatively well defined although the actual quantities of the various components will be species/individual dependent the antibodies would be expected to be 20% and albumin 60% of the total protein concentration. In order to selectively separate the group of antibodies from non-antibody proteins the first chromatographic step would be the use of immobilized Protein A. The serum was diluted with binding buffer, 0.01 *M* Tris · HCl, pH 8.0 in the ratio 1:5 (v/v) and filtered prior to pumping through the PL-AFC Protein A column. The polyclonal antibodies are selectively retained and after equilibrating the column with binding buffer eluted by reducing the pH, 0.1 *M* glycine, pH 2.5. Following the affinity separation the pH of the eluted polyclonal antibody fraction was adjusted to neutrality to maximise the recovery of biological activity. The purity of the bound fraction or the presence of antibodies in the unbound fraction can be determined using anion exchange or if high purity is required residual albumin and transferrin can be removed (Fig. 6). Using the high speed/fast flow PL-SAX 4000 Å material exposure to potentially deactivating conditions can be minimised as fractionation is accomplished in 90 s.

The enzyme amyloglucosidase was fractionated from *Aspergillus niger* cell culture filtrate which had a total protein concentration as determined by UV at 280 nm of 395 mg/ml. The crude cell culture filtrate was diluted with the low-strength component of the mobile phase, eluent A : 0.01 *M* Tris · HCl, pH 8.0, and filtered prior to the chromatographic fractionation. No other sample preparation was carried out. Using

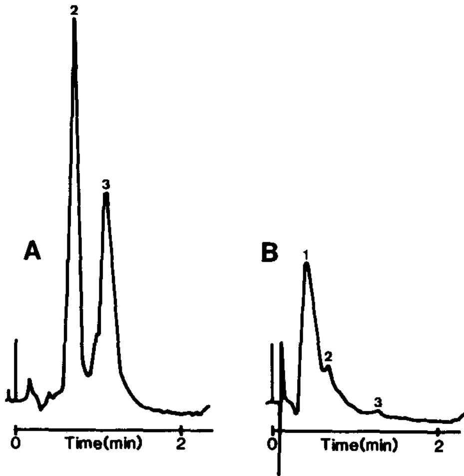

Fig. 6. Anion-exchange separation of the bound (B) and unbound (A) fractions from a Protein A antibody purification. Eluent A, 0.01 *M* Tris·HCl, pH 8.0; eluent B, A + 0.5 *M* NaCl, pH 8.0; gradient, linear 0–100% B in 2 min; flow-rate, 4.0 ml/min; detection, UV at 280 nm; column, PL-SAX 4000 Å 8 μm, 50 × 4.6 mm I.D.; PEEK hardware. Peaks: 1 = antibodies; 2 = transferrin; 3 = albumin.

an analytical column, PL-SAX 4000 Å 8 μm, 50 × 4.6 mm I.D. PEEK column and a total protein load of 36 μg baseline resolution of two components was achieved in less than 2 min. As it is known that the enzyme amyloglycosidase occurs in two forms with molecular weights of 99 000 daltons and 112 000 daltons of the same amino acid composition but with different carbohydrate content[22] an enzyme activity measure-

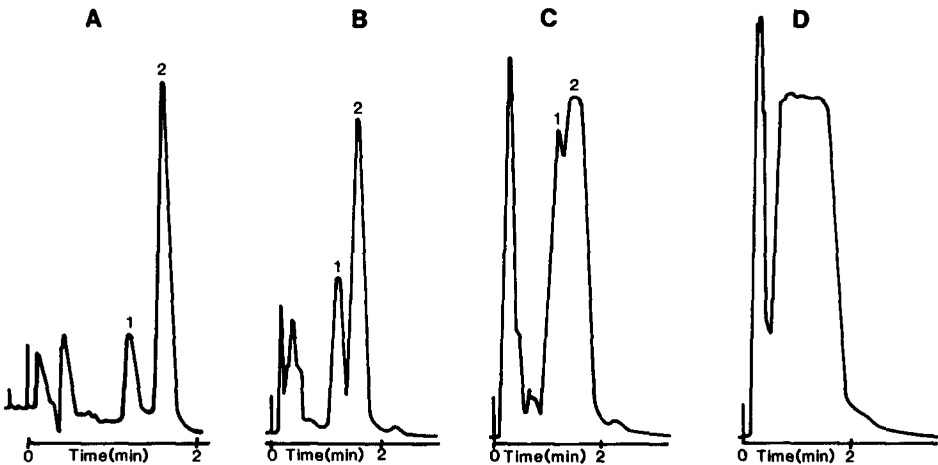

Fig. 7. Anion-exchange separation of two isoenzymes of amyloglucosidase from *Aspergillus niger* cell culture filtrate. (A) 36 μg of protein; (B) 3.6 mg of protein; (C) 10 mg of protein; (D) 20 mg of protein. Eluent A, 0.01 *M* Tris·HCl, pH 8.0; Eluent B, A + 0.5 *M* NaCl, pH 8.0; gradient, linear 0–100% B in 2 min; flow-rate, 4.0 ml/min; detection, UV at 280 nm; column, PL-SAX 4000 Å 8 μm, 50 × 4.6 mm I.D.; PEEK hardware.

ment was carried out on both peaks, by adding an aliquot of the fractions to a 1% starch solution heated to 60°C. After a 10-min hydrolysis the amount of glucose produced was determined using the dinitrosalicylic acid assay[23]. Enzyme activity was confirmed in both peaks 1 and 2. Using the analytical size column baseline resolution of the two isoenzymes can be achieved with loadings upto 1.4 mg; however, for the purification of total enzyme a column load of 20 mg can be chromatographed with a 2-min gradient development time and 30 s re-equilibration (Fig. 7). This would enable 480 mg of protein to be fractionated per hour with an analytical 50 × 4.6 mm I.D. column.

CONCLUSION

From this initial evaluation of the high-speed, fast flow matrix both as a reversed-phase material, PLRP-S 4000 Å and in the derivatized form as a strong anion exchanger, PL-SAX 4000 Å, it would appear to be a more universal solution to the speed/capacity/resolution triangle. When used with short gradient development times and high linear velocities resolution and loading are comparable to those achieved under conventional HPLC conditions for wide pore matrices.

The equipment requirements for the use of these materials are not as severe as with the non-porous matrices due to the higher loadings involved although the requirement on the pumping system and gradient former are similar. The work reported here was carried out using an unmodified analytical UV detector with a 10 μl cell volume and 10 mm path length.

It is anticipated that the main application areas for these material will be in the analysis of very large biomolecules under conventional HPLC conditions, the rapid preparative fractionation of biologically active molecules in mg–g quantities, high-speed purity determination of fractions from a large-scale LC process and the optimization of product yields from fermentation broths and process separation systems.

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