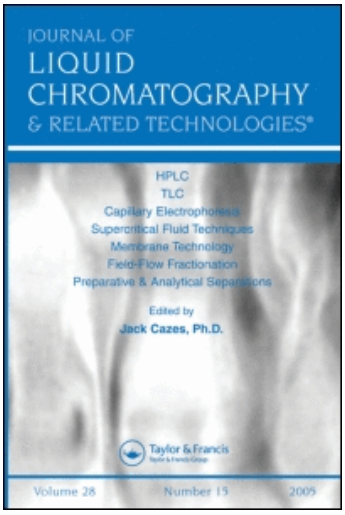

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Biomolecule Separations with Two New HPLC ion-Exchange Columns

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BIOMOLECULE SEPARATIONS WITH TWO NEW HPLC ION-EXCHANGE COLUMNS

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ABSTRACT

Two new HPLC packing materials, Hydrophase™ HP-SAX and HP-SCX, based upon a macroporous hydrophilic polymer, have been developed for biomolecule separations. Hydrophase HP-SAX was developed by attaching quaternary nitrogen groups onto the surface of the base polymer for anion-exchange separations. HP-SCX was formed by attaching sulfonyl groups to the base polymer for cation-exchange chromatographic separations. Both HP-SAX and HP-SCX have high efficiency/theoretical plate ratings, broad pH stability range, tolerate high pressures (up to 2250 psi), and can be operated at high flow rates. Data are presented which illustrate the resolution characteristics of the resins at highflow rates, up to 3 mL/min. Additional experiments demonstrate protein/enzyme loading, separation and recovery. The resolution and recovery analyses using these polymeric materials reveal the versatility and reliability of HP-SAX and HP-SCX columns.

INTRODUCTION

High performance ion-exchange chromatography is a widely used technique for the isolation of biomolecules, including nucleic acids, pro-

teins, and peptides [1], with increasing emphasis on preparative applications [2]. With this separation method a mixture of ionic biomolecules passes over and/or through an insoluble matrix with cationic or anionic sites. The insoluble column matrix is equilibrated with an aqueous buffer/eluent. After sample injection, the separation is developed by passing the eluent through the column by hydrodynamic pressure. Separations progress by dynamic ionic interactions between solute biomolecules and ionic matrix. To complement the existing Hydrophase group of columns [3,4], two new column phases, HP-SAX and HP-SCX, were developed which provide additional flexibility in the selection of the appropriate ion-exchange column for biomolecule separations. The HP-SAX or strong anion-exchange column was formed by covalent attachment of quaternary nitrogen groups to the hydrophilic polymer core. The HP-SAX column displays anion-exchange character up to pH 12. The HP-SCX or strong cation-exchange column was constructed by bonding sulfonyl groups to the core polymer. HP-SCX column displays cation-exchange properties down to pH 2. Both columns are potentially useful over the pH range of 2 to 12. The data presented here show that these columns can be operated at high flow rates while maintaining good resolution of protein mixtures. They have a broader useful pH range than silica-based columns, and permit excellent recoveries of trace amounts of proteins and enzymatic activities from complex mixtures. A preliminary report on these two ion-exchange columns was presented [5].

EXPERIMENTAL

Materials

All proteins, including myoglobin (bovine blood), cytochrome C (horse heart), ovalbumin (chicken egg), yeast extract, lysozyme (chicken egg), α-chymotrypsinogen (bovine pancreas), hexokinase and glucose-6-phosphate dehydrogenase (yeast extract), RNase A (bovine pancreas), transferrin (bovine), catalase (bovine liver), gliadin (wheat), and oxytocin, plus cytidine, tryptophan, Tris, $NADP^+$, glucose, glucose-6-phosphate, magnesium chloride, disodium ATP, *Micrococcus lysodeikticus*, and the Sigma

Protein assay kit were purchased from Sigma Chemical (St. Louis, MO, USA). Hydrochloric acid and sodium hydroxide were obtained from Fisher Scientific (Santa Clara, CA, USA). Bis-Tris was obtained from Research Organics (Cleveland, OH, USA). Sodium chloride and potassium phosphate were from EM Science (Gibbstown, NJ, USA). Sodium azide was acquired from Aldrich (Milwaukee, WI, USA). Deionized glass distilled water was used to prepare buffers and sample solutions.

Apparatus and Columns

An EM Science/Hitachi (Gibbstown, NJ, USA) liquid chromatographic system was used for this study. This included an L-6200 ternary gradient pump, L-4200 UV-Visible detector and D-2500 Chromato-Integrator. Rheodyne Model 7125 injector (Rheodyne, Cotati, CA, USA) and various Hamilton LC syringes (Rainin Instrument Co., Emeryville, CA, USA) also were used. A Spectronic 1201 spectrophotometer from Milton Roy (Rochester, NY, USA) was used for determination of protein concentrations and enzyme assays. The HPLC columns, HP-SAX (polymeric strong anion-exchange, 10 μm) and HP-SCX (polymeric strong cation-exchange, 10 μm), were each 100 mm x 7.8 mm stainless steel columns.

Procedures

Eluent buffers were prepared at room temperature, degassed under vacuum, and filtered through 0.45 μm membrane prior to use. Protein and other test samples were freshly dissolved in suitable buffers and stored at 4°C when not in use. All experiments were performed at room temperature. In the enzyme purification studies, 1 mL fractions were collected from the column effluent and assayed for activity according to published methods for hexokinase [6] and glucose-6-phosphate dehydrogenase [7] with the Spectronic 1201. Other analysis conditions are listed with the tables and figures.

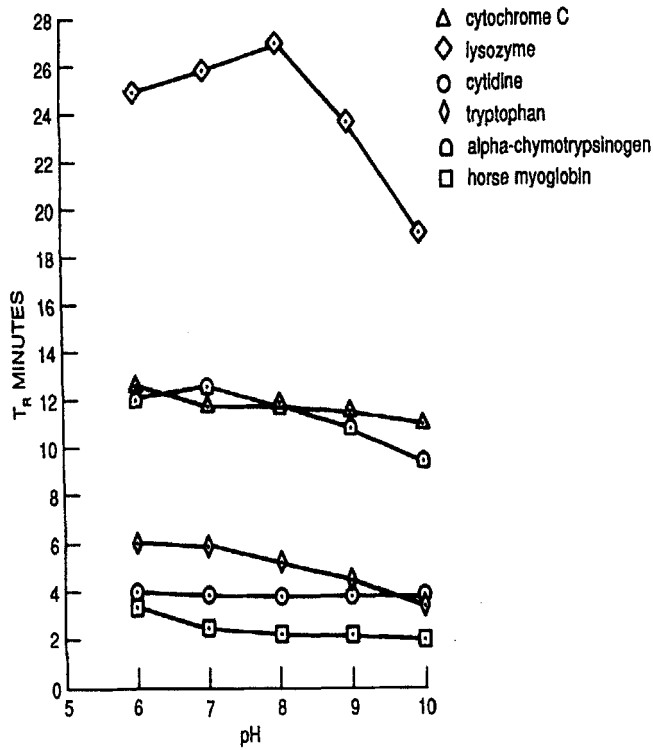

FIGURE 1. Plot of retention time vs. pH with HP-SCX column. *Analysis conditions:* Column: Hydrophase HP-SCX; Eluent: A: 5 m*M* sodium phosphate, various pH values, B: A + 0.5 *M* NaCl; Gradient: 100% A to 100% B in 15 min, then 100% B for 15 min; Flow rate: 1.0 mL/min; Temperature: ambient; Detection: UV at 280 nm, except cytidine at 260 nm; Injection: 20 μL.

RESULTS AND DISCUSSION

Analysis with HP-SCX

Versatility, which includes stability over a broad pH range, is an important characteristic for ion-exchange resins. Figure 1 illustrates the change in retention time of six distinctive biomolecules on the HP-SCX column over the pH range of 6 to 10. The retention times of most of these mole-

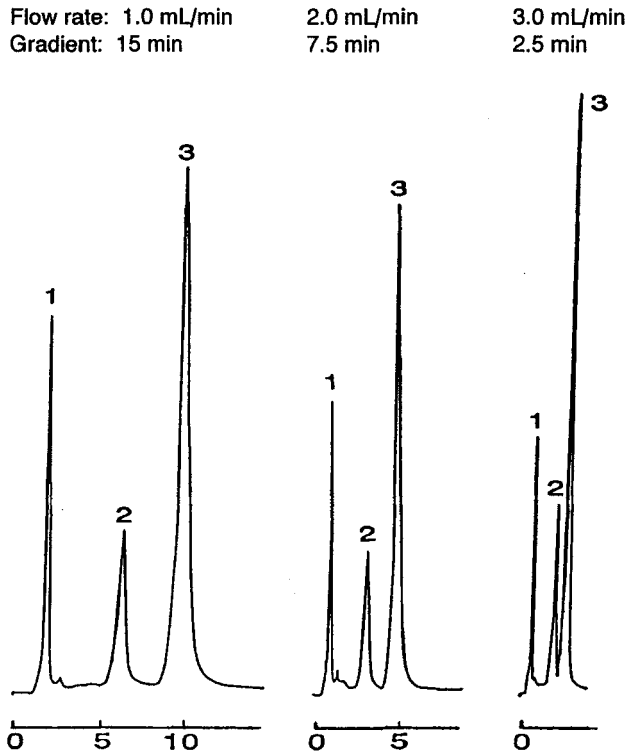

FIGURE 2. Protein separation with HP-SCX. *Analysis conditions:* Column: Hydrophase HP-SCX; Eluent: A: 20 m*M* BisTris, pH 6.02, B: A + 0.5 *M* NaCl; Gradient: 100% A to 100% B times indicated; Flow rate: as indicated; Temperature: ambient; Detection: UV at 280 nm; Injection: 20 μL; Sample: 1. ovalbumin (0.08 mg), 2. myoglobin (0.06 mg), 3. cytochrome C (0.11 mg).

cules do not change markedly, but all decrease or remain the same. Lysozyme, a highly basic protein (pI = 10.55) [8], shows the greatest reduction in retention time over the range tested, indicating that choice of proper pH can be an important factor for optimizing separations. In Figure 2 the rapid chromatographic resolution of three proteins is depicted. Virtual baseline separation and recovery was achieved in less than 4 minutes. This is possible because the HP-SCX column can be operated

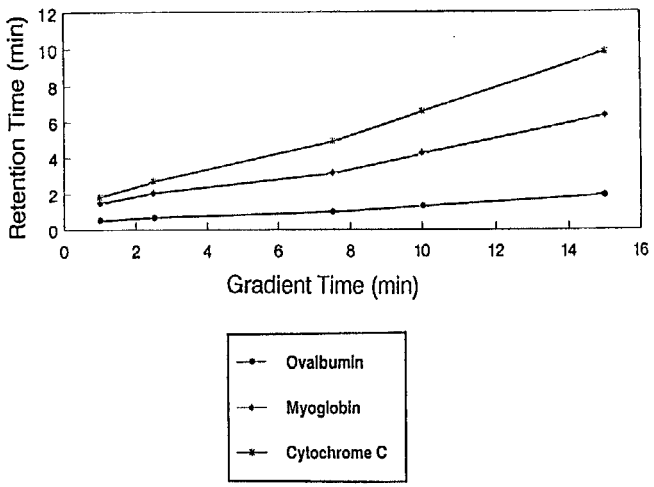

FIGURE 3. Replot of chromatograms from Figure 2, protein peak retention time vs. sodium chloride gradient time.

at flow rates of 3 mL/min or higher, and tolerates high pressures (up to 2250 psi) without loss of performance. This is graphically shown in Figure 3, where even at high flow rates with very short, steep gradients, the resolution of the three proteins is retained. An important objective of biochromatography is recovery of biological activity after the purification process. An example of this with the HP-SCX column is pictured in Figure 4. Hexokinase was isolated from a yeast extract. Activity measurements revealed a purification factor of 11.3 and recovery of 87% of the initial activity after a single chromatographic run. Using high flow rates with the HP-SCX column, enzymatic recoveries of lysozyme are listed in Table 1. Greater than 95% activity was recovered at all flow rates.

Analysis with HP-SAX

To complement ion-exchange analysis with the HP-SCX column, the HP-SAX (strong anion-exchanger) is suitable for resolution of complex,

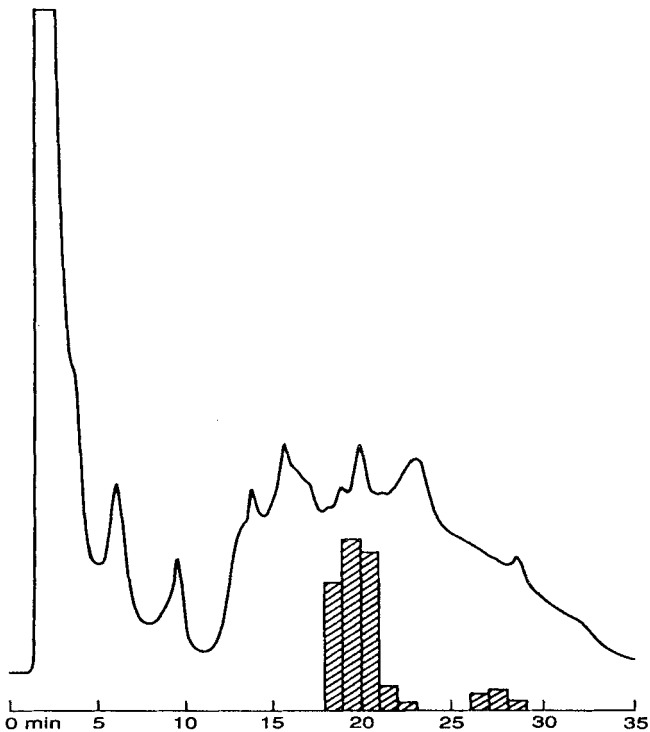

FIGURE 4. Separation of hexokinase from yeast extract. *Analysis conditions:* Column: Hydrophase HP-SCX; Eluent: A: 20 m*M* BisTris, pH 4.7, B: A + 0.5 *M* NaCl; Gradient: 0 to 100% B in 30 min; Flow rate: 1.0 mL/min; Temperature: ambient; Detection: UV at 280 nm; Injection: 500 μL yeast extract (4.93 mg protein); Shaded area: enzymatic activity.

more acidic mixtures. Figure 5 shows a plot of retention time vs. pH for the same six biomolecules depicted in Figure 1. The pattern of retention time with increasing pH is different for these molecules on the HP-SAX column with one exception. All retention times increase or remain unchanged as the eluent pH is raised from 6 to 10. The retention time of horse myoglobin initially declines because the pI of the protein is 7; then retention time gradually increases as the molecule develops an increas-

TABLE 1.
Lysozyme Recovery with HP-SCX Column

Flow rate	Gradient time	Percent Recovery*
1.0 mL/min	20 minutes	97.4 ±1%
2.0 mL/min	10 minutes	98.4 ±1%
3.0 mL/min	5 minutes	98.5 ±1%

Analysis conditions: Column: HP-SCX; Eluents: A: 20 m*M* Bis-Tris, pH 6.0, B: A + 1.0 *M* NaCl; 0% to 100% B in times indicated; Flow rate: as indicated; Temperature: ambient; Detection: UV at 280 nm; Injection: 20 μL of lysozyme stock solution 10 mg/mL. *Percent recovery is based upon assay of an aliquot of major peak recovered from column vs. activity of aliquot from stock solution; activity determined by measuring decrease in O.D. at 450 nm of 0.015% (w/v) *Micrococcus lysodeikticus* suspension in 0.066 *M* sodium phosphate, pH 6.24.

ingly negative charge. Figure 5 thus supports again the idea that selection of the proper pH is important for optimal separation and recovery of biomolecules. As with the cation-exchange studies, speed of separation and recovery can be critical using anion-exchange chromatography. The high speed separation and recovery of three proteins with the HP-SAX column is illustrated in Figure 6. Baseline resolution is attained in less than 6 minutes. The effects of decreased gradient time on protein retention and resolution for those shown in Figure 6 is graphically expressed in Figure 7. The lines do not intersect/overlap, indicating that the resolution was maintained.

Mass recovery of protein and other biomolecules is, of course, vital to the overall performance of an anion-exchange column. Recoverablility of ovalbumin from the HP-SAX column is given in Table 2. Even at the level

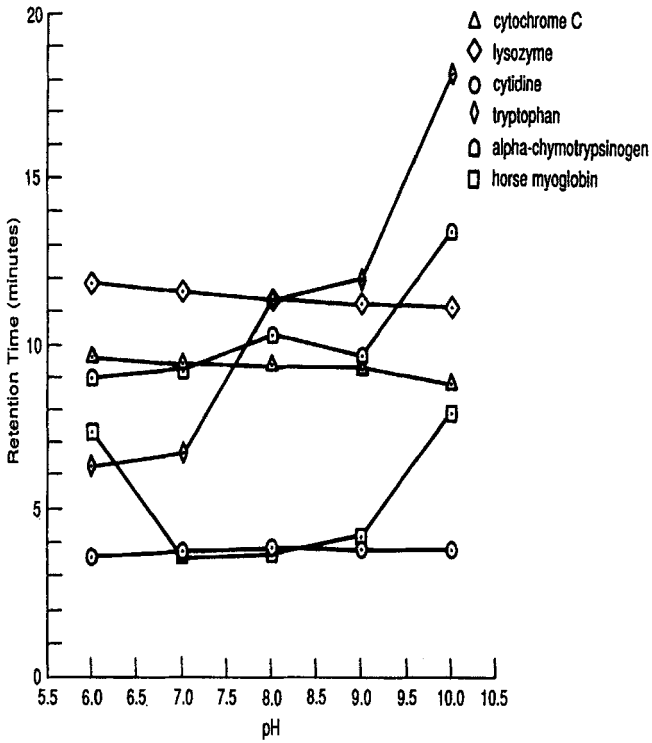

FIGURE 5. Retention time vs. pH for 6 biomolecules on Hydrophase HP-SAX. *Analysis conditions:* Column: Hydrophase HP-SAX; Eluent: A: 5 m*M* sodium phosphate, various pH values, B: A + 0.5 *M* NaCl; Gradient: 100% A to 100% B in 15 min, then 100% B for 15 min; Flow rate: 1.0 mL/min; Temperature: ambient; Detection: UV at 280 nm, except cytidine at 260 nm; Injection: 20 μL.

of 4 milligrams injected, recovery still exceeded 82%. Thus, recovery remained high even with a 200-fold increase in the amount of protein injected onto the column.

An additional test for the recoverability of proteins from the HP-SAX columns was performed. A series of increasingly hydrophobic proteins

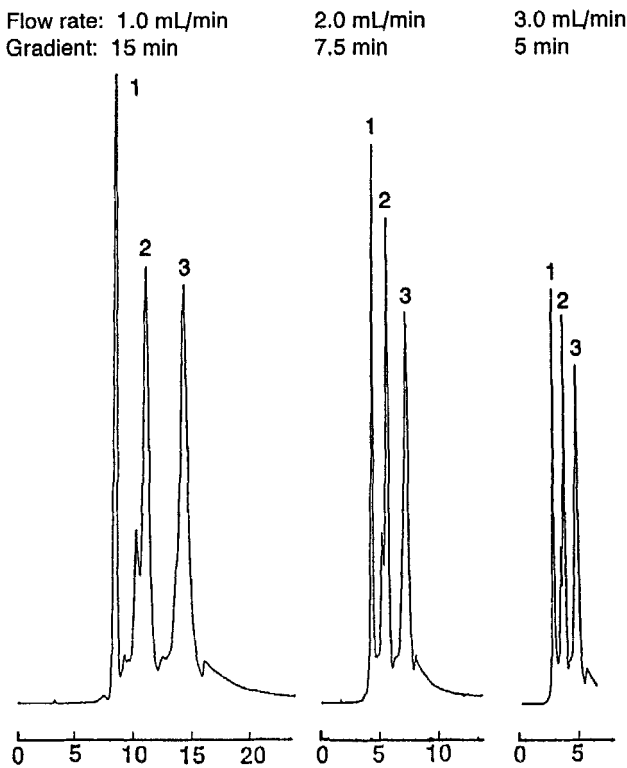

FIGURE 6. Protein separation with HP-SAX. *Analysis conditions:* Column: Hydrophase HP-SAX; Eluent: A: 5 m*M* BisTris, pH 8.35, B: A + 0.5 *M* NaCl; Gradient: 100% A to 50% B in times indicated; Flow rate: as indicated; Temperature: ambient; Detection: UV at 280 nm; Injection: 100 μL; Sample: 1. myoglobin, 2. cytochrome C, 3. ovalbumin.

were injected and analyzed. The results, listed in Table 3, show complete or high recoveries of the entire group.

Another type of protein study with the HP-SAX column is illustrated in Figure 8, recovery of enzymatic activity from a complex sample. Sixty-three percent of glucose-6-phosphate dehydrogenase activity was recov-

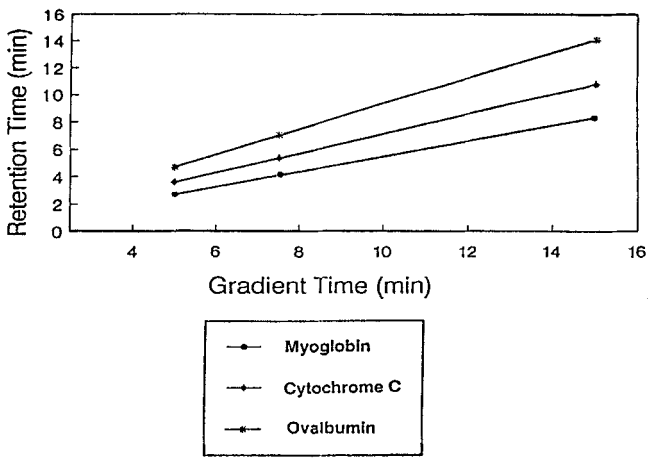

FIGURE 7. Replot of chromatograms from Figure 6, protein peak retention time vs. sodium chloride gradient time.

TABLE 2.
Protein Mass Recovery Using HP-SAX Column

Ovalbumin Injected (mg protein)	Percent Recovery* (%)
0.02	91.9
0.10	87.5
0.40	83.7
2.00	83.1
4.00	82.4

Analysis conditions: Column: HP-SAX; Eluent: A: 5 m*M* Tris, pH 8.3, B: (A) + 0.25 *M* NaCl; Gradient: 0% to 100% B in 15 min; Flow rate: 1.0 mL/min; Temperature: ambient; Detection: UV at 280 nm; Injection: 200 μL of ovalbumin stock solutions. *Percent recovery is based upon the total area of integrated chromatographic peak vs. control injection.

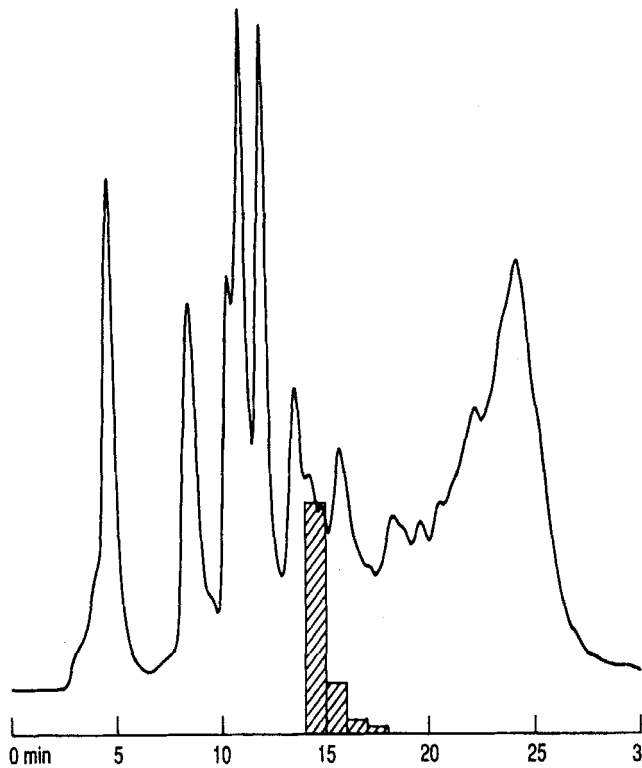

FIGURE 8. Separation of glucose-6-phosphate dehydrogenase from yeast extract. *Analysis conditions:* Column: Hydrophase HP-SAX; Eluent: A: 20 m*M* Tris, pH 8.0, B: A + 0.5 *M* NaCl; Gradient: 0 to 100% B in 30 min; Flow rate: 1.0 mL/min; Temperature: ambient; Detection: UV at 280 nm; Injection: 500 μL yeast extract (5.3 mg protein); Shaded area: enzymatic activity.

ered with a purification factor of 13.5. This compares well to previously reported results with another HPLC anion-exchange column [9].

CONCLUSIONS

A 100 x 7.8 mm column packed with Hydrophase HP-SAX or HP-SCX polymer can be used for analytical separations (microgram level) or semi-

TABLE 3
Mass Recovery of Hydrophobic Protein Series Using HP-SAX

Protein Injected	(μg)	Hydrophobicity*	% Recovery#
RNase A	5	780	100
RNase A	20	780	100
Transferrin	5	930	100
Transferrin	20	930	100
Chymotrypsinogen A	5	1010	92.0
Chymotrypsinogen A	20	1010	84.3
Catalase	5	1110	100
Catalase	20	1110	70.9
Gliadin	4	1203	100
Oxytocin	5	1290	100
Oxytocin	20	1290	100

Analysis Conditions: Column: HP-SAX; Eluent: A: 50 m*M* Tris, pH 8.0, B: (A) + 1.0 *M* NaCl; Gradient: 0% to 100% B in 20 min; Temperature: ambient; Detection: UV at 280 nm; Injection: 20 μL. *Average values: G. D. Fasman, ed. (1976) *Handbook of Biochemistry and Molecular Biology*, CRC Press, Boca Raton, FL. #Recovery is based on total area of chromatographic integrated peak(s) vs. control injections.

preparative (subgram levels) methods. The base polymer is stable in aqueous buffers and high concentration salt solutions often used in ion-exchange chromatography of biomolecules. Separations can be performed over the pH range of 2 to 12 with either the HP-SAX or HP-SCX, depending upon the nature of the sample. Good recovery rates of both protein mass and enzymatic activity have been noted with each of the columns. These results suggest that these two new ion-exchange materials have great potential utility and versatility in biochemical studies.

REFERENCES

1. Barth, H. G., Barber, W. E., Lochmuller, C. H., Majors, R. E. and Regnier, F. E., Column liquid chromatography, Anal. Chem., 60, 387R-435R, 1988.

2. Huang, J.-X. and Guiochon G., Applications of preparative high-performance liquid chromatography to the separation and purification of peptides and proteins, J. Chromatogr., 492, 431-469, 1989.

3. Kitagawa, N., Ion-excange chromatography of proteins on a polyethyleneimine-grafted hydrophilic polymer for high-performance liquid chromatography, J. Chromatogr., 443, 133-141, 1988.

4. Kitagawa, N., New hydrophilic polymer for protein separations by HPLC, in *Techniques in Protein Chemistry*, Hugli, T. E., ed., Academic Press, New York, 1988, pp. 348-356.

5. Nelson, N. F. and Kitagawa, N., Biomolecule separations with two new HPLC ion-exchange columns, Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Paper No. 532, New York, NY, March 6, 1990.

6. Bergmeyer, H. U., ed., Hexokinase from yeast, in *Methods of Enzymatic Analysis*, 3rd edition, Vol. II, VCH Publishing, New York, 1988, pp. 222-223.

7. Deutsch, J., Glucose-6-phosphate dehydrogenase, in *Methods of Enzymatic Analysis*, 3rd editioin, Vol. III, Bergmeyer, H.U., ed., VCH Publishing, New York, 1988, pp. 190-197.

8. Gelfi, C., Bossi, M. L., Bjellqvist, B. and Righetti, P. G., Isoelectric focusing in immobilized pH gradients in the pH 10-11 range, Biochem. Biophys. Methods, 15, 41-48, 1987.

9. Swartz, M. E., Tomany, M. J., Phillips, D. J. and Tarvin, T. L., Isolation and purification methodologies using new silica-based ion exchange media, Biochromatogr., 2, 38-45, 1987.