ELSEVIER

Journal of Chromatography A, 944 (2002) 169–177

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Reversed-phase poly(styrene–divinylbenzene) materials optimised for large scale preparative and process purification of synthetic peptides and recombinant proteins

Linda L. Lloyd*, Mark I. Millichip, Jennifer M. Watkins

Polymer Laboratories Ltd., Essex Road, Church Stretton, Shropshire SY6 6AX, UK

Abstract

Rigid macroporous copolymers of styrene and divinylbenzene have been designed for large-scale preparative and process-scale purification of synthetic peptides and recombinant proteins. The polymeric particles are mechanically stable and hence able to operate at the required high linear velocities. The pore size and pore morphology has been optimised to enable unhindered solute diffusion whilst providing maximum available surface area to enhance loading capacity. A 100 Å pore size has been developed for synthetic peptides and a 300 Å pore size for recombinant proteins. Precise control of particle size, within the range 10 to 20 μm, is possible which together with the very narrow particle size distribution enables maximum resolution/loading to be obtained within the pressure limits of the instrumentation being used. The chemical stability of the polymer enables cleaning in place with 1 *M* sodium hydroxide without particle dissolution or a deterioration in selectivity. These materials can be packed into compression hardware and are manufactured as single lots up to 100 kg (300 l) batch size.

Keywords: Preparative chromatography; Stationary phases, LC; Poly(styrene–divinylbenzene); Peptides; Proteins

1. Introduction

Polymeric particles have long been used for the separation of biomolecules [1,2] but these original materials are semi-rigid porous particles and hence have limited mechanical stability. This necessitates their use in a large particle format and under low-pressure, low-flow conditions. Whilst these materials are extremely useful for the early stages of purification of a recombinant product, their large particle size and inherent low performance makes them unsuitable for the high-performance, polishing stage of a purification protocol. To overcome this limited mechanical stability, materials based on silica particles have been developed, and used, for high-performance separations of biomolecules [3–5] including preparative fractionation of peptides [6]. However, such silica-based materials have limited chemical stability which can reduce the lifetime of the column packing, restrict the cleaning regimes available and severely reduce the pH range at which the purification can be performed. The requirement for stringent cleaning in place and depyrogenation is considered essential for preparative and process columns used for purification of peptide- and protein-based pharmaceuticals, the additional benefit of enhanced resolution/solubility can often also be achieved at pH values outside the normal working range of silica-based packings. To address these issues small particle sized polymeric materials have been developed for analytical separations and small

*Corresponding author.

E-mail address: linda.lloyd@polymerlabs.com (L.L. Lloyd).

0021-9673/02/$ – see front matter
PII: S0021-9673(01)01238-9

scale fractionations [7] but the production of these materials on a large scale for process work has not been possible. Much work has been carried in the area of polymer chemistry with a view to designing polymeric media, which can be used for high-performance liquid chromatography (HPLC) [8–10]. Some methacrylate-based polymeric materials have been commercialised but issues relating to mechanical stability and 1 *M* NaOH compatibility remain.

Poly(styrene–divinylbenzene)-based particles for biomolecule reversed-phase HPLC [11] have gained acceptance for their use in the analysis of biological macromolecules including peptides, proteins and oligonucleotides. A number of commercial large-scale macroporous poly(styrene–divinylbenzene) products for process chromatography have been introduced but they have been restricted to large inefficient particles with a broad particle size distribution [12]. However, they have demonstrated the suitability of this type of materials for preparative and process scale purifications of synthetic peptides and recombinant proteins. With the advent of peptide–protein pharmaceuticals the requirement is now for the purification of such molecules at the multi-kg level necessitating the use of columns with I.D.s in the range 45 to 60 cm. Thus there is a need for a range of higher efficiency poly(styrene–divinylbenzene) materials where the pore size and pore size distribution has been optimised for the purification of small synthetic peptides or recombinant proteins and are sufficiently mechanically stable to enable stable packed beds to be obtained in process scale columns.

This paper reports on the chromatographic characteristics of a range of poly(styrene–divinylbenzene) materials, PLRP-S, which have been developed for the process purification of peptides and recombinant proteins.

2. Experimental

2.1. Gel permeation chromatography system

A modular isocratic Knauer HPLC system (Knauer, Berlin, Germany) fitted with a Rheodyne 7125 injector equipped with a 20-μl injector loop (Polymer Labs., Church Stretton, UK) was used. The detector was a variable-wavelength UV–Vis monitor operated at 254 nm. Tetrahydrofuran (THF) was used as the eluent and polystyrene standards with a narrow polydispersity (Polymer Labs.) used as the calibrants. The column, 300×7.5 mm I.D., was calibrated at a flow-rate of 1.0 ml/min.

2.2. Mechanical stability

To assess the particle permeability and mechanical rigidity the materials were packed into 150×4.6 mm I.D. columns and methanol–water (70:30, v/v) was pumped through the column using a Haskel air-driven pump Model MCP-71 (Sunderland, UK) at constant pressure. The flow-rate was monitored at 500 p.s.i. pressure increments using an analytical flow meter (G.J.C. Instruments, Merseyside, UK) (1 p.s.i.=6894.76 Pa).

2.3. Reversed-phase HPLC

A quaternary low-pressure mixing system consisting of a Model LC 1150 pump, a PL-DG 804 degasser and an LC 1200 UV–Vis detector set at 220 nm for the peptide analysis and 254 nm for the protein analysis (Polymer Labs.) was used. A manual Rheodyne 77251 injector fitted with a 20-μl loop (Polymer Labs.) was used to inject the peptide–protein samples. Peptide and protein reversed-phase HPLC was carried out using 250×4.6 mm I.D. and 150×4.6 mm I.D. columns, respectively.

2.4. Dynamic loading capacity

Frontal loading was used to determine the binding capacity. A dilute 1 mg/ml solution of the required protein in binding eluent, 0.1% trifluoroacetic acid (TFA) in acetonitrile (ACN)–water (95:5, v/v) was pumped through a 50×4.6 mm I.D. column at the required flow-rate of 0.5 to 5.0 ml/min. The protein breakthrough curve was established by monitoring the column eluent at 256 nm. The volume of protein solution required to saturate the column was determined at 20% of the adsorption of the protein solution. The column packing was washed using a strong eluent, 0.1% TFA in ACN–water (95:5, v/v), and conditioned using the binding eluent before repeating the frontal loading experiment.

2.5. Eluents and chemicals

The water used for eluent preparation was purified using an Elgastat Prima RO system coupled to an Elgastat UHP system (Elga, High Wycombe, UK) and the eluent additives were of analytical or HPLC grade (Fisher, Loughborough, UK). With the exception of the decapeptide test mix (Spi, Alberta, Canada) all the peptides and proteins used as chromatographic test probes were of high purity (Sigma, Poole, UK).

3. Results and discussion

3.1. Particle characteristics

Macroporous copolymers of styrene and divinylbenzene have long been used in liquid chromatography [13,14]. Their suitability for such applications is determined by the pore size and pore size distribution of the macroporous polymeric particles. The ability to perform under HPLC conditions requires both the pore morphology and mechanical rigidity and also uniformity of particle size. High-performance separations require small rigid particles able to operate under conditions of high eluent flow-rate and pressure. For large-scale preparative and process high-performance separations the ability to manufacture, reproducibly, on a multi-kg single batch scale is of paramount important if efficient large columns, 45 cm and 60 cm I.D., are to be produced using single batches of media. With these requirements in mind much work has been carried out with a view to optimising the suspension polymerisation process to make it reproducible and scaleable and to provide a range of materials with optimised pore sizes, pore size distributions and particle size for HPLC [15]. Suspension polymerisation production plant and methodology has been developed to produce high efficiency particles with narrow particle size distributions in batch sizes in excess of 300 l whilst accurately controlling the pore morphology. Two nominal pore sizes have been produced, PLRP-S 100 Å and PLRP-S 300 Å, with particle sizes in the range 10 to 20 μm. Optical microscopy shows that the particles produced are spherical, Fig. 1, and have a narrow particle size distribution. The width of the particle size distribution at 1/2 height is of the order of 2.2 μm for the 10 μm products and 3.3 and 4.5 μm for the 10–15 and 15–20 μm batches, respectively.

3.1.1. Pore size and pore size distribution

The pore size and pore morphology of the media will effect the biomolecule diffusion into the porous structure and also the available surface area for interaction. It was shown by Lewis et al. [16] that for silica based reversed-phase materials wide pore silica is required as the base particle for protein sepa-

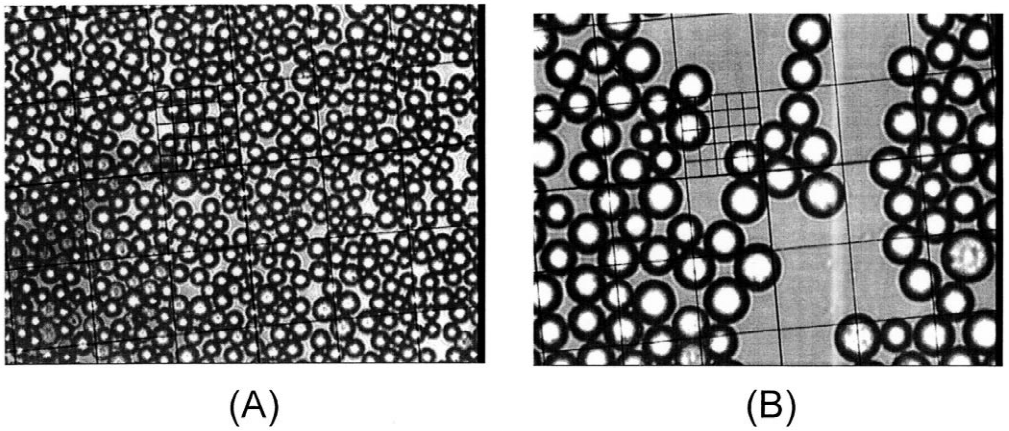

Fig. 1. Optical microscopy images. Representative sample of PLRP-S 100 Å, 10–15 μm (A) ×10 (one small square is equivalent to 10 μm), (B) ×20 (one small square is equivalent to 5 μm).

rations. Gel permeation chromatography (GPC) is a form of liquid chromatography where there is no interaction with the column packing and hence the separation mechanism is dependent only on the hydrodynamic diameter of a solute in the eluent. Therefore, a molecular size calibration curve can be produced, molecular mass of a series of solutes of the same chemical type vs. elution time, which is related to the pore size and pore size distribution of the packing materials [17]. The GPC calibration curves for the two pore sizes, 100 Å and 300 Å, are shown in Fig. 2. The larger pore size of the PLRP-S 300 Å is clearly evident from the increase in the molecular mass resolving range. This is the material which has been designed for the analysis of proteins where improved access is required for the larger molecules. The PLRP-S 100 Å has a smaller pore size and increased surface area/capacity for the smaller peptides.

3.1.2. Mechanical rigidity

The mechanical rigidity of the particles and packed bed stability of the columns were assessed under chromatographic conditions. A plot of column pressure vs. flow-rate was produced for the two pore sizes in methanol–water (70:30, v/v) eluent, Fig. 3.

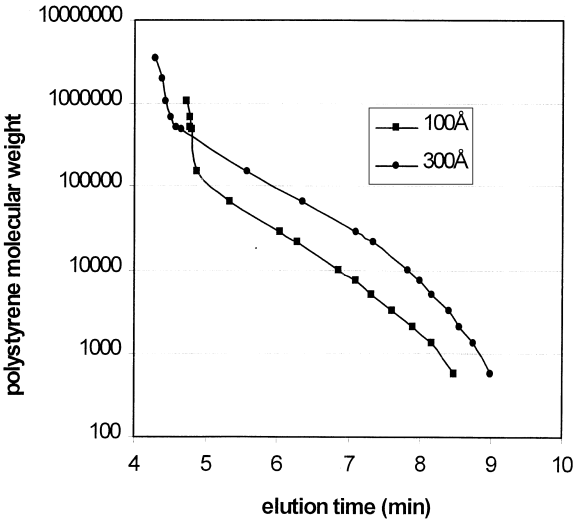

Fig. 2. Gel permeation calibration curves. Eluent: tetrahydrofuran, calibrants: polystyrenes, column: 300×7.5 mm, flow-rate: 1.0 ml/min. ●=PLRP-S 300 Å and ■=PLRP-S 100 Å.

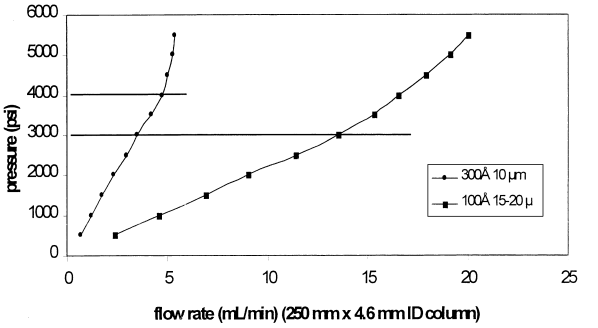

Fig. 3. Column pressure vs. flow-rate. Eluent: methanol–water (70:30, v/v), column: 250×4.6 mm I.D., ●=PLRP-S 300 Å, 10 μm and ■=PLRP-S 100 Å, 10–15 μm.

The two curves are off-set as two different nominal particle sizes were used, 10 μm for the 300 Å and 10–15 μm for the 100 Å particles. The increased permeability of the 10–15 μm particle packed bed is clearly illustrated. The two columns were packed at 3000 p.s.i. pressure and as would be expected if the particles were mechanically stable a linear increase in flow-rate with increasing pressure is observed up to this point. Above this pressure the gel bed begins to repack and deviation from linearity is observed. It is only at a pressure of 5000 p.s.i. that the curve for the 300 Å wider pore material is beginning to approach a vertical, the point at which the particles compress and a further increase in pressure does not result in increased flow-rate. This particle compression point is not observed over the range evaluated for the small pore 100 Å material. From this it can be concluded that the particles, both 100 and 300 Å pore sizes, are mechanically stable and sufficiently rigid to operate under HPLC conditions of pressure and flow-rate.

3.2. Chromatographic performance

3.2.1. Separation selectivity

In order to assess the suitability of the PLRP-S materials for the purification of synthetic peptides it is necessary to assess the selectivity and the possibility of any non-specific surface interactions by using a series of specifically designed peptide standards. Such a series of synthetic peptides has been proposed by Mant and Hodges [18]. These standards have been used for evaluating the suitability of

HPLC materials for the reversed-phase analysis of peptides, resolution and non-specific interactions. A sample of these five decapeptides was used to evaluate the PLRP-S 100 Å and 300 Å materials. Fig. 4 shows the separation of the mixture achieved using the PLRP-S 100 Å material. The five peptides are well resolved using both pore sizes and the peaks obtained are sharp and symmetrical indicating no non-specific interactions. As would be expected, due to the identical chemistry of the two pore sizes, the peptide elution order is the same for both the 100 and 300 Å pore sizes but a lower percentage of organic modifier, acetonitrile, is required for elution from the wide pore material. It can therefore be concluded that the surface is free from ion-exchange type functionalities which would cause peak tailing due to the secondary interaction with charged amino acid residues. The performance and resolution obtained with this polymeric HPLC material is comparable to silica-based reversed-phase materials used for peptide analysis/purification.

3.2.2. Batch to batch reproducibility

In preparative and process chromatography batch to batch reproducibility is critical if a robust protocol is to be established. With this in mind the quality control procedures for the finished media involves a separation of four standard peptides which range in size from the small peptide, oxytocin, to insulin and for the PLRP-S 300 Å the separation of six reference proteins is also performed. The standard peptide separation obtained with a PLRP-S 300 Å 10–15 μm material together with a plot showing the calculated selectivity factors for five consecutive production batches of media are shown in Fig. 5. The corresponding information for the separation of the standard proteins obtained for the same batches of media is shown in Fig. 6. For the separation of both peptides and proteins excellent batch to batch reproducibility is observed.

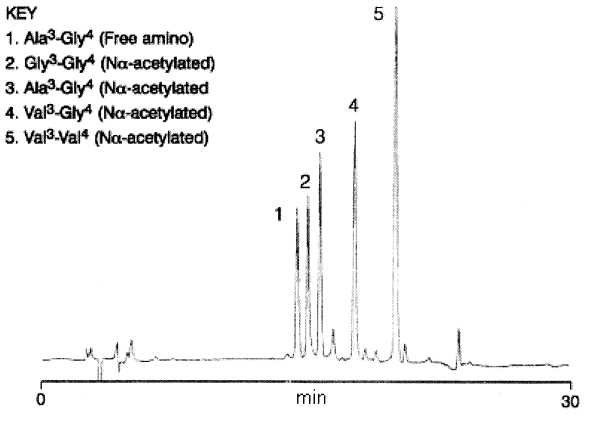

Fig. 4. Separation of five decapeptides. Gradient from 1 to 30% acetonitrile containing 0.1% TFA in 30 min at a flow-rate of 1.0 ml/min, column PLRP-S 100 Å, 250×4.6 mm I.D. Peaks: 1=Ala^3–Gly^4 (free amino), 2=Gly^3–Gly^4 (*N*-acetylated), 3=Ala^3–Gly^4 (*N*-acetylated), 4=Val^3–Gly^4 (*N*-acetylated), 5=Val^3–Val^4 (*N*-acetylated).

3.2.3. Adsorbent capacity

Static binding studies can be used to compare the maximum solute adsorption capacities of porous particles but the data obtained does not reflect the situation experienced in an HPLC purification process, i.e., solute uptake from a flowing eluent with restricted diffusion. For this reason frontal loading curves were generated using the 10–15 μm particles for the two pore sizes, PLRP-S 100 Å and 300 Å, as a function of increasing eluent flow-rate/linear velocity. Two proteins were used for this study, lysozyme, which has a molecular mass of 14 300 and bovine serum albumin with a molecular mass of 68 000. In Fig. 7 the frontal loading curves for lysozyme using both the 100 Å (A) and 300 Å (B) are shown as a function of flow-rate. The curves obtained with the 300 Å material are sharper indicating that there is less hindered diffusion with the 300 Å wider pore material. With peptides, which have a smaller solution size, the frontal loading curves obtained with the PLRP-S 100 Å material would be sharper as their diffusion would not be hindered. With the PLRP-S 300 Å material the curve shape is maintained as the flow-rate increases. The lysozyme binding capacity of the two pore sizes at low linear velocity (0.5 ml/min flow-rate with a 4.6 mm I.D. column) is very similar, 25 mg/ml of packed bed for the 100 Å and 30 mg/ml packed bed for the 300 Å but as the linear velocity increases the capacity decreases more rapidly for the 100 Å particle, Fig. 8. Increasing the size of the protein, bovine serum albumin, sees the capacity of the 100 Å material fall to 2 mg/ml packed bed and for the 300 Å 12 mg/ml

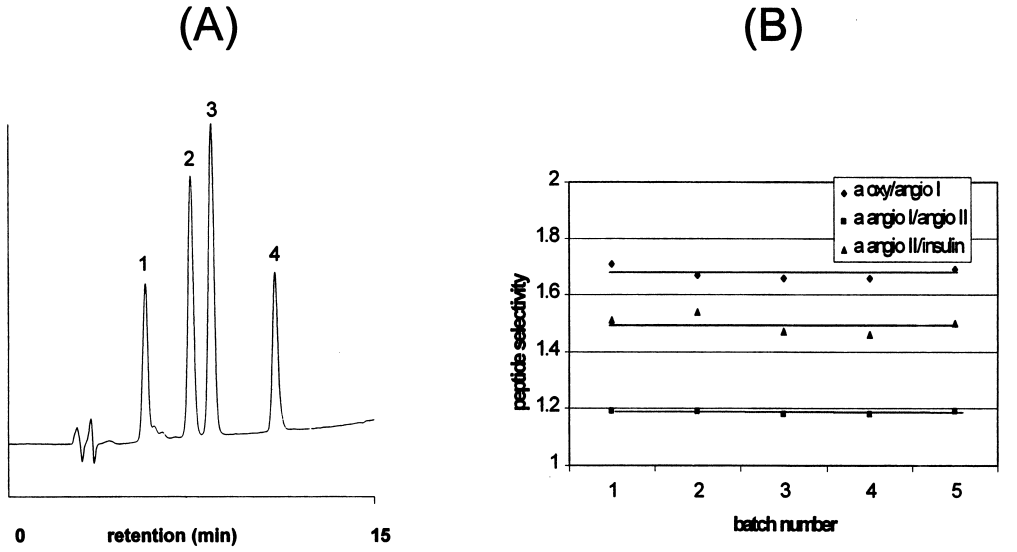

Fig. 5. Separation of four standard peptides (A) and the plot of selectivity vs. batch number for five consecutive batches of PLRP-S 300 Å, 10–15 μm product (B). Gradient from 20 to 50% acetonitrile containing 0.1% TFA in 15 min at a flow-rate of 1.0 ml/min, column 250×4.6 mm I.D. Peaks: 1=oxytocin, 2=angiotensin II, 3=angiotensin I, 4=insulin.

packed bed loading was obtained. This is due to the bovine serum albumin being unable to permeate the pore of the 100 Å material and only the external surface of the particles is available for adsorption.

3.2.4. Alkaline stability

For the purification of biomolecules, peptides and proteins, cleaning in place and depyrogenation are essential to maintain column performance and mo-

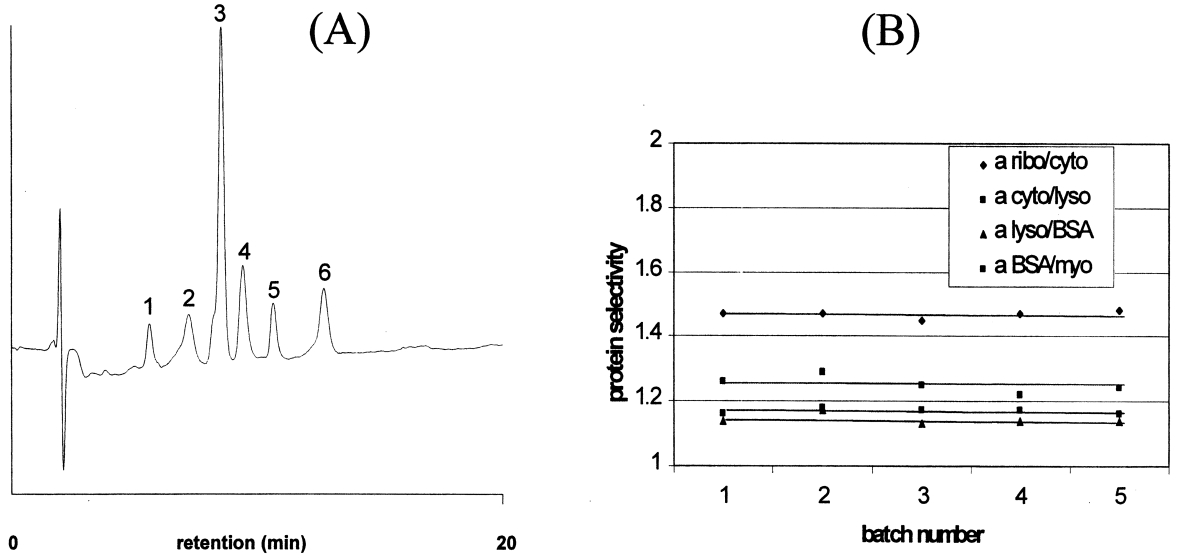

Fig. 6. Separation of six standard proteins (A) and the plot of selectivity vs. batch number for five consecutive batches of PLRP-S 300 Å, 10–15 μm product (B). Gradient from 20 to 80% acetonitrile containing 0.1% TFA in 20 min at a flow-rate of 1.0 ml/min, column 150×4.6 mm I.D. Peaks: 1=ribonuclease A, 2=cytochrome *c*, 3=lysozyme, 4=bovine serum albumin, 5=myoglobin, 6=ovalbumin.

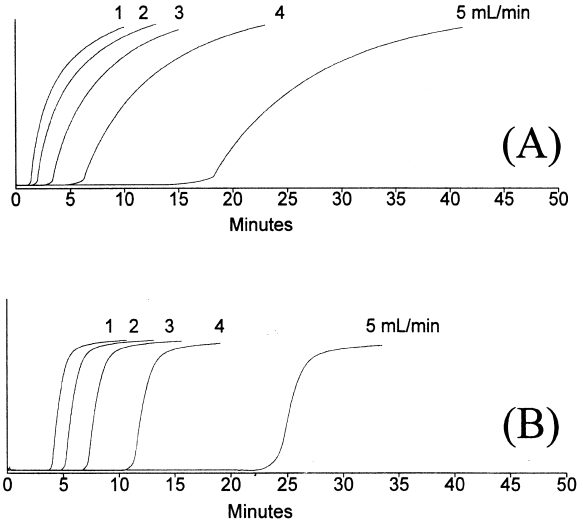

Fig. 7. Lysozyme frontal loading curves obtained with 10–15 μm PLRP-S 100 Å (A) and 10–15 μm PLRP-S 300 Å (B). Column: 50×4.6 mm I.D., protein solution: 1.0 mg/ml 0.1% TFA in 1% acetonitrile. Flow-rate: 1.0, 2.0, 3.0, 4.0 and 5.0 ml/min.

lecular integrity [19]. Poly(styrene–divinylbenzene) materials are extremely chemically stable being resistant to both strong acids and alkalis. When used as matrices for reversed-phase chromatography there is no possibility of dissolution of the base particle and as the interaction is with the surface and not with a bonded hydrophobic ligand there is also no possibility of functional group stripping as has been observed with some silica-based materials with prolonged use of eluents containing 0.1% TFA [20].

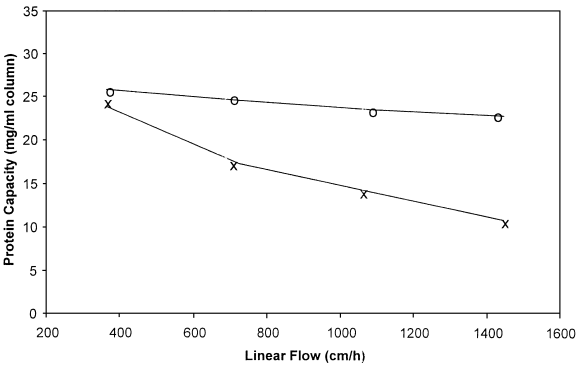

Fig. 8. Plot of linear velocity vs. lysozyme capacity for the PLRP-S materials. ○=PLRP-S 300 Å, 10 μm and ×=PLRP-S 100 Å, 10–15 μm.

Sodium hydroxide is often used for cleaning/depyrogenation and so to assess the alkali stability of the PLRP-S media in packed column format, for cleaning in place, the standard peptide and protein separation was performed before and after flushing the column with 240 column volumes of 1 *M* sodium hydroxide. It can be seen, Fig. 9, that there is no deterioration in column performance or separation selectivity, after this cleaning in place has been accomplished. The column packed bed is stable and the particle characteristics are unchanged.

3.2.5. Peptide selectivity

Reversed-phase analysis of peptides and proteins conventionally uses an acidic ion pairing agent with the most commonly used being TFA at a concentration of 0.1% in both the organic and aqueous components of the eluents. TFA is reported to improve the solubility of the biomolecules and also increase retention and improve peak shape, both broadening and symmetry, by pairing with the positively charged and polar groups in the amino acid sequence. Where the HPLC material exhibits non-specific interactions, such as is the case with residual silanol groups on the surface of some silicas, these interactions are also suppressed by the use of TFA. As the PLRP-S materials are macroporous copolymers of styrene and divinylbenzene and the reversed-phase interaction is with the surface of the hydrophobic polymer it would be expected that lower concentrations of TFA could be used whilst maintaining peak shape. The separation of the closely related group of small peptides, angiotensins, was used to investigate the effect of TFA concentration. Using the conventional concentration of TFA, 0.1%, it can be seen in Fig. 10A that the peak shapes are good but angiotensin II and III co-elute. As the concentration of TFA is reduced the retention is decreased and at 0.02% angiotensin II and III are beginning to be resolved. By further reducing the concentration of TFA resolution of the three angiotensins is obtained but the peaks are broader although still symmetrical.

Proteins and peptides contain both basic and acidic amino acids and therefore in reversed-phase HPLC it would be expected that chromatographic selectivity could be altered by changing the pH at which the separation is performed. PLRP-S material being

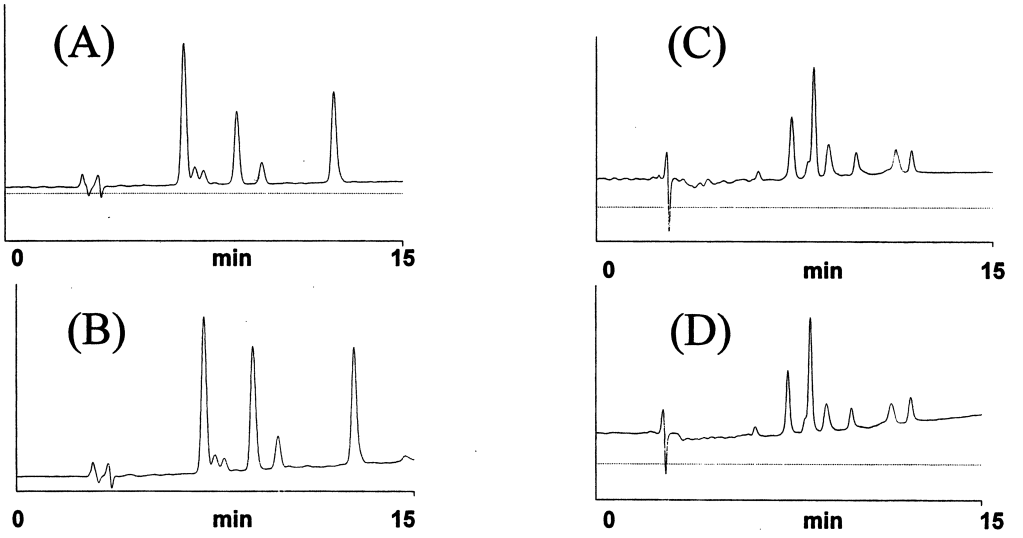

Fig. 9. Separation of four standard peptides before (A) and after (B) flushing with 240 column volumes of 1 *M* NaOH and of six standard proteins before (C) and after (D) flushing with 240 column volumes of 1 *M* NaOH. Column: PLRP-S 300 Å, 10–15 μm, 250×4.6 mm I.D. Peptides are oxytocin, angiotensin II, angiotensin I and insulin and the proteins are ribonuclease A, cytochrome *c*, lysozyme, bovine serum albumin, myoglobin and ovalbumin.

stable over the entire pH range it is possible to fully exploit the ability to manipulate the peptide–protein charge and hence selectivity. It would be expected that the separation of angiotensin II and III could be manipulated by changes in the eluent pH as angiotensin II has an additional C-terminal aspartyl residue compared to angiotensin III. This amino acid has an acidic side chain which is ionised at pH 10. Therefore, if the pH of the eluent was above 10 the aspartyl residue would have the effect of reducing the reversed-phase interaction of angiotensin II and resolution would be achieved. This effect can clearly

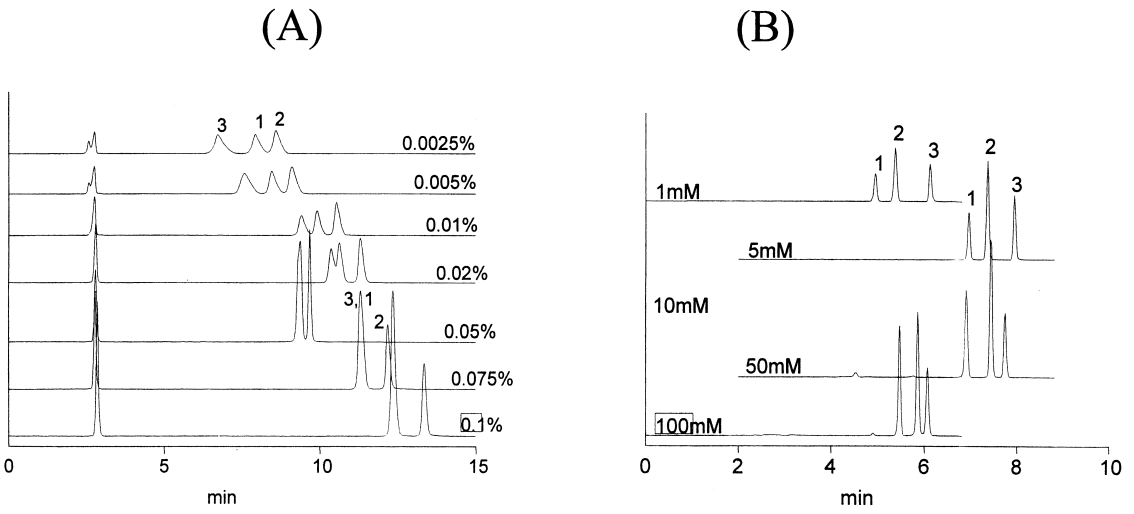

Fig. 10. Effect of type and concentration of eluent additives on the selectivity of a peptide separation (A) TFA, (B) ammonium hydroxide. Peaks: 1=angiotensin II, 2=angiotensin I, 3=angiotensin III.

be seen in Fig. 10B where ammonium hydroxide was used to control pH. Using ammonium hydroxide to control the pH of the eluent resolution of the three angiotensins is achieved and the peak shapes are good, sharp and symmetrical.

4. Conclusions

A range of macroporous poly(styrene–divinylbenzene) materials have been designed for the large scale, high-performance preparative and process fractionation of synthetic peptides and recombinant proteins. The materials have been produced in two pore sizes, 100 Å optimised for the synthetic peptides and 300 Å for the recombinant proteins and in particle sizes ranging from 10 to 20 μm. The particles are uniform in size and shape and mechanically stable to enable operation under HPLC conditions of pressure and flow-rate. The production process is scaleable and robust and hence excellent batch to batch reproducibility is obtained and batches of 100 kg size can be readily produced.

The ease of manufacture of these high-performance macroporous polymers and the ability to control pore morphology gives the flexibility to consider the manufacture of media specifically designed for the purification of a specified peptide or protein and by control of particle size optimisation for maximum throughput with a given chromatography plant.

References

[1] H.A. Sober, E.A. Peterson, J. Am. Chem. Soc. 76 (1954) 1711.
[2] E.A. Peterson, Cellulosic Ion Exchangers, Elsevier, Amsterdam, 1970.
[3] K. Tsuiji, J.H. Robinson, J. Chromatogr. 112 (1976) 663.
[4] S.H. Chang, K.M. Gooding, F.E. Regnier, J. Chromatogr. 112 (1976) 103.
[5] F.E. Regnier, R. Noel, J. Chromatogr. Sci. 14 (1976) 316.
[6] K. Larsson, W. Herrmann, P. Moller, D. Sanchez, J. Chromatogr. 450 (1988) 71.
[7] L.L. Lloyd, J. Chromatogr. 544 (1991) 201.
[8] J.J. Jagodzinski, G.T. Marshall, B.J. Poulsen, G. Raza, W.A. Rolls, J. Chromatogr. 591 (1992) 89.
[9] T. Ohtani, Y. Tamura, M. Kasai, T. Uchida, Y. Yanogihara, K. Noguchi, J. Chromatogr. 515 (1990) 175.
[10] K. Hosoya, S. Maruya, K. Kimata, H. Kinoshita, T. Araki, N. Tanaka, J. Chromatogr. 625 (1992) 121.
[11] K.A. Tweeton, T.N. Tweeton, J. Chromatogr. 359 (1986) 111.
[12] P.G. Cartier, J.J. Maikner, K.C. Deissler, J.R. Fisher, presented at the 16th International Symposium on Column Liquid Chromatography, Baltimore, MD, 14–19 June 1992.
[13] J.C. More, J. Polym. Sci. 2 (1964) 835.
[14] J.V. Dawkins, T. Stone, G. Yeadon, Polymer 18 (1977) 1179.
[15] F.M.B. Coutinho, R.C.A. Cid, Eur. Polym. J. 26 (1990) 1185.
[16] R.V. Lewis, A. Fallon, S. Tein, K.D. Gibson, S. Underfriend, Anal. Biochem. 104 (1980) 153.
[17] J.V. Dawkins, M. Hemming, Makromol. Chem. 176 (1975) 1795.
[18] C.T. Mant, R.S. Hodges, LC–GC 4 (1986) 250.
[19] C.T. Wehr, R.E. Majors, LC–GC 5 (1987) 942.
[20] J.L. Glajch, J.J. Kirkland, J. Kohler, J. Chromatogr. 384 (1987) 81.