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SEPARATION OF PROTEINS ON A POLYMERIC FLUOROCARBON HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY COLUMN PACKING

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SUMMARY

A rigid, inert fluorocarbon resin has been evaluated as an high-performance liquid chromatography packing for the separation of proteins and peptides. Samples are separated with gradient elution of the mobile phase from aqueous buffer to acetonitrile. The selectivity and retention of the fluorocarbon resin packing for proteins are similar to that of alipathic hydrocarbon-bonded silica packings.

INTRODUCTION

Modern reversed-phase liquid chromatography is a high-resolution, high-speed method for separation and purification of proteins and peptides with molecular weights of up to 50 000 dalton. The most common stationary phases are short-chain aliphatic hydrocarbons that are bonded to a silica support[1–5]. Although these bonded silicas give highly efficient separations, the bonded stationary phase is not stable outside the pH range of 2–8. Even within this pH range, a slow loss of stationary phase has been shown to occur[6], causing changes in column retention and selectivity as well as contamination of the eluate.

Polymeric HPLC packings do not suffer from the chemical stability problems of the bonded silica packings. Recently, cross-linked polystyrene–divinylbenzene resins have been developed as high-performance liquid chromatography (HPLC) packings for reversed-phase chromatography of proteins[7]. Although these resins do not have the mechanical stability or sample capacity of the bonded silica packings, they are more stable chemically and can be used with mobile phases from pH 0 to 13[7].

Fluorocarbon resins have also been used for reversed-phase HPLC. Fluorocarbon stationary phases, bonded to silica supports, give HPLC column packings that have a selectivity similar to the corresponding aliphatic hydrocarbon-bonded silica packings but are less retentive[8–12]. Since these fluorocarbon phases are bonded to the silica support, their use must still be restricted to mobile phases in the pH 2–8 range. Fluorocarbon polymeric packings do not have this disadvantage and should be stable at any pH. Since fluorocarbon liquids are used as blood substitutes and are considered to be less denaturing to protein than are hydrocarbon liquids[13], fluorocarbon polymers could also be more compatible with proteins than hydrocarbon

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polymers. There is some evidence for this, since fluorocarbon resins have been used as HPLC column packings for the immobilization of enzymes with retention of biological activity[14,15]. Fluorocarbon resins have also been chemically modified and studied as HPLC packings for ion-exchange and reversed-phase separations of low-molecular-weight compounds[16–18]. Although fluorocarbon resins have been tested as sorbents for the extraction of organic pollutants[19], proteins[20], and nucleic acids[21] from aqueous solutions, they have not been used previously as HPLC packings for the resolution of protein mixtures. This paper presents a study of a unique, rigid, fluorocarbon polymeric packing for the HPLC separation and isolation of proteins and peptides.

EXPERIMENTAL

A DuPont (Wilmington, DE, U.S.A.) 8800 liquid chromatograph, equipped with a six-port Rheodyne (Cotati, CA, U.S.A.) injection valve and a DuPont Model 8800 Spectro UV spectrometric detector were used for experimental measurements. The chromatograms were obtained at 220 nm, unless otherwise noted. Either a Hewlett-Packard (Palo Alto, CA, U.S.A.) Model 85 computer or a Nelson Analytical (Cupertino, CA, U.S.A.) Model 760 interface with a Hewlett-Packard 220 computer was used for data reduction and storage of chromatograms. A Varian (Palo Alto, CA, U.S.A.) Model 219 UV spectrophotometer with 3-ml quartz cuvettes was used to measure UV absorbance of standard and eluent samples in the resin capacity tests. Surface area measurements were obtained with a Micromeritics (Norcross, GA, U.S.A.) Model 2100 surface area analyzer. Mercury porosimetry measurements were made with a Micromeritics Model 9200 analyzer.

A proprietary DuPont fluorocarbon resin was used for these studies. The resin is porous, with an average pore diameter of 330 Å and a surface area of 5 m^2/g. The particle size of the resin is between 20 and 45 μm with a median value of 35 μm. The resin was either dry-packed or slurry-packed into 8 cm × 6.2 mm I.D. columns. The dry-packed columns had to be topped after being washed with methanol in order to obtain a stable packing bed. The resin was packed as a slurry in butanol into 8 cm × 6.2 mm I.D. columns; the slurry-packed columns were stable without topping. Because the resin is very hydrophobic, the packed columns had to be first conditioned by washing with twenty column volumes of a water-miscible organic solvent, such as methanol or ethanol, before use with aqueous solvents. Once conditioned, the columns could be used for long periods, up to four weeks of constant operation, without reconditioning. However, both the dry-packed and slurry-packed columns had to be conditioned again if they were allowed to dry. We routinely flushed the columns each morning with ten column volumes of methanol or ethanol if they had not been used overnight. The columns were stable up to pressures of 150 bar (2200 p.s.i.); above that value the columns sometimes showed irreversible compression with a resulting back-pressure increase and drop in performance. A column backpressure of 10 bar was typical for a mobile phase of water at a flow-rate of 1 ml/min.

Commercial columns of 6-μm, C_8 bonded, 150-Å silica, Zorbax® Bio Series PEP/RP-1, (DuPont) and 5-μm, C_3 bonded, 300-Å silica (Ultrapore RPSC; Beckman, Berkley, CA, U.S.A.) were also used for the study. The PEP/RP-1 column was 8 cm × 6.2 mm I.D.; the Ultrapore RPSC was 7.5 cm × 4.6 mm I.D. The protein

standards of ovalbumin, lysozyme, myoglobin, ribonuclease A, glucagon, amylase, melittin, carbonic anhydrase, and insulin (bovine pancreas) were obtained from Sigma (St. Louis, MO, U.S.A.). The organic solvents, acetonitrile and methanol, were HPLC-grade from Fisher Scientific (Pittsburgh, PA, U.S.A.). The trifluoroacetic acid (TFA) used as mobile phase additive was also obtained from Fisher Scientific. The reagents used in the Bradford protein assay were from BioRads Labs. (Richmond, CA, U.S.A.).

RESULTS AND DISCUSSION

The fluorocarbon resin was first tested in the chromatographic separation of proteins by comparing it with C_3 bonded silica packing. A standard mixture of four proteins (Table I) was chromatographed on a dry-packed 8 cm × 6.2 mm I.D. column of the fluorocarbon resin with a mobile phase gradient from 0.1% TFA in water (pH 2) to 0.1% TFA in acetonitrile–water (90:10, v/v). The resulting chromatogram is shown in Fig. 1, where it is compared with a similar chromatogram from a C_3 bonded silica column (Ultrapore RPSC), with the identical sample and mobile phase conditions. The selectivity and retention of both columns for these proteins are remarkably similar for such different column packing materials. The peak-width of the fluorocarbon resin column is broader than that of the bonded silica, as is expected when a 3.5-μm packing is compared with a 5-μm packing. Similar retention and selectivity in protein separation is also observed when the fluorocarbon resin column is compared with the PEP/RP-1 column of C_8 bonded silica eluted with a gradient of ethanol or acetonitrile in 0.1 *M* potassium dihydrogen phosphate (pH 2) aqueous buffer.

This similarity in chromatographic properties is interesting since the fluorocarbon resin is chemically more stable than bonded silica packings and should have no mobile phase pH limitations. This was further investigated by chromatographing a standard mixture, containing the proteins and peptides shown in Table II, on a column of the fluorocarbon resin with the standard 0.1% TFA (pH 2) mobile phase and gradient elution with acetonitrile. The same protein mixture was also chromatographed on the same column with the same gradient elution profile but with aqueous mobile phases as pH 1 and pH 11. The resulting chromatograms are compared in Fig. 2. The best selectivity for the widest range of proteins was obtained with TFA modifier at pH 2. At pH 1, the peptides and small proteins were separated with good peaks shapes, although the higher-molecular-weight proteins tailed. At pH 11, the peptides were again well separated although the three largest proteins were not eluted;

TABLE I

PROTEINS FOR COMPARISON OF COLUMN PACKING

Peak	*Protein*	*mol.wt.*	*pI*
1	Insulin (bovine pancreas)	5700	5.4
2	Glucagon	3550	6.6
3	Lysozyme	14 400	11
4	Myoglobin	17 800	7.3

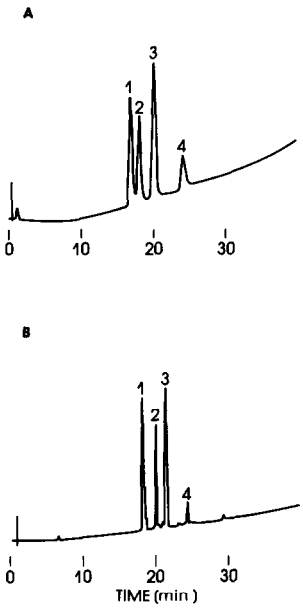

Fig. 1. Comparison of column packings for the separation of proteins. (A) 35-μm fluorocarbon resin, 8 cm × 6.2 mm I.D. (B) 5-μm C_3 bonded silica, Ultrapore RPSC, 7.5 cm × 4.6 mm I.D. Conditions: mobile phase from 0.1% TFA in water (pH 2) to 0.1% TFA in acetonitrile–water (90:10, v/v) in 45 min at 1 ml/min and detection at 220 nm (0.64 a.u.f.s.). Peak identity: see Table I. Sample size: 25 μg each.

these proteins were strongly retained but could be eluted from the column if the mobile phase gradient was run at pH 2. The use of mobile phases at pH 1 and pH 11 did not adversely affect column life or performance.

A variety of other protein samples, ranging from large proteins, such as amylase (200 000 dalton), to very basic peptides, such as melittin, have been chromatographed with good peak shapes. However, proteins larger than 200 000 dalton, in general, gave poor peak shape or were not eluted from columns of the fluorocarbon polymer.

TABLE II

PROTEINS AND PEPTIDES FOR COMPARISON OF MOBILE PHASES

Peak	*Protein*	*mol.wt.*	*pI*
1	Tetraglycine	300	
2	Enkephalin (leucine)	555	
3	Enkephalin (methionine)	634	
4	Insulin chain A	2530	
5	Angiotensin I	1417	
6	Ribonuclease	13 700	9.3
7	Insulin (bovine pancreas)	5700	5.4
8	Lysozyme	14 400	11
9	Carbonic anhydrase	29 000	6
10	Ovalbumin	45 000	4.6

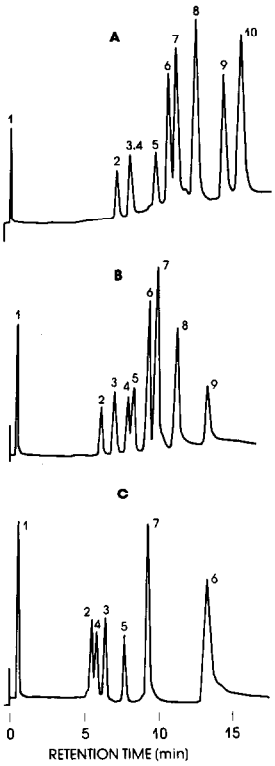

Fig. 2. Effect of mobile phase pH on the separation of proteins. Column: 35-μm fluorocarbon resin, 8 cm × 6.2 mm I.D. Conditions: aqueous buffer to acetonitrile–buffer solution (70:30, v/v) in 20 min at 2 ml/min and detection at 220 nm (0.64 a.u.f.s.). (A) 0.1% TFA (pH 2). (B) 0.1 *M* sulphuric acid (pH 1). (C) 0.1% ammonium hydroxide (pH 11). Peak identity: see Table II. Sample size: 25 μg each.

The chemical stability of the fluorocarbon resin should be an advantage in its use as an HPLC packing. In order to study this further, a stability test was conducted over a 30-day period with a dry-packed 8 cm × 6.2 mm I.D. column of this resin. A standard mixture, containing four proteins (Table I), was injected fifteen times a day under the chromatographic conditions shown in Fig. 3; the mobile phase was pumped continuously and the column was not conditioned during this time. The temperature of the column was held at 50°C in order to make the test more severe. The chromatograms of the first sample injection on the first day and the 465th sample injection on the 30th day are compared in Fig. 3. There was no significant change in either retention time or peak efficiency during the 30-day period in which 40 000 column volumes of phase had been pumped through the column.

The capacity of the fluorocarbon resin for adsorbing proteins was determined in a sample load test. A sample containing insulin at 10 mg/ml in 0.1 *M* potassium dihydrogen phosphate (pH 2) was injected into a 8 cm × 6.2 mm I.D. column containing approximately 2.5 g of the fluorocarbon resin and then chromatographed with a mobile-phase gradient from 0.1 *M* potassium dihydrogen phosphate (pH 2) to acetonitrile–0.1 *M* potassium dihydrogen phosphate (60:40, v/v) at a flow-rate of

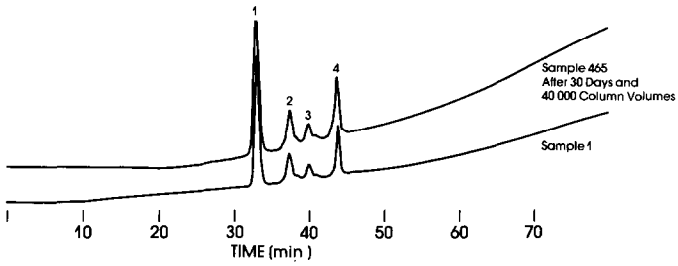

Fig. 3. Stability study of fluorocarbon resin packing. Column: 35-μm fluorocarbon resin, 8 cm × 6.2 mm I.D. Conditions: 0.1% TFA (pH 2) in acetonitrile–water (10:90, v/v) to 0.1% TFA in acetonitrile–water (90:10, v/v) in 80 min at 1 ml/min and 50°C with detection at 220 nm (0.64 a.u.f.s.). Peak identity: see Table I.

2 ml/min. Samples of increasing size were chromatographed, and the retention time and peak-width were measured. A comparison study was also made on the PEP/RP-1 column containing approximately 3 g of C_8 bonded, 150-Å Zorbax® silica (surface area of 140 m^2/g). The peak-width of the insulin peak is plotted against the sample size in mg of insulin per g of column packing in Fig. 4. The results show that the peak-width for both columns does not increase significantly until sample loads reach about 1 mg protein/g of column packing. The "dynamic capacity" of a packing is often defined as the sample load at which the peak efficiency decreases by 75% (or the band-width doubles). By this definition, the dynamic capacity of the fluorocarbon resins with gradient mobile phase conditions is approximately 2–3 mg of protein/g of packing.

Absolute capacity tests of the fluorocarbon resins were made in small columns, 2 cm × 4 mm I.D., containing measured amounts of packing. The columns were conditioned with methanol and equilibrated with 0.1 *M* potassium dihydrogen phosphate (pH 2) in water. Samples of known amounts of either bovine serum albumin (BSA) or insulin (bovine pancreas) were introduced into the columns, and the eluent was trapped and the absorbance at 280 nm was measured to determine the amount

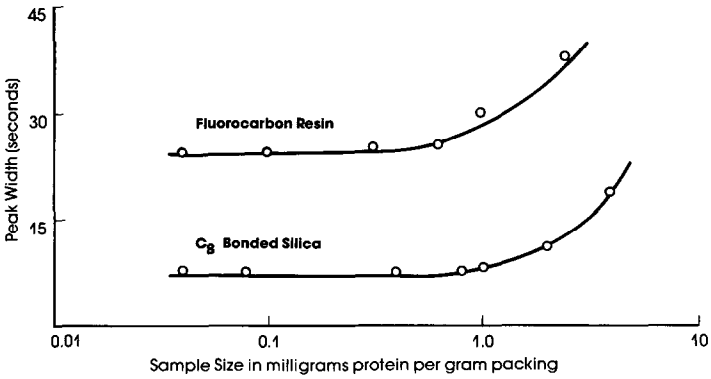

Fig. 4. Comparison of protein sample size *versus* peak width for C_8 bonded silica (5 μm) and fluorocarbon (35 μm) resin packings. Conditions: 0.1% TFA in water (pH 2) to 0.1% TFA in acetonitrile–water (70:30, v/v) in 20 min at 2 ml/min and detection at 280 nm.

of unretained protein. The absolute capacity of protein that could be retained on the fluorocarbon packing was then calculated to be 6 mg of protein/g of packing.

The recovery of protein samples injected into an HPLC column is a function of the type of packing, the type of protein, and the mobile-phase conditions. Based on our results, the mobile phase conditions used in chromatogram A in Fig. 1 appeared to give the best recovery and were used for this study. Two protein standards were tested separately with an 8 cm × 6.2 mm I.D. packed column of the fluorocarbon resin. The column was conditioned with methanol, equilibrated with the aqueous mobile phase, and a 500-μg sample of the protein was injected. The sample was then eluted from the column under the conditions shown in Fig. 1. The eluted sample was then trapped and measured to determine the protein content by the Bradford test[22]. Samples of 500 μg that had not been chromatographed were also measured by the same test and the results were compared to give a recovery value. For the two proteins tested, the average percent recovery for lysozyme was 90.6%, and for carbonic anhydrase 99.3%.

CONCLUSION

A unique, rigid fluorocarbon resin has been tested as an HPLC packing for the separation and isolation of proteins and peptides. The fluorocarbon resin can be slurry-packed into stable HPLC columns (8 cm × 6.2 mm I.D.) that can be used at pressures up to 150 bar or 8 ml/min mobile-phase flow. The columns need to be conditioned with a water-miscible organic solvent before use, because of the hydrophobic nature of the resin. Protein and peptide samples can be separated with the column by reversed-phase chromatography, using an aqueous buffered mobile phase and gradient elution with acetonitrile or ethanol. The retention and selectivity of the fluorocarbon packing are similar to packings of C_3 or C_8 hydrocarbon-bonded silicas. The dynamic capacity of the packing for insulin was measured to be 2–3 mg/g of packing.

The major advantages of this fluorocarbon packing are its chemical stability and the purity of eluted samples. The packing can be used with acids and bases without adverse effect, and columns have been exposed to over 40 000 column volumes of pH 2 mobile phase at 50°C without significant change in retention or peak efficiency for protein standards. Sample purity is maintained, since there is no bonded stationary phase that can be stripped off to contaminate the eluate.

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