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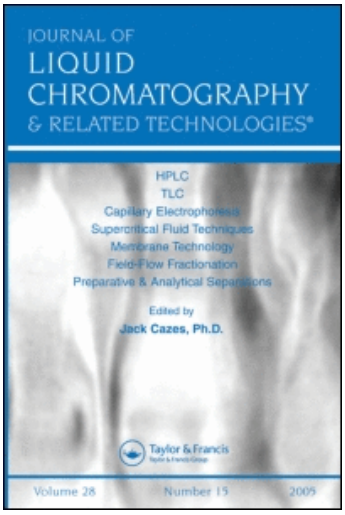

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Preparative Hydrophobic Interaction Chromatography of Proteins Using Ether Based Chemically Bonded Phases

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PREPARATIVE HYDROPHOBIC INTERACTION CHROMATOGRAPHY OF PROTEINS USING ETHER BASED CHEMICALLY BONDED PHASES

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ABSTRACT

This paper examines the use of 15-20 micron wide-pore silica-based ether bonded phases for the preparative hydrophobic interaction chromatography of proteins. In particular, silyl ethers are immobilized on large particle silica in an analogous manner to previously developed ether bonded 5 um analytical supports. The preparative supports are reproducibly prepared and exhibit constant chromatographic retention for at least five months of continual use. Preparative columns can be operated for protein chromatography with peak shapes and capacity as predicted by the Snyder gradient elution model. Moreover, similar retention times are obtained relative to those on the 5 um analytical columns, enabling the direct transition and scale-up of separation. Gradient optimization is seen to directly parallel that performed on 5 um bonded ether analytical columns. Acceptable chromatographic resolution was obtained with sample capacity of >15 mg protein/ml column volume using a repetitive injection technique. A column clean-up strategy is examined for rapid and safe removal of contaminants. An illustrative example of use of the bonded ether preparative columns is made by application to soybean trypsin inhibitor purification. Initial results are presented on a column-switching method for the analytical monitoring of preparative separation.

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INTRODUCTION

The research area of bonded phase synthesis for high performance liquid chromatographic (HPLC) biopolymer separation and analysis continues to see rapid development. A wide variety of columns are available to separate biomacromolecules via size exclusion (SEC), ion exchange (IEC), reversed phase (RPLC) and affinity modes of LC. Attention has now turned to the development of new procedures (e.g., metal chelate (1), mixed-mode (2)) for HPLC of various biopolymers (i.e., nucleic acids, proteins, etc.), re-definition of existing HPLC modes, applications and extension to the preparative mode. At the same time, advances continue in alternative separation strategies, e.g., capillary electrophoresis (3).

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A relatively new mode of HPLC, hydrophobic interaction chromatography (HIC), as developed by us (4) and other groups (5-10) yields efficient protein separation with selectivity supplementing that offered by other widely-used modes of SEC, IEC and RPLC. More importantly, unlike RPLC, separation is achieved via the use of aqueous salt solutions near neutral pH which significantly lessens the potential for unfolding and denaturation (and mass loss) of the protein species (11). These characteristics follow from the use of hydrophilic ligands (i.e., ethers, glycols, polyamines, etc.) immobilized on silica or hydrophilic polymer supports. Thus, it appears likely that the HIC technique will find use in scaled-up preparative purification of active protein. In fact, several papers on this subject have recently appeared in the literature (12,13).

In this paper, we examine the capability of ether bonded silica-based phases for preparative HIC of proteins. Silyl ethers are reproducibly attached to 15-20 micron, 300 Å pore diameter silica gel for use as stable packing in HIC. Direct translation of protein separation from 5 um based analytical columns to 15-20 um preparative columns is achieved for ease of scale-up in preparative HIC. The bonded ether columns possess high loading capacity for protein which is a function of sample preparation and

method of injection. A post-separation column clean-up method is proposed for rapid and safe removal of contaminants. An illustrative application of the preparative HIC columns to the purification of soybean trypsin inhibitor provides a practical example of their use. Lastly, initial results are presented on a column-switching method for the analytical monitoring of preparative separation. Taken together, these results demonstrate that HIC on ether bonded phases is a useful and powerful LC mode for purification of active biopolymer samples.

EXPERIMENTAL

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Equipment and Chemicals

The gradient HPLC system (Beckman Instruments, Inc., San Ramon, CA, U.S.A.) consisted of two Model 110B pumps fitted with preparative heads, a Model 340 mixer and injector, a Model 165 variable wavelength detector (with a 5 mm preparative flow cell) and a Model 427 integrator. A gradient delay volume of 6.66 ml was measured and subtracted from all chromatographic data presented.

Analytical (4.6 mm ID x 10 cm) and semi-preparative (10 mm ID x 25 cm) Spherogel CAA HIC columns containing 5 micron, 300 Å pore diameter silica-based bonded ether packing for HIC were obtained from Beckman Instruments, Inc. (San Ramon, CA, U.S.A.). In one instance, bulk 5 micron ether bonded HIC support was packed into 4.6 mm ID x 2 cm analytical columns for use in the coupled column LC system. Preparative bonded ether bulk packing based on 15-20 um, 300 Å pore sized Vydac silica was obtained from Cambridge Analytical/Separations (Boston, MA, U.S.A.) and packed into 4.6 mm ID x 10 cm or 21.2 mm ID x 15 cm stainless steel tubes following standard slurry procedures. Elemental analysis for surface coverage was performed by MultiChem Labs. (Lowell, MA, U.S.A.). The precision of percent carbon (%C) data for a given phase is ca. 3% relative standard deviation (RSD).

Cytochrome c (CYT, type VI, from horse heart), ribonuclease A (RNase, type III-A, from bovine pancreas), lysozyme (LYS, grade I,

from chicken egg white), ∝-chymotrypsin (CHT, type VII, from bovine pancreas), ∝ -chymotrypsinogen A (CHTG, Type II, from bovine pancreas) and two grades of soybean trypsin inhibitor ("pure" STI, type I-S and "crude" STI, type II-S, crude, soluble powder) as well as ammonium acetate and grade III ammonium sulfate were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and used as received. Reagent-grade H_3PO_4 and HPLC-grade 2-propanol were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). HPLC-grade water was prepared in-house.

Chromatographic Procedures

Mobile phases were prepared by adding the correct weight of salt and buffer to a volumetric flask containing HPLC-grade water previously degassed by aspirator vacuum. The pH was adjusted to 6.0 with glacial acetic acid and a small amount of HPLC-grade water (degassed) was added to the mark. Mobile phases containing high salt concentrations were not allowed to remain in the column or pumps for long periods of time. Protein solutions were freshly made up in water at 5-10 mg/ml concentrations unless otherwise indicated. A pre-column (4.6 mm ID x 6 cm) containing the same bonded phase as the HPLC column was placed between the pumps and injector.

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RESULTS AND DISCUSSION

In this paper, we describe results obtained on large particle (15-20 um) wide-pore (300 Å) silica-based bonded ether supports for preparative hydrophobic interaction chromatographic (HIC) separation of proteins. In previously published work (4,14), we had developed the bonded ether chemistry on 5 micron, 300 Å Vydac silica and optimized gradient design for successful HIC of active protein. We have continued to use the same bonding chemistry and ether ligand for the synthesis of preparative phases in this work. We will first examine characteristics of the large particle bonded phase and then turn to a discussion of operational aspects of preparative HIC for protein separation.

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Characteristics of 15-20 Micron Bonded Ether HIC Supports Phase Evaluation

In this section, results are described on the use of 15-20 um bonded ether support packed in analytical sized 4.6 mm ID x 10 cm columns in comparison with similarly sized columns containing 5 um bonded ether support. Wide diameter columns will be discussed separately. Table I presents characteristics of the 5 um and 15-20 um bonded phases, while Table II (lines 1 and 2) lists experimental data on analytical-sized columns containing these packings.

Obvious differences exist in support surface area as well as the bonded phase coverage. However, despite this lower coverage on the larger particles, sensitive size exclusion chromatographic (SEC) tests with the basic protein lysozyme (LYS) under low ionic strength mobile phase (4) conditions revealed no charge effects, i.e., good coverage of silanols is achieved. Moreover, the phases

TABLE I

Characteristics of Bonded Ether Phases

$\equiv Si\ (CH_2)_3\ O\ (CH_2 - CH_2 - O)_2 - CH_3$

d_p* (um)	Surface* Area (m^2/g)	Pore* Size (Å)	%C**	Coverage*** ($\mu mol/m^2$)	S+	Relative++ Cost
5	74	300	4.0	6.2	6.2	1
15-20	146	300	5.96	4.9	7.9	0.3

* Data reported by manufacturer on unbonded silica.
** %C of bonded phase by elemental analysis, corrected for %C in unbonded silica.
*** Coverage is calculated assuming an average reaction of three ethoxy groups per silane molecule.
\+ S = slope of log k' vs. concentration fraction of ammonium sulfate, from ref. (4). Average of S values for CYT, RNase and LYS.
++ Comparative cost of synthesized phase.

TABLE II

Characteristics of Ether Bonded HIC Columns

d_p (um)	ID (mm)	L (cm)	Packing* (g)	V_O** (ml)	ΔP+ (psi)	F (ml/min)	Protein++ Mass Loaded (mg)	Relative Cost
5	4.6	10	1	1.2	535	1.0	20	1
15-20	4.6	10	1	1.2	55	1.0	20	0.8
15-20	21.2	15	32	38.3	100	21	670	5#

* Amount of support contained in column.
** Void volume measured with uracil, an unretained solute.
\+ Δ P measured for indicated column at indicated flow rate in 0.5 M NH_4OAc, pH 6.0 at 25 °C.
++ Representative mass of protein loaded on indicated column, see Figure 7C and text.
\# Relative cost of a similarly sized column containing 5 um bonded ether particles is 9.5.

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exhibit good stability (see later). Differences in silanol content or reactivity between the two silicas may account for the observed variation in coverage. Finally, the 15-20 um bonded phase may be prepared for ca. one-third the cost of 5 um based supports reflecting primarily the decreased pricing of large particle silica.

Figure 1 provides chromatograms of a standard five-component protein mixture on the 15-20 um column as compared with the 5 um analytical column. The separation was obtained on each column under identical conditions, i.e., 20 minute linear gradients at 1.0 ml/min flow rates using equal sample sizes. Note the excellent resolution obtained on the 5 um column for this sample. The peak preceeding CHTG (peak 5) corresponds to a derivative of CHTG, tentatively identified as neo-chymotrypsinogen (15), and occurs to variable extent in commercial CHTG samples. This peak has also been observed to grow at the expense of the CHTG peak with aging of the sample. Table II (lines 1 and 2) provides

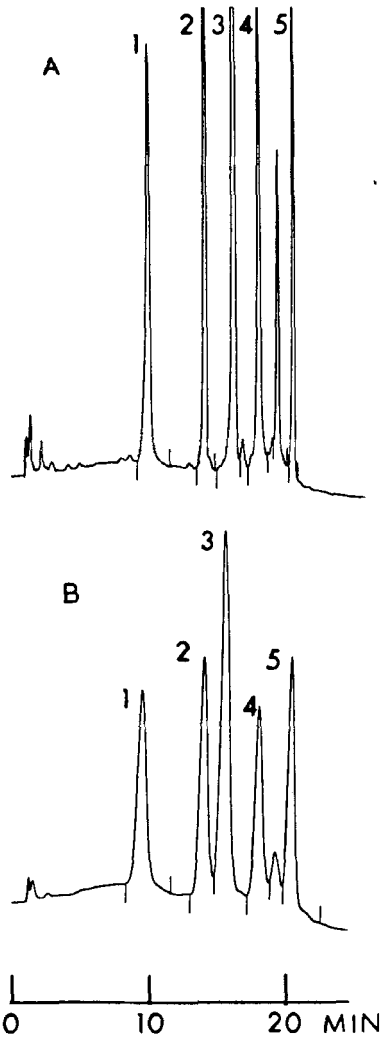

FIGURE 1 Effect of support particle diameter in HIC protein separation. Standard proteins (1 = CYT, 30 ug; 2 = RNase, 90 ug; 3 = LYS, 30 ug; 4 = CHT, 30 ug; 5 = CHTG, 30 ug) were chromatographed on a 4.6 mm ID x 10 cm ether column of indicated particle diameter at a flow rate of 1.0 ml/min using a 20 minute linear gradient at 25 °C from 3.0 M ammonium sulfate, 0.5 M ammonium acetate, pH 6.0 to 0.5 M ammonium acetate, pH 6.0. Detector 280 nm, 0.1 a.u.f.s. A. 5 micron particles. B. 15-20 micron particles.

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additional characteristic data on these columns. Of particular importance is the constancy in protein retention time between the two columns, which facilitates transfer of separation between the two phases. This retention constancy occurs in spite of the two times greater surface area of the larger particles and is probably due to the use of gradient elution, i.e., change in k' during separation. Data accumulated on other large particle silicas suggests that significant increases in retention are seen once support surface area rises above ca. 250 m^2/g (16).

As seen in Table I, the similarity in S values (17) (i.e., the slopes of log k' vs. concentration fraction of ammonium sulfate) measured on these columns (14) in the range of 6 to 8 further confirms this observation. Furthermore, in accordance with theory (14), the 15-20 um column provides proportionately less (by dp^{-1}) peak capacity (defined as gradient time t_G divided by $4\sigma_t$ averaged for RNase, LYS, CHT and CHTG (14): 7.4 at 15-20 um, assuming average dp of 17.5 um vs. 23 at 5 um) and peak height (for CHTG normalized for peak area: 1.6 at 15-20 um vs. 5.0 at 5 um), but with reduced pressure drop and at a cost savings (see Table II). These relationships of peak capacity and height to dp also indicate that these columns are equivalently packed. The excellent correspondence of HIC results on 15-20 um bonded supports to Snyder's gradient LC model (17) provides a rational approach for optimization of separation similar to that developed for the 5 um analytical supports (14).

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Column Reproducibility and Stability

Table III presents data for 15-20 um column-to-column reproducibility and data accumulated on the 5 um columns. Each phase was prepared separately using the same batch of silane and Vydac silica and identical bonding reaction conditions. The %C data show a precision of 0.89 %RSD and indicate the phases are identical in terms of %C when compared to the precision of the analysis (ca. 3% RSD). Similarly, the precision in chromatographic retention data under HIC conditions for well-retained proteins is excellent (ca. 2%) and the results compare

TABLE III

15-20 Micron Bonded Ether Column Reproducibility

Conditions as in Figure 1B.

Batch*	%C**	V_g (ml)+ CYT	RNase	LYS	CHT	CHTG
1	6.03	3.1	7.9	9.2	12.1	14.6
2	5.98	3.3	8.0	9.3	12.3	14.8
3	5.92	3.6	8.2	9.5	12.5	15.1
4	5.91	3.3	8.2	9.6	12.3	14.8
Mean	5.96	3.4	8.1	9.4	12.3	14.8
RSD, %	0.89	6.1	1.9	1.9	1.3	1.4
Mean++, 5um	4.0	3.9	8.0	10.1	12.2	14.7
RSD, %	2.5	7.4	1.9	0.7	0.9	0.9

* The four bonded phases were prepared separately using the same batch of silane and silica.

** %C of bonded phase by elemental analysis, corrected for %C in unbonded silica.

+ V_g = gradient elution volume corrected for gradient delay volume.

++ Mean and precision of data obtained on four separately prepared batches of 5 um bonded ether support.

TABLE IV

15-20 Micron Bonded Ether Column Stability

Conditions as in Figure 1B.

Volume Mobile Phase Passed (L)	V_g (ml)* RNase	LYS	CHT	CHTG
0	8.2	9.5	12.6	15.0
13.5	7.9	9.4	12.3	14.8
30.7	7.9	9.4	12.4	14.8
55.1	8.1	9.6	12.3	14.7
Mean	8.0	9.5	12.4	14.8
RSD, %	1.9	1.0	1.1	0.8

* V_g = protein elution volume corrected for gradient delay volume.

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well with that obtained on 5 um analytical columns under the same conditions. Cytochrome c exhibits a higher deviation in retention volume as it is just retained (i.e., at the front of the gradient) on these phases.

A frequently cited concern of chromatographers is the operational stability of the hydrophilic bonded phase in use for separation. Therefore, we next studied protein retention constancy on the 15-20 um bonded phase as a function of usage under typical HIC conditions. Table IV demonstrates that retention does not vary by more than ca. 2% RSD with use of 55 liters of mobile phase. This usage is equivalent to 5.7 months of daily operation.

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Sample Capacity

An important consideration in preparative HIC is sample capacity, i.e., amount of solute that can be loaded onto the column to the point where separation is compromised. In analytical chromatography, this limit is often specified as that sample size resulting in a reduction in k' or plate count by 10% (18,19). However, for preparative LC work, the column can still provide acceptable separation (20). Thus, loading capacity in preparative LC is that limit where sample size compromises resolution to the extent that throughput and/or purity goals are not maintained. It should be noted that in overloaded analytical LC, where real preparative work is conducted, the equation for resolution is not applicable and column performance must be evaluated empirically for each application.

Previously, we had studied sample capacity on ether based HIC analytical columns by examining gradient retention volume as a function of protein sample size (14). Ca. 13 mg of a four-component protein mixture could be injected before significant shifts (i.e. 10%) in retention occurred. However, successful resolution of 4-8 mg of each protein in one run on the 10 cm column was possible due to the wide separation of the peaks.

Previous assessments of sample capacity in HIC have differed in the method of characterization, the sample components, matrix,

and concentration and HIC separation conditions. Using a dynamic measure of column capacity based on changes in chromatographic resolution, Schmuck (13, 21, 22) and Regnier (23) measured sample capacities of ca. 8 mg/ml on propyl and butyrate polyamine-based HIC columns. Kato, et al. could load ca. 200 mg of protein (3.7 mg/ml capacity) on preparative Phenyl-5PW columns (13 um, 21.5 mm ID x 15 cm) before slight decreases in resolution were noted using 60 or 120 minute gradients (12). Significantly greater sample capacity was measured by use of a column breakthrough technique, e.g. 66 mg/ml ovalbumin on a propyl HIC support (13, 22) or by a static hemoglobin binding assay, e.g. 150-180 mg/ml on a series of polyamine based HIC supports (6) and in one case 403 mg/g support on poly (aspartamide) HIC phases (10). In general, although methods differ, it is evident that the HIC separation technique exhibits a high loading capacity for protein.

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We decided to examine further the variables governing sample capacity in HIC on bonded ether columns. Figure 2 presents protein HIC chromatograms representing successively greater injected amounts of a 100 mg/ml protein solution containing CYT, RNase, LYS and CHTG on a 5 um ether 4.6 mm ID x 10 cm analytical column. Minor shifts in retention are evident with some loss in resolution between RNase and LYS as load is increased beyond 3 mg, however, separation is still achieved for the proteins even at the highest sample size used, i.e. ca. 15 mg protein/ml column volume. As sample injection volume increased beyond ca. 5% of the column volume (ca. 60 ul), a peak appeared at the void volume which due to the red color could be tentatively identified as CYT (see Figure 2C-F). Note also that a retained peak is present in the chromatogram for CYT. As the protein sample is made up in water (a strong solvent in HIC), this result suggested that large sample sizes lead to incomplete mixing with the high salt content of mobile phase A for partial elution of the more hydrophilic proteins. While salt could be added to the sample, care must be taken not to precipitate proteins in the vial. Higher concentrations of protein are often too viscous to be injected.

For chromatographing large samples on the HIC column, we determined that a repetitive injection technique could be

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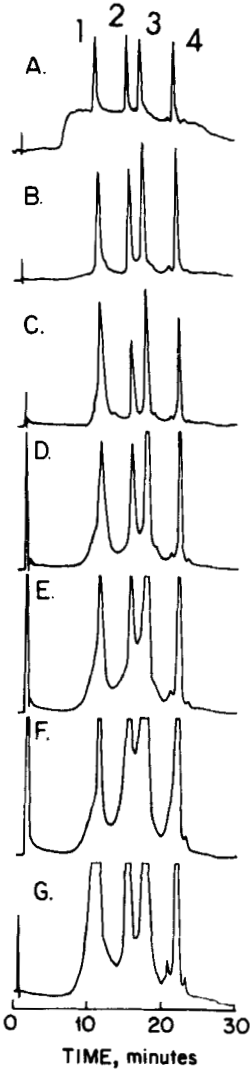

FIGURE 2 Effect of sample size on HIC of proteins using a 5 um bonded ether analytical column. All chromatographic conditions as in Figure 1A, except as noted below. Successively greater injections were made of a 101 mg/ml protein solution containing the four proteins CYT(1):RNase(2):LYS(3):CHTG(4) in the mass ratio 1.3:3.2:1.1:1.0. A. 1 ul injection, 101.3 ug load, detection 280 nm at 0.1 a.u.f.s.; B. 5 ul, 507 ug, 280 nm, 0.2 a.u.f.s.; C. 30 ul, 3.04 mg, 254 nm, 2.0 a.u.f.s.; All the following runs were detected at 254 nm, 2.0 a.u.f.s. D. 70 ul, 7.09 mg; E. 100ul, 10.1 mg; F. 170 ul, 17.2 mg; G. six 30 ul injections, 18.2 mg.

employed. Figure 2G shows a separation resulting from six 30 ul injections of protein onto the column while isocratic in mobile phase A. Improved resolution and the absence of a significant void peak could be obtained for this 18 mg load relative to the case where all sample was injected at one time (i.e., Figure 2F). Figure 3 presents a 21 mg load of five proteins chromatographed on a 4.6 mm ID x 10 cm analytical-sized column containing 15-20 um bonded ether phase. Thirty 20 ul injections of a 35 mg/ml protein solution were made with the column running isocratically in 100% A solvent. In comparing Figure 3 with Figure 1B, we see that similar retention and resolution are obtained for both the high and small loads of protein. No significant increases in pressure drop were noted during the injection procedure. Furthermore, the absence of ghosting in the blank gradient run after the separation suggested good recovery of protein mass. We have previously shown that protein mass is not lost to the ether column for significantly long incubation times under conditions of high retention (i.e., isocratic operation in 100 %A) (14). While the 21 mg protein load represents a 17.5 mg/ml capacity, it is clear that further increases in load can be tolerated by the column before resolution is seriously degraded. In fact, using the repetitive injection method, we could load and retain 120 mg of LYS (sixty 20 ul injections of a 100 mg/ml solution) onto the 15-20 um column. Subsequent blank gradients run after elution of the protein exhibited minimal amounts of LYS. Thus, it is evident for ether based HIC columns that sample introduction plays an important role in attaining high column capacity.

We next examined in brief, the "absolute" column loading capacity via a method similar to that used by Schmuck, et al. (13). A dilute protein solution (2 mg/ml LYS in water) could be mixed on-line with a high salt-containing mobile phase (A = 3.43 M $(NH_4)_2SO_4$, 0.17 M NH_4OAc, pH 6.0; 80/20 salt solution/protein solution) such that the protein is retained on the column. Ca. 440 mg of LYS could be placed on the 4.6 mm ID x 10 cm column of 15-20 um ether phase without breakthrough and represents at least a 367 mg/ml column volume or 440 mg/g support capacity. By

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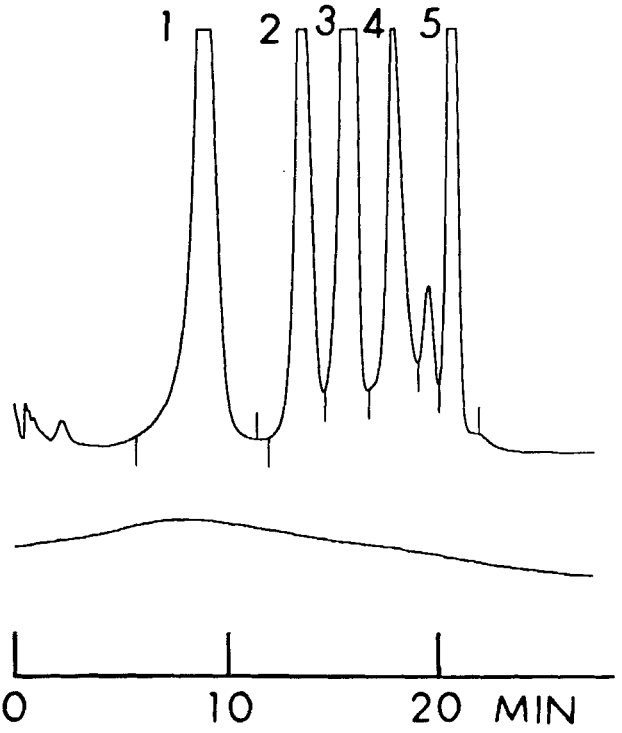

FIGURE 3 Preparative HIC protein separation on 15-20 um ether column. All chromatographic conditions as in Figure 1B, except as noted below. Thirty 20 ul injections of a 35 mg/ml protein solution loaded 21 mg onto the column. The column was run isocratically in 100% A as the injections were made. A blank gradient was run after the separation and is shown below the preparative separation. Proteins: 1. CYT, 3 mg; 2. RNase, 9 mg; 3. LYS, 3 mg; 4. CHT, 3 mg; 5. CHTG, 3 mg with detection at 255 nm at 2.0 a.u.f.s.

running the usual descending salt gradient, the protein was recovered, and a subsequent blank gradient suggested minimal mass loss (<5% of peak area in the first run). A precipitate appeared to form upon elution of the LYS peak from the LC detector but re-dissolved by dilution with additional mobile phase. This effect may have potential for easier collection and concentration of protein. In practice, this loading technique is rather time-

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consuming (43 hrs. to load 438 mg), however, the results illustrate the high loading capacity of these columns for protein.

Preparative Columns Containing 15-20 Micron Bonded Ether Support

The results of the previous section also suggest that the use of larger column sizes may facilitate the chromatography of greater amounts of protein (by allowing larger samples to be injected). One approach (18) is simply the preparation of larger (wide) columns packed with the same 5 um analytical packing. This strategy is valuable in those preparative separations where poor selectivity must be compensated with a greater number of plates for resolution. It should be noted that the use of gradient elution in HIC can provide an alternative strategy to column length changes in isocratic operation (14). Figure 4 illustrates the use of 5 micron bonded ether particles in a 10 mm ID x 25 cm column for preparative HIC of proteins. Twenty 50 ul injections of sample placed 140 mg of a five-component protein mixture onto the column. Acceptable resolution was obtained with a 96 minute linear gradient. While high mg load separations are achieved, this approach suffers from the relatively greater cost of 5 um silica as well as greater difficulty in packing such material in large diameter columns.

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It was logical to next examine wide-bore columns packed with the 15-20 um bonded ether support for preparative protein HIC. Accordingly, we prepared 21.2 mm (1") ID x 15 cm columns packed with the 15-20 um ether bonded support and studied their characteristics for preparative separations. These columns could be produced for the same cost as the 5 um, 10 mm ID x 25 cm columns cited above. Figure 5B presents a standard protein separation on such a column while in Figure 5A the same separation is shown on the 5 um analytical column. Note that for both separations, a 40 minute linear gradient was used with a linear velocity of 0.14 cm/sec. Relative to the analytical column, detection is two times less sensitive on the preparative column, while a 50 fold greater mass (0.18 mg protein on the 5 um column vs. 9 mg on the 15-20 um column) of protein was loaded on the 1"

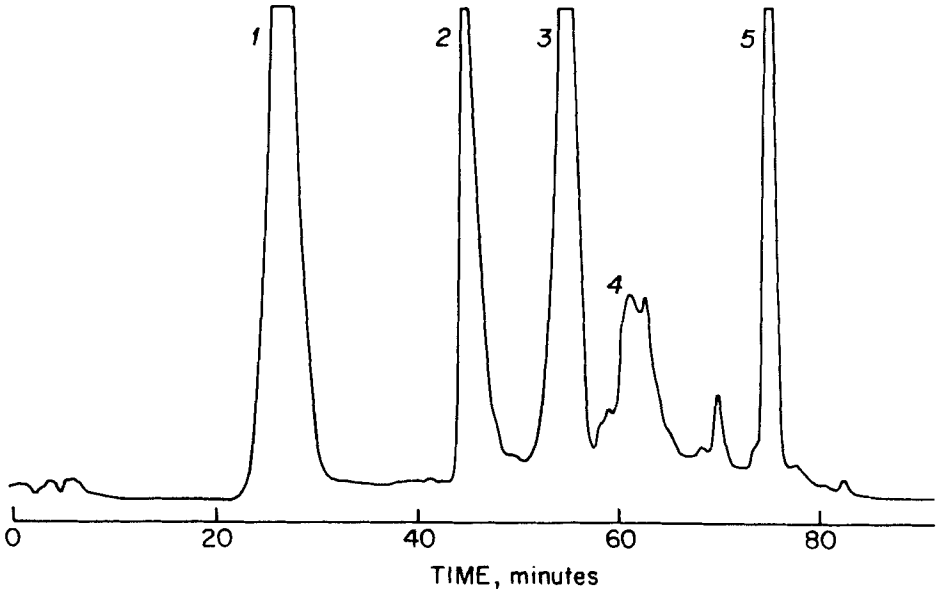

FIGURE 4 Preparative HIC protein separation on a 10 mm ID x 25 cm column of 5 um ether support. Gradient conditions as in Figure 1A, except a 96 minute linear gradient and flow rate of 2.5 ml/min were used. Detection was at 254 nm wtih 2.0 a.u.f.s. Twenty 50 ul injections placed 20 mg each of CYT (1), LYS (3), CHT (4) and CHTG (5) and 60 mg of RNase (2) for a total load of 140 mg of protein.

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HIC column. The 9 mg load on the 15-20 um column is ca. 2.4 times greater than that predicted (by ratio of column ID) for a direct comparison of peak shape characteristics on the two columns. Even so, comparison of peak capacity (as defined earlier, 33 on the 5 um column vs. 11 on the 15-20 um preparative column) on the two columns is in inverse proportionality to particle diameter. Interestingly, the gradient conditions used provide a $\bar{k}'$ value, i.e., solute k' as it passes the midpoint of the column, on the preparative column that is two times less than obtained on the analytical column. However, the proportionality observed above suggests this difference in retention to have minimal influence on the peak capacity acheived on the preparative column. The results

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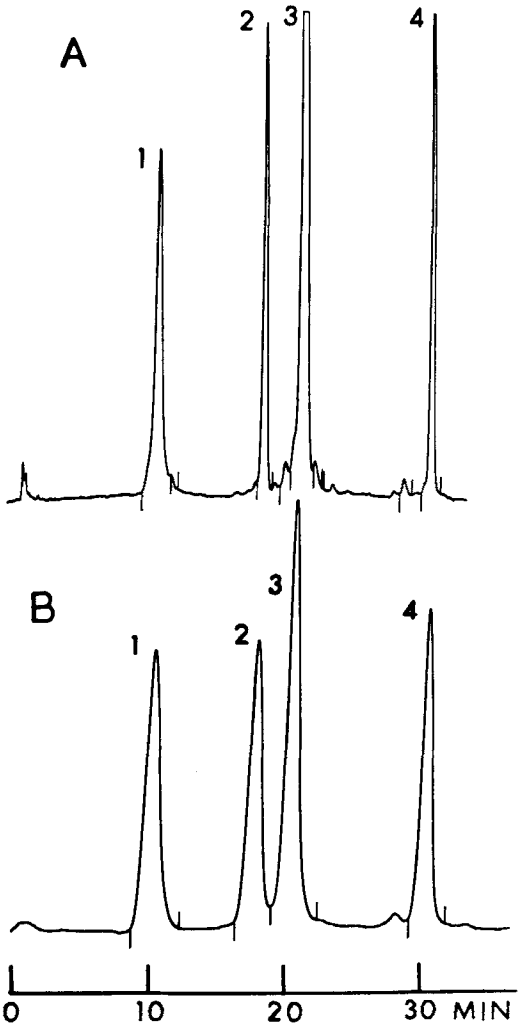

FIGURE 5 Comparison of 5 um ether analytical column with 15-20 um ether preparative column for standard protein HIC separation. All conditions as in Figure 1A, except as noted below. A. 5 um ether phase in 4.6 mm ID x 10 cm column, 40 minute linear gradient was used with detection at 280 nm at 0.05 a.u.f.s. Proteins: 1. CYT, 30 ug; 2. RNase, 90 ug; 3. LYS, 30 ug; 4. CHTG, 30 ug. B. 15-20 ether phase in 21.2 mm ID x 15 cm column with 40 minute gradient run at a 21 ml/min flow rate and detection at 280 nm, 0.1 a.u.f.s. Proteins: 1. CYT, 1.5 mg; 2. RNase, 4.5 mg; 3. LYS, 1.5 mg; 4. CHTG, 1.5 mg.

also suggest that the greater load has not influenced resolution to any significant degree and further that the two columns are equivalently packed. Likewise, after correction for the change in detector sensitivity and the factor of 2.4, relative peak height (for CHTG normalized for peak area, 3.0 on the 5 um column vs. 1.0 on the 15-20 um column) is also inversely proportional to the particle diameter. Separate measurements using uracil (an unretained marker) revealed a reduced plate height, $h \cong 2.8$ at a reduced velocity of $\nu = 3.4$. Additional characteristics for these columns are indicated in Table II (line 3).

By further use of repetitive injection, scale-up of separation to high mg loads is accomplished. Figure 6 shows a chromatogram obtained on the 15-20 um 1" column using a 40 minute gradient for the separation of 324 mg of four proteins. No significant shifts in protein retention are noted as compared to the chromatogram in Figure 5B and acceptable resolution is realized. As much as 1 g of LYS could be loaded, retained and eluted successfully from this 1" column.

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Purification of Soybean Trypsin Inhibitor (STI)

The purification of STI provides an illustrative example of the use of 1" 15-20 um preparative columns for HIC protein separation. Figure 7A presents a separation of a commercial "pure" STI sample on an analytical 5 um HIC column of the ether phase. Previous studies including RPLC techniques have shown peaks 1 and 2 to be active STI (4,24). Figure 7B shows a commercially available "crude" STI sample on the same analytical column. Despite the injection of a larger sample, note the presence of more peaks, particularly in the tail end of the chromatogram. The manufacturer indicates the "crude" STI to exhibit a specific activity ca. 1.6 times less than that of the "pure" STI. Figure 7C results from the injection of 667 mg of the crude STI sample onto the 1" preparative column with a "heart-cut" fraction collected as indicated. The absence of ghost peaks in a blank gradient run after the preparative separation suggests good mass recovery is obtained. Re-injection of the collected fraction

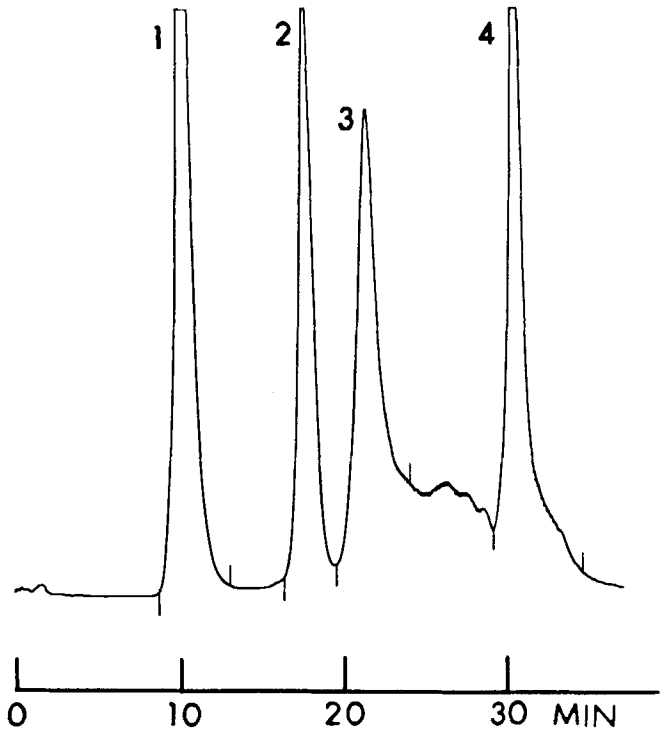

FIGURE 6 Preparative HIC separation of standard proteins on 21.2 mm ID x 15 cm column of 15-20 um ether phase. All conditions as in Figure 5B. Three 0.5 ml injections placed 324 mg of total protein on the column, consisting of 60 mg each of CYT (1), LYS (3) and CHTG (4) and 144 mg of RNase (2).

into the analytical column yields a profile at least as good as that provided by the "pure" sample; compare Figure 7D with 7A. Note that all the chromatographic profiles of STI in Figure 7 A-D compare well in terms of retention constancy. Significantly more STI could be obtained by collection of the entire peak from the preparative column.

Column Clean-Up

Another area of concern in preparative LC involves procedures to be used for column clean-up, especially with regard to the

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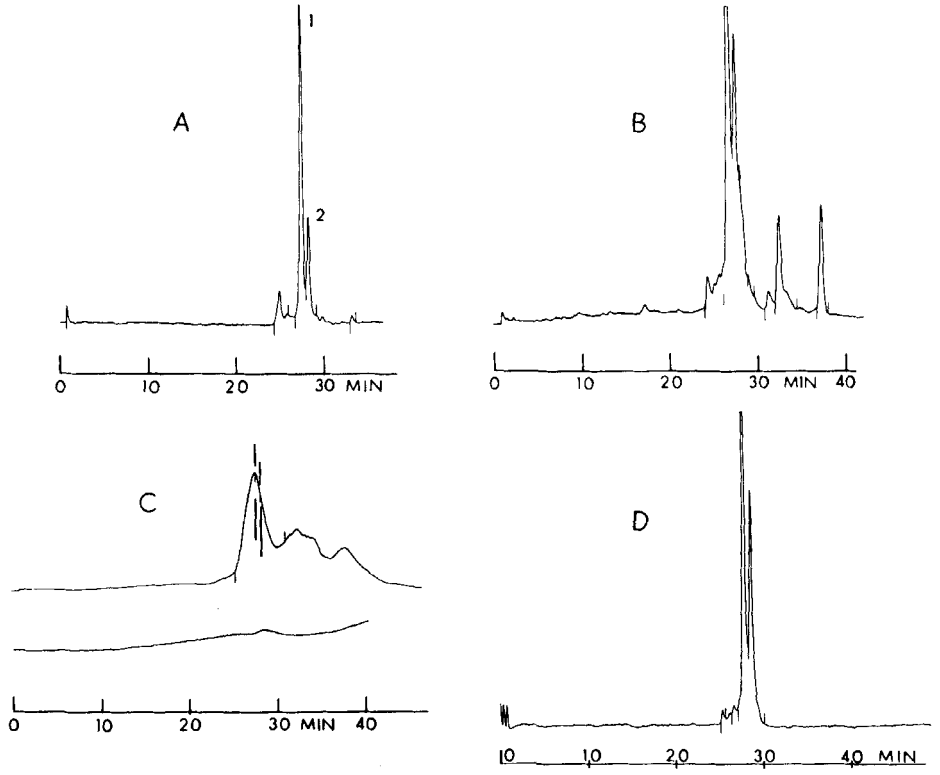

FIGURE 7 Soybean trypsin inhibitor purification and analysis by ether HIC columns. All chromatographic conditions as indicated in Figure 5. A. 150 ug of "pure" STI run on 5 um 4.6 mm ID x 10 cm analytical column. Peaks 1 and 2 discussed in text. B. 500 ug of "crude" STI injected on 5 um analytical column. C. Preparative separation of 667 mg of crude STI on 15-20 um 21.2 mm ID x 15 cm column with detection at 280 nm, 2.0 a.u.f.s. A fraction was collected at the indicated lines. A blank gradient run at 280 nm, 0.1 a.u.f.s. after the preparative separation is shown beneath the STI separation. D. 75 ul of the preparative fraction were re-injected into the 5 um analytical HIC column.

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stability of packings based on silica gel. While the biochemist is perhaps more familiar with the use of dilute NaOH washes on agarose-based phases, these strongly basic conditions can quickly degrade silica-based LC supports (25). We had previously indicated that the use of typical RPLC protein elution conditions (i.e., 10 mM H_3PO_4 with gradients in propanol) will clean ether-based HIC columns of contaminants not eluting in the salt gradients (4). As the blank gradient in Figure 7C indicated no further material eluting under HIC conditions, we decided to run the clean-up conditions on this column. Figure 8A shows that the use of an isocratic wash with 10 mM H_3PO_4 (pH 2.2) can remove strongly retained material. Several five minute gradients to 80% 2-propanol in 10 mM H_3PO_4 (Figure 8B) will also remove strongly bound material. This column had been used extensively prior to the STI purification and no attempt was made to identify the eluted material. The prolonged use of these washing conditions has not been observed to degrade the column performance for protein HIC. These mobile phases therefore provide rapid clean-up of the preparative HIC columns in a pH region (i.e., pH 2.2) which is compatible with the use of silica-based packings.

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Analytical Monitoring of Preparative Separation

Having previously established the retention time of the desired protein, it is particularly important during the course of the scaled-up preparative HIC separation to know when to collect the purified component of interest (i.e., start and stop of the peak). In this regard, we devised a simple method based on column-switching concepts (18, 26) to analyze via an analytical column the effluent of the preparative column. The LC system employed is diagrammed in Figure 9 and consists of a gradient and an isocratic HIC system interconnected via a switching valve. In this experiment, portions of protein peaks from the preparative chromatogram run on the gradient system could be switched onto the isocratic system for analysis.

A short (2 cm) analytical column provides an acceptable protein separation under gradient conditions as indicated in Figure 10A and thus, isocratic elution conditions may be arranged

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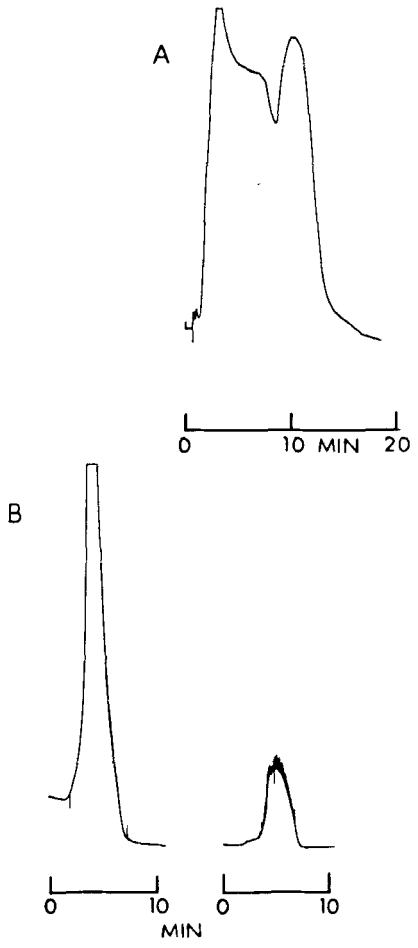

FIGURE 8 Column clean-up of the 15-20 um ether preparative (21.2 mm ID x 15 cm) column. A. An isocratic mobile phase of 10 mM H_3PO_4 in water was used with detection at 280 nm at 0.1 a.u.f.s. B. Two successive 5 min. gradients from 10 mM H_3PO_4 to 80% 2-propanol (total H_3PO_4 concentration = 10 mM) with detection at 280 nm, 0.1 a.u.f.s.

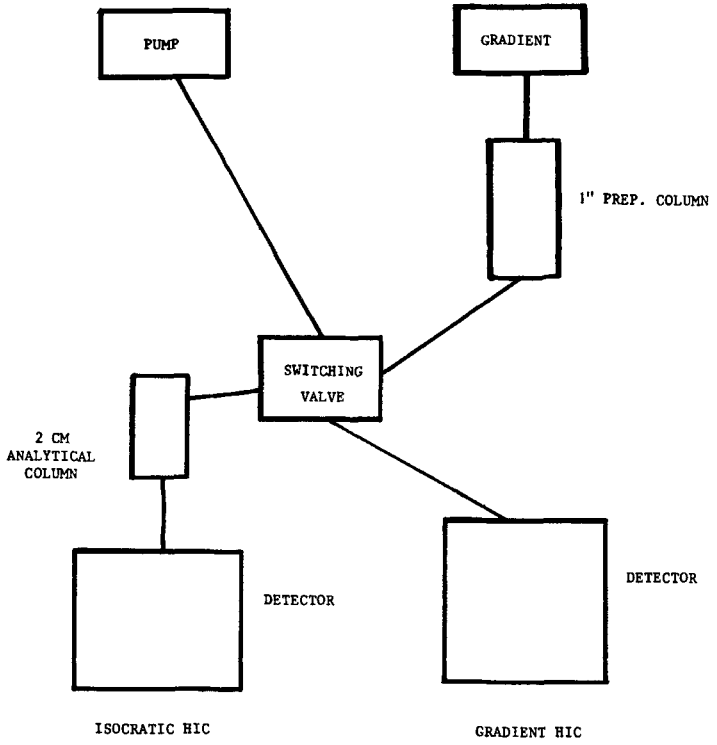

FIGURE 9 Schematic diagram of the experimental set-up for the analytical monitoring of preparative HIC separation. See text for explanation.

so as to provide resolution of neighboring peaks (see Figure 10B). LYS and RNase are separated with k' values of 8.1 and 0.7, respectively. Figure 10D shows a chromatogram of isocratic protein separation of fractions switched from the less than optimal gradient separation indicated in Figure 10C. The time axis in Figure 10D is from the start of the gradient on the preparative column, and the switching times are indicated by "I" and "II" in Figures 10 C and D (note the baseline disturbances). While it is evident that I is a pure RNase peak, fraction II contains a significant amount of LYS as well as RNase. By means of the appropriate automation, holding coils and timing, this

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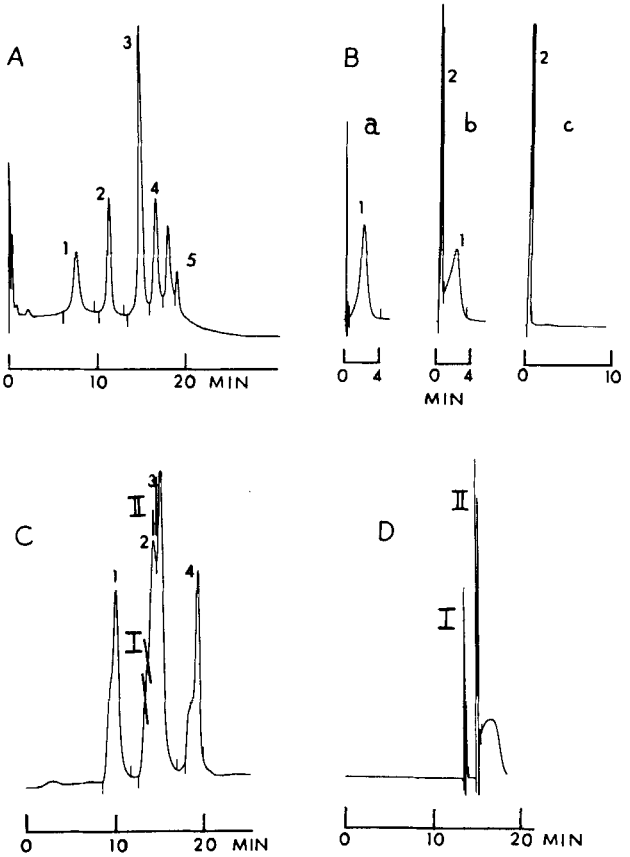

FIGURE 10 Analytical monitoring of preparative HIC separation. A. Standard protein separation on 5 um ether phase in 4.6 mm ID x 2 cm column. All conditions and proteins as in Figure 1A. B. Isocratic separation of RNase and LYS on 5 um 4.6 mm ID x 2 cm column. Mobile phase: 1.65 M ammonium sulfate, 0.5 M ammonium acetate, pH 6.0 with detection at 280 nm, 0.1 a.u.f.s. Injections of a. LYS (1), b. LYS (1) and RNase (2), c. RNase (2). C. 3.9 mg of standard protein mixture injected on 15-20 um 21.2 mm ID x 15 cm column with 20 minute gradient time used. All other conditions as in Figure 6. Fractions of peaks are switched to the short 2 cm column as indicated by "I" and "II" on the chromatogram. D. Peak fractions "I" and "II" from the preparative column. Run was started at the same time as in Figure 10C. Chromatographic conditions as in Figure 10B. Breaks in the baseline indicated the switching of the fractions.

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analytical monitor could be used to control fraction collection (i.e., signal the start and end of collection of a desired peak). The length of the short analytical column could be adjusted so as to provide the fastest analysis, i.e., more complex (more peaks) samples to be purified on the gradient side require greater column length on the isocratic side.

Alternative analytical detectors (e.g., fluorescence, UV at a second wavelength) could provide sensitive monitoring of fractions to supplement the information gained on the less sensitive preparative detector. In fact, sufficient studies could even remove the need for a preparative detector in the gradient HIC separation. Finally, an analytical gradient LC could be programmed to step to any of the desired isocratic solvent compositions so as to analyze the protein peaks of interest. In addition, sets of preparative and analytical LC columns, e.g., RPLC, IEC, HIC, could be linked via column switching so as to provide purification with analysis of collected fractions. Mobile phase composition may be matched to minimize the impact of ionic strength, pH, etc. on the succeeding separation steps (26). Several workers have already published results on coupled column approaches for multi-profiling of biological samples (27). Further development of these ideas is under study in our laboratory.

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CONCLUSIONS

In this paper, we have addressed several aspects of preparative HIC of proteins employing bonded ether silica-based columns. The bonded phase is reproducibly prepared in a stable manner on 15-20 micron wide-pore silica gel. These large particle supports may be packed in narrow or wide bore columns for characterized HIC separation of proteins. The preparative columns provide similar retention for protein as that obtained on 5 micron analytical columns for the same gradient conditions and linear velocity. Thus, separation can be easily transferred and directly scaled-up from the analytical column to the more expensive

preparative columns. Furthermore, the preparative columns possess high loading capacity for protein (>15 mg/ml) when used with the proper sample introduction techniques. We have used the purification of soybean trypsin inhibitor as an illustrative application of the bonded ether column (analytical and preparative) approach to preparative HIC. By means of dilute H_3PO_4 and 2-propanol washes, column clean-up after separation may be achieved in a rapid and safe manner for these silica-based columns. Finally, preliminary results are presented on a column switching procedure for the analytical monitoring of the preparative separation. Further work in this area as well as in the synthesis and use of bonded ion-exchange ether based protein separation columns will be subsequently reported.

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REFERENCES

1. El Rassi, Z. and Horvath, Cs., J. Chromatogr., 359 (1986) 241.

2. Kennedy, L.A., Kopaciewicz, W. and Regnier, F.E., J. Chromatogr., 359 (1986) 73.

3. Jorgenson, J.W., Anal. Chem., 58 (1986) 743A.

4. Miller, N.T., Feibush, B. and Karger, B.L., J. Chromatogr., 316 (1985) 519.

5. Kato, Y., Kitamura, T. and Hashimoto, T., J. Chromatogr., 298 (1984) 407.

6. Fausnaugh, J.L., Pfannkoch, E., Gupta, S. and Regnier, F.E., Anal. Biochem., 137 (1984) 464.

7. Gooding, D.L., Schmuck, M.N. and Gooding K.M., J. Chromatogr., 296 (1984) 107.

8. Goheen, S.C. and Matson, R.S., J. Chromatogr., 326 (1985) 235.

9. Chang, J.E., El Rassi, Z. and Horvath, Cs., J. Chromatogr., 319 (1985) 396.

10. Alpert, A.J., J. Chromatogr., 359 (1986) 85.

11. Miller, N.T., Feibush, B., Corina, K., Powers-Lee, S. and Karger, B.L., Anal. Biochem., 148 (1985) 510.

12. Kato, Y., Kitamura, T. and Hashimoto, T., J. Chromatogr., 333 (1985) 202.

13. Gooding, D.L., Schmuck, M.N., Nowlan, M.P. and Gooding, K.M., J. Chromatogr., 359 (1986) 331.

14. Miller, N.T. and Karger, B.L., J. Chromatogr., 326 (1985) 45.

15. Cohen, S.A., Anderson, D.B., Thimot, N.Z., Tarvin, T.L., and Bidlingmeyer, B.A., Lecture No. 4.3, Ninth International Symposium on Column Liquid Chromatography, Edinburgh, UK, 1985.

16. Miller, N.T. and Shieh, C.H., unpublished results.

17. Larmann, J.P., DeStefano, J.J., Goldberg, A.P., Stout, R.W., Snyder, L.R. and Stadalius, M.A., J. Chromatogr., 255 (1983) 163.

18. Snyder, L.R. and Kirkland, J.J., Introduction to Modern Liquid Chromatography - Second Edition, Wiley, New York, 1979, p. 616.

19. Verzele, M. and Geeraert, J. Chromatogr. Sci., 18 (1980) 559.

20. a. Verzele, M. and Dewaele, C., LC, Liq. Chromatogr., HPLC Mag. 3 (1985) 22.
    b. Sitrin, R., DePhillips, P., Dingerdissen, J., Erhard, K. and Filan, J., LC, Liq. Chromatogr., HPLC Mag. 4 (1986) 530.

21. Schmuck, M.N., Gooding, K.M. and Gooding, D.L., J. Liq. Chromatogr., 7 (1984) 2863.

22. Schmuck, M.N., Gooding, K.M. and Gooding, D.L., LC, Liq. Chromatogr., HPLC Mag., 3 (1985) 814.

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23. Fausnaugh, J.L., Kennedy, L.A. and Regnier, F.E., J. Chromatogr., 317 (1984) 141.

24. Cohen, S.A., Dong, S., Benedek, K. and Karger, B.L., Symposium Proceedings, Fifth International Symposium on Affinity Chromatography and Biological Recognition, Chaiken, I., Wilchek, M. and Parikh, S., eds., Academic Press, New York, 1983, p. 479.

25. Wehrli, A., Hildenbrand, J.C., Keller, H.P., Stampfli, R. and Frei, R.W., J. Chromatogr., 149 (1978) 199.

26. Tapuhi, Y., Miller, N.T. and Karger, B.L., J. Chromatogr., 205 (1981) 325.

27. Kopaciewicz, W. and Regnier, F.E., Anal. Biochem., 129 (1983) 472.

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