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MULTIMODAL LIQUID CHROMATOGRAPHY COLUMNS FOR THE SEPARATION OF PROTEINS IN EITHER THE ANION-EXCHANGE OR HYDROPHOBIC-INTERACTION MODE

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SUMMARY

Several high-performance stationary phases suitable for protein chromatography were synthesized. Columns packed with these materials could be operated independently in either the anion-exchange or hydrophobic-interaction mode. Two approaches were used to prepare these materials. In the first method, a polyamine was adsorbed on the surface of macroporous silica and then crosslinked with a multifunctional oxirane. The hydrophobicity of the crosslinking agent and the extent of interconnection were used to modulate the electrostatic and solvophobic interactions. The second approach also utilized a crosslinked polyamine stationary phase; however, the forces of interaction were attenuated through controlled acylation of surface amines with a small anhydride molecule.

The resolving ability of these columns, functioning in either mode, was comparable to commercial high-performance liquid chromatographic columns, designed to operate by a single retention mechanism. Column selectivity for proteins was completely different in each mode. Protein fractions collected from a multimodal column, operated in the anion-exchange mode, could be further purified by rechromatographing them on the same column in the hydrophobic-interaction mode. Utility of the multimodal column was demonstrated with the fractionation of several cytochromes and ferredoxins from the cyanobacterium *Microcystis aeruginosa*.

INTRODUCTION

Liquid chromatography columns are generally classified according to their primary mode of separation; as size-exclusion, ion-exchange, reversed-phase, hydrophobic-interaction or bioaffinity columns. Although useful, this classification can lead to the misconception that these columns operate by a single mechanism. Retention by more than one mechanism is common for chromatographic media with multiple species of organic and inorganic functional groups. Silanol contributions in reversed-phase chromatography (RPC)[1], hydrophobic interactions in ion-exchange chromatography (IEC)[2], electrostatic effects in size-exclusion chromatography (SEC)[3], and hydrophobic interactions in affinity chromatography[4] are all examples of mixed-mode contributions to retention. However, mobile phases are usually se-

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lected so as to maximize the predominant stationary phase solute interaction and to minimize all others.

Adsorption of biopolymers at surfaces has been described in IEC[5], hydrophobic-interaction chromatography (HIC)[6] and RPC[7] as a cooperative process, occuring between similar ligands (ionic or lipophilic) on the solute and stationary phase. Cooperative adsorption may be explained in terms of concentration effects at the surface of a chromatographic packing. Subsequent to adsorption at a single site, the local concentration of solute functional groups is increased enormously. This concentration effect plays the dual role of increasing the probability of adsorption at additional functional groups in the molecule and of decreasing the probability of desorption.

Adsorption in cooperative mixed-mode retention will be very similar to the cooperative processes already identified in IEC[5], HIC[6] and RPC[7] the principal difference being that a mixture of dissimilar stationary phase ligands participates in the adsorption process. The separation of both polypeptides and polynucleotides on ion-exchange columns that are weakly hydrophobic provides examples of the exploitation of mixed mode retention[8–10].

A refinement of mixed-mode retention not previously examined is the design of chromatographic media that segregate the various retention mechanisms occuring within a mixed-mode column. These columns would behave in such a way that by varying the mobile phase, they could be induced to operate in any one of multiple independent modes and be in essence "multimodal columns". Identification of the various retention mechanisms could be achieved in the same way as non-ideal behavior in SEC[3]. Plots of k' *versus* ionic strength would identify electrostatic interactions at low ionic strength, whereas hydrophobic effects would be observed at high ionic strength. Using columns that are capable of both electrostatic and solvophobic interactions, it has been shown[3] that these plots are concave and drop to zero at intermediate salt concentrations.

In this paper we describe the design and application of chromatographic media with both weakly hydrophobic and cationic functionalities, which can operate, depending on the mobile phase, in either the anion-exchange or hydrophobic interaction mode. An increasing salt gradient (0 to 0.5 M sodium chloride) fractionates proteins by a predominantly ionic mechanism, while a decreasing salt gradient (from 1.7 to 0 M sodium sulfate) resolves proteins by hydrophobic interactions. The advantage of multimodal chromatographic media is the wider range of selectivity that may be achieved with a single column. One column could potentially replace two in the protein purification process.

MATERIALS AND METHODS

Materials

Vydac 101TPB 5.5-μm silica was a gift from The Separations Group (Hesperia, CA, U.S.A.). 1,4-Butanediol diglycidyl ether (BUDGE), N,N-diisopropylethylamine (DIEA), and pivalic (trimethylacetic) anhydride (PA) were purchased from Aldrich (Milwaukee, WI, U.S.A.) and N,N-dimethylformamide (DMF) from Fisher Scientific Company (Fairlawn, NJ, U.S.A.). Polysciences (Warrington, PA, U.S.A.) supplied Polyethyleneimine-18 (PEI-18). Triethylamine (TEA) was purchased from Mal-

linckrodt (Paris, KY, U.S.A.). The SynChropak Propyl column was obtained from SynChrom (Linden, IN, U.S.A.). The Pharmacia Mono Q column was purchased from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.).

All proteins were purchased from Sigma (St. Louis, MO, U.S.A.). The crude extract from *M. aeruginosa* was provided by Dr. D. W. Krogmann (Purdue University, W. Lafayette, IN, U.S.A.). All buffers and solvents were of analytical-reagent grade.

Instrumentation

Two pumping systems were used for chromatographic evaluations: a Varian Vista 5500 liquid chromatograph (Varian, Walnut Creek, CA, U.S.A.) and an LDC Constametric I and IIG system with a Gradient Master (Laboratory Data Control, Riviera Beach, FL, U.S.A.). Absorbance was monitored at 254 nm by a Varian UV or a Spectroflow 773 detector (Kratos, Ramsey, NJ, U.S.A.). A Varian 634 series UV–VIS spectrophotometer was used to measure concentrations in the picric acid and Hb-binding assays.

Chromatographic separations of colored proteins were monitored by a HP-1040A high-speed spectrophotometric detector, equipped with a HP-85B personal computer, a HP 9121 micro flexible disc drive, and a HP 7470A plotter (Hewlett-Packard, Palo Alto, CA, U.S.A.).

Synthesis of the packing materials

Multimodal media were synthesized by two routes, both of which are modifications of the method developed by Alpert and Regnier[11] for adsorbed polyamine coatings. An extensively crosslinked stationary phase (Fig. 1, phase V) was synthesized as follows. An amount of 1 g silica was suspended and agitated in 10 ml of a 1% (w/v) PEI-18 solution in methanol. The mixture was left at room temperature for 1 h without further agitation. The supernatant was collected and used to wash the polyamine-coated silica after isolation in a sintered glass funnel. This was then dried under vacuum for 30 min. Crosslinking was achieved in 10 ml of a 25% (v/v) BUDGE solution in methanol, kept at room temperature for 12 h. At the end of this period, 2 ml of TEA was added, and the mixture was heated for 30 min at 70°C. The coated and crosslinked silica was then collected in a sintered glass funnel, washed with TEA and methanol, dried under vacuum, and stored in a dessicator.

The anhydride-derivatized supports (Fig. 1, phase IV) were synthesized by a two step procedure. PEI-18 was adsorbed and crosslinked as previously described; however, a solution of only 5% (v/v) BUDGE was used. An amount of 1 g of this material was weighed into a 50-ml round-bottom flask, heated at 100°C for 30 min, and cooled in a dessicator under vacuum. Dry DMF (5 ml), 250 μl of DIEA and 0.5 ml of PA were then added. The mixture was thoroughly agitated and left at room temperature for 12 h. The flask was then heated over steam for 30 min. The derivatized silica was filtered in a sintered-glass funnel, washed with methanol, dried under vacuum for 30 min, and stored in a dessicator.

Cyanobacterium extract fractionation

The crude *M. aeruginosa* extract was partially processed by centrifugation for 10 min at 10 000 *g* to remove cellular debris, and then filtering the supernatant

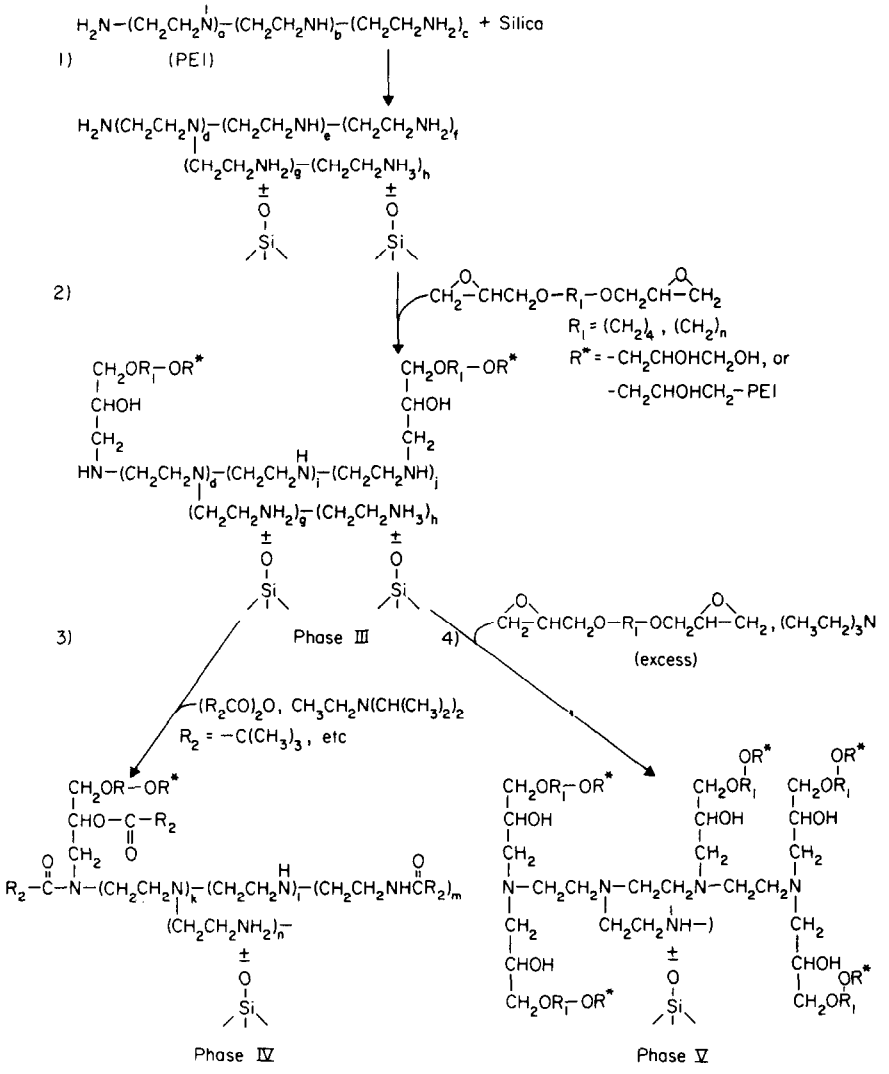

Fig. 1. Synthetic routes for multimodal polyamine-coated stationary phases. The reaction steps are numbered as 1–4. Roman numerals designate intermediary and product phases.

through an Amico 20 000 MW cutoff membrane (Amicon Corporation, Lexington, MA, U.S.A.).

Static load capacity measurements

Picric acid ion-pairing capacity (IPC) and ionic hemoglobin binding assays (Hb_{iec}) were performed to quantitate stationary phase surface amines and ionic protein load capacity, respectively, as described by Kopaciewicz *et al.*[10].

Chromatographic evaluations

Portions of the coated silicas were packed into 5 × 0.41 cm or 25 × 0.41 cm I.D. columns for evaluation[12]. Conditions for anion-exchange chromatography (AEC) were: 20-min linear gradient from 0.01 *M* Tris–HCl (pH 8) to 0.25 or 0.5 *M* sodium chloride in 0.01 *M* buffer (pH 8) at a flow-rate of 1 ml/min. Conditions for HIC were: 20-min linear descending salt gradient from 1.7 *M* sodium sulfate in 0.01 *M* phosphate (pH 7.5) to 0.01 *M* buffer (pH 7.5) at a flow-rate of 1.5 ml/min.

Samples for use in either the anion-exchange or hydrophobic-interaction modes were prepared in the low-salt eluent. Ovalbumin (OVA, 8 mg/ml) and soybean trypsin inhibitor (STI, 15 mg/ml) were used as test materials for AEC. Columns exhibiting HIC behavior were evaluated with a mixture of ribonuclease A (RNAse), lysozyme (LYZ), ovalbumin (OVA), and α-chymotrypsinogen (α-CHYGN) (10, 5, 20, and 5 mg/ml, respectively).

Application

The *M. aeruginosa* extract, containing less than 0.1 mg/ml total protein, was chromatographed on a 25 × 0.41-cm column, packed with material V (Fig. 1, Phase V) (100 μl-injections). HIC was performed with a 20-min linear descending salt gradient from 1.7 *M* sodium sulfate in 0.01 *M* phosphate (pH 7.5) to 0.85 *M* sodium sulfate in 0.01 *M* buffer (pH 7.5), followed by a step to 100% 0.01 *M* buffer (pH 7.5) at a flow-rate of 1 ml/min. Chromatographic conditions for AEC were: 20-min linear gradient from 0.01 *M* Tris–HCl (pH 8) to 0.125 *M* sodium chloride in 0.01 *M* buffer (pH 8) followed by a step to 0.5 *M* sodium chloride in 0.01 *M* buffer (pH 8) at a flow-rate of 1 ml/min.

RESULTS AND DISCUSSION

Synthesis of the stationary phase

The segregation of ionic and solvophobic retention mechanisms was controlled by varying the ionic and hydrophobic composition of the stationary phase. Kopaciewicz *et al.*[10] have reported that the ionic retention of proteins, chromatographed on adsorbed PEI coatings, could be modulated by altering either the ligand density or the physiochemical environment surrounding the amines (hydrophobic and steric factors). These alterations of the stationary-phase character were achieved either by varying the polyamine concentration or by using different crosslinking reagents.

In our work, ionic stationary phase–solute interaction was successfully attenuated by "steric bulking" via extensive crosslinking (Fig. 1, phase V), or by the controlled acylation of stationary-phase amines (Fig. 1, phase IV). Exhaustive derivatization of the polyamine bonded phase (phase III) with a multifunctional oxirane was accomplished as follows. Adsorbed amines were first crosslinked into a stable layer with a 25% methanolic solution of BUDGE at room temperature. At the conclusion of this step, some of the primary and secondary amines in the coating are ion-paired with surface silanols (Fig. 1, phase III). These amines, which were responsible for establishing the initial adsorbed layer, were then displaced by addition of TEA. Further derivatization with oxirane occurs during subsequent heating of the reaction mixture. The hydrophobic character of the coating is controlled by the chemical nature of the "R" group of the crosslinking reagent. In this case (with BUDGE),

four methylene groups are added; however, other hydrophilic or hydrophobic oxiranes may be used.

As stated earlier, the ionic contribution to retention was also substantially reduced by decreasing the ligand density through acylation of stationary-phase amines (Fig. 1, phase IV). Exhaustive acylation with PA in the presence of DIEA reduced the picric acid ion-pairing capacity by 30%, indicating a reduction in surface amines. (The DIEA releases primary and secondary amine groups that were previously ion-paired with surface silanols and sequesters acid functionalities generated during the reaction). Additional hydrophobicity was also imparted to the bonded phase with the trimethylacetate moiety. By using other acylating agents, as well as by controlling the extent of this reaction, one can prepare stationary phases with a range of hydrophobic and ionic character.

Fig. 1, phase IV, illustrates the possibility of esterification of the secondary hydroxyls in addition to acylation of amines. Under acidic conditions, these esters could be hydrolyzed. In order to circumvent any potential problems in this respect, a sterically hindered anhydride, PA, was used. (The hydrolysis rate of the pivalate ester is 10% of that of the acetate derivative[13].)

Characterization of stationary phases

Characterization of the polyamine coatings was achieved by a combination of elemental analysis, small-molecule (picric acid) and protein-binding assays (Table I). Carbon and nitrogen analysis data obtained from these materials are expressed as a C/N ratio. Compared to phase III, the C/N ratios for phases IV and V are significantly higher. This would be expected, since phase III is the intermediate material from which the remaining stationary phases are synthesized (Fig. 1). The picric acid assay was used to detect accessible non-ionized primary, secondary, and tertiary amines. Since amides do not ion-pair with picric acid, the decrease in ion-pairing capacity (IPC) was used to estimate the extent of acylation. Although phases III, IV, and V contain approximately the same amount of nitrogen, IPC values were substantially lower for phases IV and V. Apparently, excessive crosslinking (phase V) decreases small-molecule binding by approximately 20%, perhaps because of steric factors. Although steric inhibition of small-molecule binding can also occur with the PA-modified phase (phase IV), reduction in picric acid ion-pairing is more the result of amine loss due to acylation. It is estimated that acylation progresses to approximately 30%.

TABLE I

CHARACTERIZATION OF STATIONARY PHASES

*Polyamine phase**	*C/N***	*IPC**** *(μmol N/g support)*	*Hb_{iec}*§ *(mg/g support)*
III	5.8	560	39
IV	6.6	370	7
V	7.6	440	32

* See Fig. 1 for chemical composition.

** Molar ratio.

*** Picric acid ion-pairing capacity (see Materials and methods).

§ Hemoglobin ion-exchange binding capacity (see Materials and methods).

Polyamine phase III is a high-performance anion-exchange medium of high load capacity (Table I). Interestingly, ionic hemoglobin-binding capacity (Hb_{iec}) was lower on phases IV and V. Again, steric factors are assumed to be responsible for decreased hemoglobin loading with phase V. However, the diminished Hb-binding capacity of phase IV reflects the loss of charged sites due to acylation. The derivatization procedures used to prepare the multimodal media (phases IV and V) described here apparently preclude high mass loading. Although reduced load capacity is of minor significance in analytical applications, it is an important consideration in preparative column applications.

Retention versus ionic strength

Plots of isocratic protein retention, expressed as the capacity factor (k') *vs.* mobile-phase ionic strength (I) for α-CHYGN, bovine serum albumin (BSA) and OVA on stationary phase V are concave (Fig. 2). The degree of segregation between ionic and hydrophobic interactions can be estimated by the equation.

$$S = H - I \tag{1}$$

where H is the ionic strength that produces a k' of 1 in the hydrophobic-interaction mode, I is the concentration that produces a k' of 1 in the ion-exchange mode and S is the measure of segregation. Values of S = 1.2, 1.8, and 2.1 were calculated for α-CHYGN, BSA, and OVA, respectively. Small values indicate that mixed mode retention is beginning to occur with regard to ionic and hydrophobic character. It should be noted that ionic and solvophobic interactions can be further segregated by

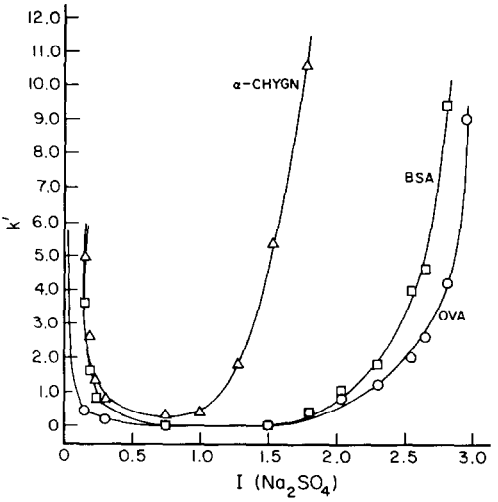

Fig. 2. The influence of ionic strength on the retention of three proteins. α-Chymotrypsinogen (α-CHYGN), ovalbumin (OVA), and bovine serum albumin (BSA) were chromatographed isocratically (pH 7.5) using sodium sulfate solutions (in 0.01 *M* phosphate buffer) of varying ionic strength. A column (5 × 0.41 cm) packed with polyamine phase V (see Fig. 1), and a flow-rate of 1 ml/min was used.

using a salt, such as sodium chloride, which has a lower molal salting-out coefficient[14]. The low ionic strength portion of the k' *vs.* I plot shows little difference relative to the displacing salt used, suggesting that the molal salting-out coefficient has a minimal influence on ion-exchange retention. However, the high-ionic strength portion is shifted to the right for salts exhibiting lower coefficients, since increased salt concentrations are required to drive the solvophobic interaction (data not shown).

Chromatographic evaluation

The chromatographic effectiveness of polyamine phases IV and V was demonstrated by comparing separations of standard protein mixtures in both the AEC and HIC modes with those obtained by using commercial columns, designed to be used in a single mode. A Mono Q strong anion-exchange column (Pharmacia Fine Chemicals) was chosen for the AEC evaluation, since it is frequently applied in biochemical research. Using OVA and soybean trypsin inhibitor (STI) as test materials, separations with a 20-min linear gradient were compared (Fig. 3). Phase V was essentially equivalent to the Mono Q column with respect to both retention and selectivity under the stated chromatographic conditions. Phase IV was substantially less retentive and required a shallower gradient slope to achieve equivalent retention times; however, its performance was also favorable. It was concluded that both phases IV and V were acceptable as AEC media.

A similar comparison was made in the HIC mode, using RNAse, OVA, LYZ and α-CHYGN as protein samples. In this case, the SynChropak Propyl column was used as a commercially available standard. Again, the resolving power of the multimodal columns was equivalent; however, their selectivity was slightly different (Fig.

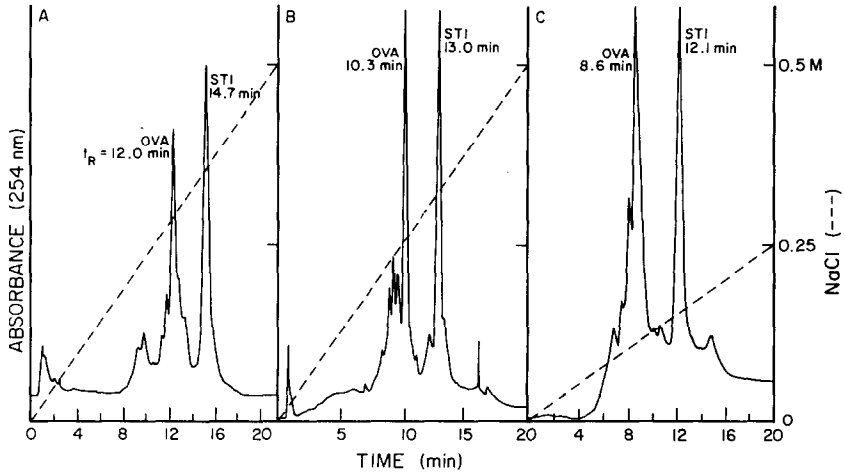

Fig. 3. Comparative anion-exchange separations. A two-component mixture [OVA, 8 mg/ml and soybean trypsin inhibitor (STI), 15 mg/ml] was chromatographed (20 μl) on (A) polyamine phase V (5 × 0.41 cm column), (B) a Pharmacia "Mono Q" strong anion-exchange column and (C) polyamine stationary phase IV (5 × 0.41 cm column). Anion-exchange chromatography was performed at pH 8 with a 20-min linear gradient from 0 to 0.25 or 0.5 *M* sodium chloride in 0.01 *M* Tris–HCl buffer at a flow-rate of 1 ml/min.

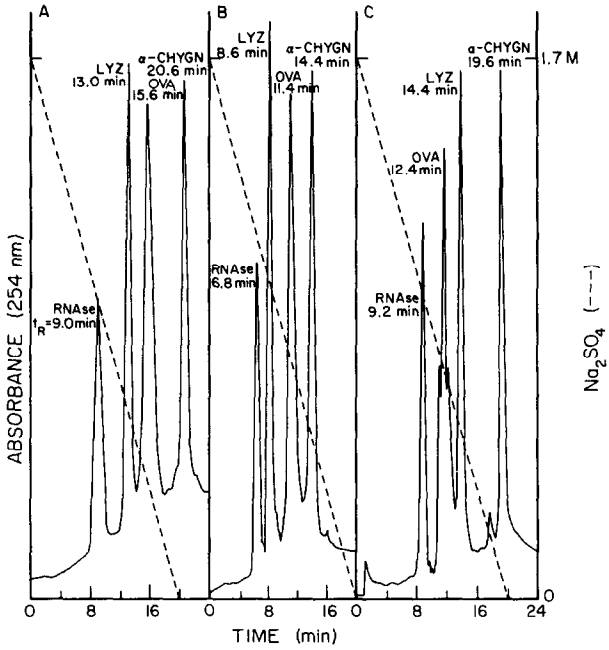

Fig. 4. Comparative hydrophobic interaction separations. A four-component mixture (RNAse, 10 mg/ml; LYZ, 5 mg/ml; OVA, 20 mg/ml, and α-CHYGN, 5 mg/ml) was chromatographed (20 μl) on (A) polyamine phase V (5 × 0.41 cm column), (B) polyamine phase IV (5 × 0.41 cm), and (C) a SynChropak Propyl column (10 × 0.41 cm). Chromatography was performed at pH 7, using a 20-min descending salt gradient from 1.7 to 0 *M* sodium sulfate in 0.01 *M* phosphate buffer at a flow-rate of 1.5 ml/min.

4). Interestingly, the elution order of LYS (p*I* = 11) and OVA (p*I* = 4.7) chromatographed on phases IV and V was inverted relative to the commercial column. This inversion of selectivity can be explained in terms of a mixed hydrophobic–electrostatic interaction in which hydrophobic effects dominate. The SynChropak Propyl column is presumably neutral, while phases IV and V contain residual positive charges. Arakawa and Timasheff[15] have shown that a salt-deficient hydration layer surrounds proteins in solutions containing high concentrations of salt. Therefore, charged groups within the contact surface area could elicit an electrostatic interaction. The existence of residual positive charges on phases IV and V probably increases the retention of OVA through a cooperative hydrophobic ionic interaction. In contrast, decreased LYZ retention may result from weak electrostatic repulsion.

Application

The potential of multimodal columns lies in the unique selectivity, elicited by each chromatographic mode. A sample isolated from a mixture on a column functioning in one mode can be rechromatographed on the same column by operating in a second mode. For example, fractions collected after ion-exchange separation could be rechromatographed on the same column in the HIC mode. All that is required between the two chromatographic steps is the addition of salt to the sample.

Fractionation of a crude extract from *M. aeruginosa* (bluegreen algae) containing cytochrome c_{553} (CYTc_{553}), cytochrome c_{550} (CYTc_{550}), and several ferre-

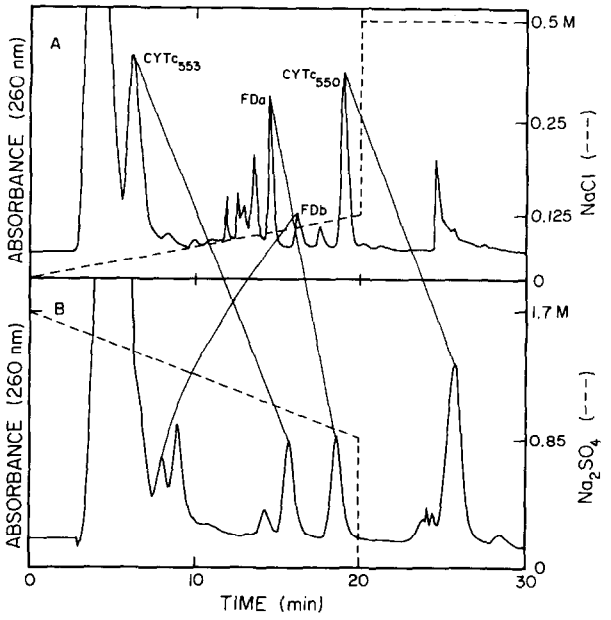

Fig. 5. Comparative selectivity of AEC and HIC for the fractionation of proteins from a crude cyanobacterium extract. The retention of CYTc_{553}, CYTc_{550}, and two ferredoxins from *Microcystis aeruginosa* was followed in AEC and HIC on a column (25 × 0.41 cm), packed with phase V. (A) AEC was performed at pH 8, using a 20-min linear gradient from 0 to 0.125 *M* sodium chloride in 0.01 *M* Tris–HCl buffer, followed by a step to 0.5 *M* sodium chloride in 0.01 *M* buffer at a flow-rate of 1 ml/min. (B) HIC was performed at pH 7.5, using a 20-min descending salt gradient from 1.7 to 0.85 *M* sodium sulfate in 0.01 *M* phosphate buffer, followed by a step to 0 *M* sodium sulfate in 0.01 *M* buffer at a flow-rate of 1 ml/min.

doxins (FDs)[16] was used to demonstrate the complementarity of AEC and HIC. The extract was first chromatographed in each mode on a 25 × 0.41 cm I.D. column, packed with phase V (Fig. 5). Protein elution was monitored with a Hewlett-Packard 1040 A scanning-diode-array detector which provided detection at multiple wavelengths and reconstruction of absorbance spectra at any point in the chromatogram. The elution positions of CYTc_{553} and CYTc_{550} were designated from these spectral data and from their elution order relative to classical AEC[17]. The peaks corresponding to FDs were also identified by this method. Since the exact type of ferredoxin was not identified, the notations "FDa" and "FDb" indicate only that they are two different molecules.

Based on the elution order of these four proteins, unique selectivities were obtained, when the extract was chromatographed on the multimodal column (phase V, 25 × 0.41 cm) by either AEC or HIC. Interestingly, the CYTc_{550} was eluted last in both modes, suggesting that this protein possesses hydrophobic and ionic character. The remaining identifiable proteins were eluted in the order CYTc_{553}, FDa, FDb, when chromatographed in the AEC mode, and FDb, CYTc_{553}, FDa in the HIC mode.

The peak containing CYTc_{553} was collected after AEC and rechromato-

graphed in the HIC mode (Fig. 6). As in the chromatogram of the crude extract, CYTc_{553} was identified as a distinct peak, eluted at 15.6 min. A three-fold increase in the A_{410}/A_{260} ratio relative to AEC demonstrated a substantial purification. Several other peaks were rechromatographed with similar results (data not shown).

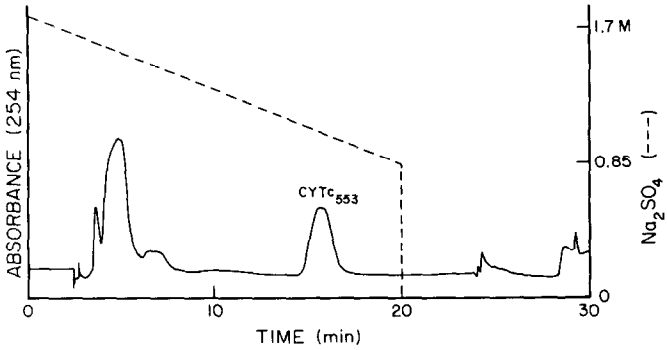

Fig. 6. Isolation of CYTc_{553} from the cyanobacterium extract. A fraction containing CYTc_{553} was collected after AEC (Fig. 5) and rechromatographed in the hydrophobic-interaction mode on the same column (phase V, 25 × 0.41 cm). AEC and HIC conditions were as described in Fig. 5. A three-fold increase in the A_{410}/A_{260} ratio, relative to AEC, indicated substantial purification.

CONCLUSIONS

It was possible to prepare AEC–HIC multimodal stationary phases by using adsorbed-coating methodology. Two synthetic strategies were employed. The first method attenuates the ionic interaction and introduces hydrophobic sites through extensive crosslinking with a weakly hydrophobic multifunctional oxirane (phase V). The second method involves the acylation of surface amines with PA, which simultaneously removes ionic sites and introduces weakly hydrophobic sites (phase IV). The predominance of either mechanism was dependent upon ionic strength. Columns were operated in the anion-exchange mode with an increasing salt gradient (0 to 0.5 *M* sodium chloride) or in the hydrophobic interaction mode with a decreasing salt gradient (1.7 to 0 *M* sodium sulfate). Since the ionic interactions between column and sample molecules were reduced in order to attain adequate segregation of electrostatic and hydrophobic modes, these column materials typically exhibited lower hemoglobin load capacities.

The multimodal columns synthesized resolved standard protein mixtures as effectively as commercial columns designed specifically to be used in a single chromatographic mode. The anion-exchange selectivity of both multimodal phases was identical to that of the Pharmacia (Mono Q) column. However, there was an inversion in the retention order of OVA and LYS, chromatographed in the HIC mode relative to the Synchropak Propyl column. Since both multimodal stationary phases exhibit a residual positive charge while the SynChropak Propyl column is presumably neutral, inverted selectivity was thought to result from secondary electrostatic interactions.

A crude extract from *Microcystis aeruginosa*, containing several cytochromes and ferredoxins, was fractionated in both the AEC and HIC mode on amine phase V. Judging from the relative retention orders of these proteins, each mode provided unique selectivity. In addition, a fraction containing CYTc_{553}, collected after AEC, was further purified by rechromatographing it in the HIC mode. This dual-mode column is advantageous not only for its selectivity, but also in sample preparation. Samples collected after AEC need only the addition of salt prior to HIC.

The ability to perform chromatography by two modes independently on the same column is potentially very useful. Since the synthetic routes for these multimodal phases differ only slightly from those utilized for "pure" anion-exchange or hydrophobic-interaction columns, two separation mechanisms can be obtained for the price of one column. The application of such multimodal stationary phases to preparative protein chromatography is expected to have a significant influence on the practice and economics of large-scale protein purification.

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REFERENCES

1 A. Nahum and Cs. Horváth, *J. Chromatogr.*, 203 (1981) 53.
2 N. K. Boardman and S. M. Partridge, *Biochem. J.*, 59 (1955) 543.
3 E. Pfannkoch, K. C. Lu, F. E. Regnier and H. G. Barth, *J. Chromatogr. Sci.*, 18 (1980) 430.
4 B. H. J. Hofstee, *Biochem. Biophys. Res. Comm.*, 50 (1973) 751.
5 W. Kopaciewicz, M. A. Rounds, J. Fausnaugh and F. E. Regnier, *J. Chromatogr.*, 266 (1983) 3.
6 H. P. Jennissen, *J. Chromatogr.*, 159 (1978) 71.
7 X. Geng and F. E. Regnier, *J. Chromatogr.*, 296 (1984) 15.
8 J. B. Crowther and R. A. Hartwick, *Chromatographia*, 16 (1982) 349.
9 R. Bischoff and L. W. McLaughlin, *J. Chromatogr.*, 296 (1984) 329.
10 W. Kopaciewicz, M. A. Rounds and F. E. Regnier, *J. Chromatogr.*, 318 (1985) 157.
11 A. Alpert and F. E. Regnier, *J. Chromatogr.*, 185 (1979) 375.
12 G. Vanacek and F. E. Regnier, *Anal. Biochem.*, 121 (1982) 156.
13 E. S. Gould, *Mechanism and Structure in Organic Chemistry*, Holt, Rinehardt and Winston, New York, 1959, p. 318.
14 W. R. Melander, D. Corradini and Cs. Horváth, *J. Chromatogr.*, 317 (1984) 67.
15 T. Arakawa and S. N. Timasheff, *Biochemistry*, 23 (1984) 5912.
16 K. K. Ho, E. H. Ulrich, D. W. Krogmann and C. Gomez-Lojero, *Biochim. Biophys. Acta*, 545 (1979) 236.
17 D. W. Krogmann, personal communication.