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WIDE-PORE SILICA-BASED ETHER-BONDED PHASES FOR SEPARATION OF PROTEINS BY HIGH-PERFORMANCE HYDROPHOBIC-INTERACTION AND SIZE-EXCLUSION CHROMATOGRAPHY

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SUMMARY

This paper examines the use of wide-pore silica-based hydrophilic ether-bonded phases for the chromatographic separation of proteins under mild elution conditions. In particular, ether phases of the following structure $\equiv Si-(CH_2)_3-O-(CH_2-CH_2-O)_n-R$, where n = 1, 2, 3 and R = methyl, ethyl or n-butyl, have been prepared. These phases can be employed either in high-performance hydrophobic-interaction or size-exclusion chromatography, depending on mobile phase conditions. In the hydrophobic-interaction mode, a gradient of decreasing salt concentration, *e.g.*, from 3 *M* ammonium sulfate (pH 6.0, 25°C), yields sharp peaks with high mass recovery of active proteins. In this mode, retention can be controlled by salt type and concentration, as well as by column temperature. In the size-exclusion mode, use of medium ionic strength, *e.g.*, 0.5 *M* ammonium acetate (pH 6.0) yields linear calibration of log (MW[η]) *vs.* retention volume. Even at 0.05 *M* salt concentration, no stationary phase charge effects on protein elution are observed. These bonded-phase columns exhibit good column-to-column reproducibility and constant retention for at least five months of continual use. Examples of the high-performance separation of proteins in both modes are illustrated.

INTRODUCTION

The application of high-performance liquid chromatography (HPLC) to the separation of biopolymers is an area under active development[1]. Much of this work has involved the chemical modification of small-particle (*e.g.*, 5–10 μm), wide-pore (*e.g.*, $\geqslant$ 300 Å) silica gels. The use of this methodology in size-exclusion chromatography (SEC) has been extensively reported[2]. A recent trend has been the transfer of other modes of macromolecule separation from agarose columns to the high-performance bonded phases, *e.g.*, affinity chromatography[3].

Since the initial work of Er-el *et al.*[4] hydrophobic-interaction chromatography (HIC) has been an important separation mode with agarose gels[5–8]. In this method, hydrophobic ligands, such as n-alkyl groups, are chemically attached to the agarose matrix, and separation is based on differences in surface hydrophobicity of proteins[9].

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Researchers have additionally found that the HIC separation of biopolymers can be achieved on unsubstituted agarose by adsorption at high ionic strength, *e.g.*, 2–3 *M* salt, followed by elution with decreasing salt concentration[10,11]. With weakly hydrophobic silica-based columns for SEC, hydrophobic retention has also been observed under certain conditions[12–16]. However, in these experiments, this retention process was viewed as deleterious to pure SEC elution. More recently, HIC as a separation mode has been accomplished with silica-based packings[17–19] and on a gel material[20].

A current popular HPLC mode of separation of proteins is reversed-phase liquid chromatography (RPLC) with *n*-alkyl-bonded ligands[21]. However, the strongly hydrophobic surface can result in denaturation of proteins upon adsorption[22], and harsh mobile phase conditions (*e.g.*, low pH, low ionic strength and organic eluents) are required for elution. It is to be noted that the use of weakly hydrophobic phases in HIC provides a milder adsorptive surface, leading to elution of proteins in an active state. It should be recognized that both HIC and RPLC involve hydrophobic retention[9]. However, it seems appropriate to use HIC a a descriptive name for the methodology in this paper, as the term is widely adopted in the separation of proteins with alkylated agarose gels and to emphasize the mild nature of the process.

In this paper, we present results obtained with synthesized uncharged hydrophilic polyether-bonded phases. Using gradient elution conditions of salt concentrations decreasing from 3 *M* ammonium sulfate, rapid separations of proteins with high recovery under mild elution conditions are achieved. These stationary phases are shown to be reproducible and stable over at least several months of continual use (over 1000 sample injections). Moreover, the bonded-ether linkages are found to be non-interacting under moderate ionic strength conditions (*e.g.*, 0.5 *M* ammonium acetate) and thus usable for SEC. These stationary phases therefore offer the possibility of protein separation by several different modes, depending on mobile phase conditions. It is to be noted that other workers have used polyether phases in the past[23–25], which however, proved to be too hydrophobic.

EXPERIMENTAL

Equipment

The gradient liquid chromatograph was composed of two Model M6000A solvent delivery pumps controlled by an M660 solvent programmer (Waters Assoc., Milford, MA, U.S.A.), a 7125 injection valve (Rheodyne, Cotati, CA, U.S.A.), a Model SF770 variable-wavelength UV detector (set at 280 nm) (Kratos, Westwood, NJ, U.S.A.) and a Model 355 recorder (Linear Instruments, Reno, NV, U.S.A.). A gradient delay volume of 5.68 ml was measured and subtracted from all chromatographic data presented. In order to exclude oxygen and thus prevent oxidation of the bonded-ether ligand, stainless-steel tubing was used throughout, and the mobile phase was continuously sparged with helium. Both systems also included a Model NBE water-bath (Haake-Büchler, Saddle Brook, NJ, U.S.A.).

Chemicals and materials

Diethylene glycol monomethyl ether (purum $\geq$ 98% by gas chromatography (GC)), 2-methoxyethanol (puriss, p.a. >99.5% (GC)), diethylene glycol monoethyl

ether (purum ⩾ 98% (GC)), diethylene glycol monobutyl ether (purum ⩾ 97% (GC)) and soybean trypsin inhibitor were obtained from Fluka (Hauppauge, NY, U.S.A.). Triethylene glycol monomethyl ether (as Poly-Solv™) was from Olin (Stamford, CT, U.S.A.). Reagent-grade sodium spheres were purchased from MCB (Norwood, OH, U.S.A.). Allyl bromide and various high-quality protein standards, as well as ammonium acetate and Grade III ammonium sulfate were obtained from Sigma (St. Louis, MO, U.S.A.) and used as received. Polystyrene molecular weight standards were obtained from Polysciences (Warrington, PA, U.S.A.). Reagent-grade sodium sulfate, HPLC-grade water and organic solvents were purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.). Hexachloroplatinic acid was from Aldrich (Milwaukee, WI, U.S.A.) and triethoxysilane from Petrarch Systems (Bristol, PA, U.S.A.).

Column hardware (blanks, fittings, etc.) was obtained from Supelco (Bellefonte, PA, U.S.A.). Silica gel (Vydac, 7 μm particle diameter, 300 Å nominal pore size, 78 m^2/g surface area (data obtained from the manufacturer)) was a gift of the Separations Group (Hesperia, CA, U.S.A.).

Synthesis

The synthesis of the appropriate silane consisted of a two-step procedure: conversion of the ether alcohol to the allyl ether by the Williamson method[26], followed by hydrosilylation to the triethoxysilane, by an adaptation of the method of Speier *et al.*[27] (see ref. 26). Purification of the allyl ethers and silanes was accomplished by silica gel column chromatography and/or fractional distillation. Purity was evaluated by gas or thin-layer chromatography. In all cases, the substances were shown to be chromatographically pure.

Table I lists the different ether columns used in this study. Bonding of the trialkoxysilanes to silica was accomplished in the presence of water (patent pending). There is a systematic increase in hydrophobicity from a tetraether (phase I) to a triether (phase II) to a diether (phase III), and variation in the alkyl substitution R (phase IV and V). In addition, four batches of phase II have been synthesized for assessment of reproducibility (see Table V). Elemental analysis for surface coverage was performed by MultiChem Labs. (Lowell, MA, U.S.A.). The precision of percent

TABLE I

CHARACTERISTICS OF ETHER-BONDED PHASES

≡Si-$(CH_2)_3$-O-$(CH_2$-CH_2-$O)_n$-R.

Phase	*R*	*n*	*% C**	*Coverage (μmol/m²)***
I	CH_3	3	4.62	4.5
II	CH_3	2	4.05	4.7
III	CH_3	1	4.46	6.5
IV	C_2H_5	2	4.32	4.6
V	*n*-C_4H_9	2	5.89	5.4

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

** Coverage is calculated assuming an average reaction of two ethoxy groups per silane molecule. See Experimental section.

carbon (% C) data for a given phase was *ca.* 3% relative standard deviation (R.S.D.). A correction is made for 0.62% C found in the blank Vydac silica gel. In the calculation of surface coverage in Table I, we approximate an average reaction of two ethoxy groups per silane molecule. The actual stoichiometry of bonding is under investigation by means of solid-phase ^{29}Si NMR spectra[28] and other methods.

Chromatographic procedures

The phases were packed into 10 cm × 4.6 mm I.D. stainless-steel tubes, following standard slurry procedures, using methanol as the driving solvent with a Model DSTV 122 air-driven pump (Haskel, Burbank, CA, U.S.A.). 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 or the appropriate pH with either glacial acetic acid or ammonium hydroxide, and a small amount of HPLC-grade water (degassed) was added to the mark. Protein solutions (5–10 mg/ml) were freshly made up in water. A pre-column (6 cm × 4.6 mm I.D.) containing the same bonded phase as the analytical column was placed between the pump and injector. Helium sparges were maintained in both the A and B solvents in order to protect the ether column from oxidation. Solutions containing high salt concentrations were not allowed to remain in the column or pumps for long periods of time. Care should be taken to maintain miscibility in all proportions, especially if organic solvents are used.

Mass and activity recovery

Lysozyme (Sigma Grade I from egg white, 3 × crystallized, dialyzed and lyophilized to powder) standards were prepared as serial dilutions in the mobile phase composition at which lysozyme is eluted from ether phase II (*i.e.*, 0.67 *M* ammonium sulfate, 0.5 *M* ammonium acetate, pH 6.0) from a 1.03 mg/ml standard (also made up in the salt solution). All solutions were made up on the day of use and kept in ice between manipulations. Using the above standards, a calibration curve was established as a plot of lysozyme concentration in μg/ml *vs.* absorbance at 280 nm on a Spectronic 710 (Bausch and Lomb, Rochester, NY, U.S.A.) spectrophotometer.

For the determination of mass recovery, 150 μl of the 1.03 mg/ml standard was chromatographed on ether phase II. The appropriate peak fraction was collected in a 10.0-ml volumetric flask and made to the mark with the salt solution. The absorbance at 280 nm (quartz cell, 1.0 cm) was measured, and the protein concentration determined from the calibration curve.

The enzymatic assay procedure for lysozyme was that of Worthington Biochemicals (Freehold, NJ, U.S.A.). An aliquot from the 1.03 mg/ml standard was diluted to *ca.* 15 μg/ml immediately prior to the assay. A 0.2-ml aliquot of this diluted solution was assayed for activity by measurement of the rate of lysis of *Micrococcus lysodeikticus* cells, as observed by a decrease in turbidity with time at 450 nm. The fractions obtained from ether phase II under HIC conditions were then surveyed for activity in a like manner. The specific activity for collected fractions was determined by dividing the activity by the mass of the fraction, measured by UV absorption, as above.

RESULTS AND DISCUSSION

In this paper, we describe the successful separation of proteins by HIC and SEC on new hydrophilic stationary phases, produced by the reaction of silica with non-ionic, neutral polyether–triethoxysilanes. As we have noted previously[26], we prefer to synthesize the ligand prior to bonding to silica in order to achieve reproducibility and control of the bonded phase itself. This practice has been followed in this work as well. We have selected a trifunctional (*i.e.*, triethoxy) silane for bonding, as it is known that the stationary phases produced are more stable toward aqueous solutions than the corresponding difunctional or monofunctional silanes[29]. We will first examine the use of these phases in HIC and then turn to SEC.

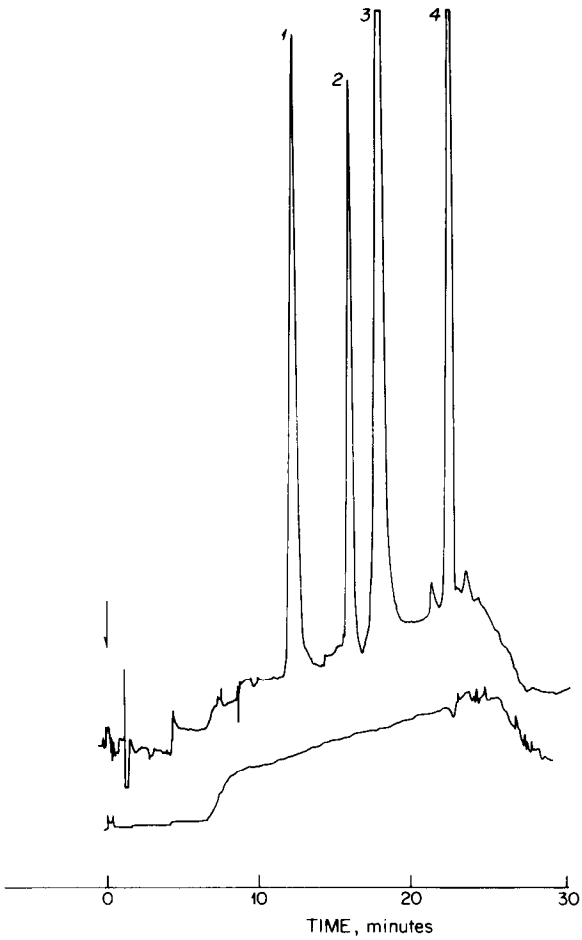

Fig. 1. Separation of standard protein mixture on ether phase II. Standard proteins (1 = cytochrome *c*; 2 = ribonuclease A; 3 = ovalbumin; 4 = α-chymotrypsinogen) were chromatographed on a 100 × 4.6 mm ether phase II column at a flow-rate of 1.0 ml/min using a 20-min linear gradient at 25.0°C from 3.0 *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., injection volume, 5 μl; protein amounts, cytochrome *c* and α-chymotrypsinogen, each 25 μg; ribonuclease A, 75 μg; ovalbumin, 100 μg.

Hydrophobic-interaction chromatography

Chromatographic characteristics. The results in this section will be based on the use of only ether phase II. Other phases will be described separately. Fig. 1 presents the high-performance separation of four standard proteins on Phase II at 25°C by the use of a 20-min salt gradient descending from 3.0 *M* ammonium sulfate with 0.5 *M* ammonium acetate (pH 6) to 0.5 *M* ammonium acetate (pH 6). A higher resolution, at the expense of time, can be obtained using a 60-min gradient (Fig. 2). Note in this figure that the chromatographic bands remain sharp, even though the average capacity factor (k') for each protein is larger by a factor of 3, relative to Fig. 1. The protein elution order is similar to that reported by others for HIC[17,20], using phenyl- and butyl-substituted phases.

A large number of proteins with a wide range of molecular weights (MW) and isoelectric points (p*I*) have been successfully eluted from this column by using the above gradient. Table II lists many of these proteins, including, where available, MW and p*I*. Successful elution of proteins has also been observed at pH 7 or 8. Moreover, we have not encountered any problems of salt precipitation, salt-related pump failure or on-column protein precipitation when using the above gradient conditions.

Many proteins are eluted as sharp bands, but a few give multiple or broadened peaks, presumably due to impurities, multiple forms of the protein, autoproteolysis, or some other factors. For example, Fig. 3 shows the separation of transferrin and soybean trypsin inhibitor (STI). It is interesting to observe the appearance of two peaks for STI, in agreement with the RPLC of this protein where two active forms have been found[30]. Fig. 4 shows the HIC of trypsin, a proteolytic enzyme, freshly made up (Fig. 4a) and after standing in sterile solution for one week (Fig. 4b). The resolving power of HIC is illustrated in these chromatograms. Finally, Fig. 5 shows the change in chromatographic peak pattern for ovalbumin upon standing in sterile solution. For a freshly made up sample a single peak is observed (Fig. 5A). After standing for two days, it shows an extra peak (Fig. 5B), and after two weeks, this peak grows to be larger than the original peak (Fig. 5C). Ovalbumin is known to

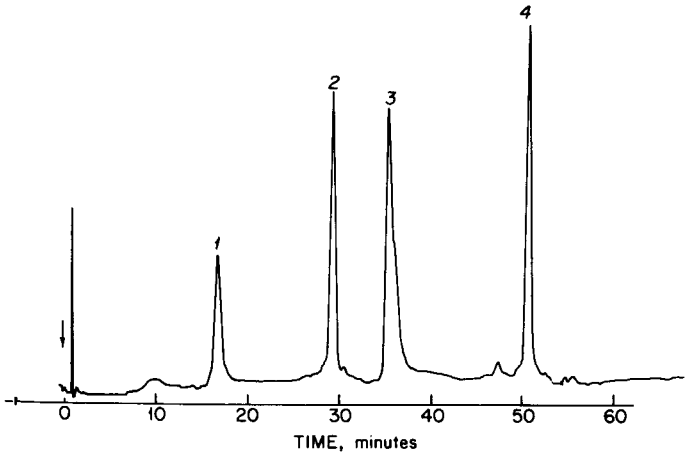

Fig. 2. Separation of standard protein mixture on ether phase II. All conditions as in Fig. 1, except that a 60-min linear gradient was employed.

TABLE II

HIC RETENTION OF PROTEINS ON ETHER-BONDED PHASE II

Mobile phase: A = 3.0 *M* ammonium sulfate, 0.5 *M* ammonium acetate, pH 6.0; B = 0.5 *M* ammonium acetate, pH 6.0; linear gradient from 0 to 100%B in 20 min; flow-rate 1 ml/min; detection at 280 nm, 0.1 a.u.f.s.; temperature 25°C. Proteins were made up individually 10–20 mg/ml in water. Injection volumes: 10–20 μl.

Protein	*MW* × 10^{-3}*	*pI**	V_g *(ml)***
Invertase (yeast)		2.7–3.7	3.4
Cytochrome *c* (equine)	12.2	10.6	5.0
Avidin (egg white)	68.0		6.5
Ribonuclease A (bovine)	13.7	9.4	8.6
β-Lactoglobulin (bovine)	35.0	5.1	9.8
Blue dextran	2000.0	–	10.0
Ovalbumin (egg)	44.0	4.7	10.2
Serum albumin (bovine)	68.0	4.4–4.8	10.3
Hexokinase (yeast)	100.0	5.9	10.7
Alkaline phosphatase (calf)	80.0	4–6	10.7
Carbonic anhydrase (bovine)	29.0	8.4–9.5	10.7
Albumin V (human)	~65.0	4.4–4.8	10.7
Albumin V, fatty acid free (human)	~65.0	4.4–4.8	10.9
Transferrin (human)	77.0	5–6	11.4
Ornithine transcarbamylase (rat)	108.0	~8	11.4
Deoxyribonuclease II (bovine)	53.0	10.3	11.6
Albumin V (pigeon)	~65.0	4.4–4.8	11.6
Lysozyme (egg)	14.0	11.0	11.8
Globulins IV-4 (human)	~160.0	–	11.8
Albumin V (chicken)	~65.0	4.4–4.8	11.8
Creatine phosphokinase (rabbit)	80.0	7	11.9
Trypsin (bovine)	23.0	10.8	12.1
Carbamyl phosphate synthetase (rat)	165.0	6.05	12.1
Trypsinogen (bovine)	24.0	9.3	12.3
Transferrin (bovine)	77.0	5.0	12.5
Myoglobin (equine)	17.5	7.1	12.7
Globulins IV (human)	~160.0	–	12.7
Trypsin inhibitor (soybean)	21.5	–	13.3
γ-Globulins II (bovine)	~160.0	–	13.4
Pepsin (hog)	34.0	< 1.0	13.9
Catalase (beef)	250.0	5.4	14.0
α-Chymotrypsinogen-A (bovine)	25.5	9.5	14.4
Hemoglobin (bovine)	64.5	6.8	14.7
γ-Globulins II (human)	160.0	–	17.4
Papain	21.0	9.6	19.1

* From refs. 16 and 47.

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

aggregate slowly in solution[31], and it is possible that the extra peak represents this aggregate.

As found by others[2], we have noted a "conditioning" period for ether-bonded phases, in which the protein peak height grows to a constant value with injection of protein solution on freshly made columns. The extent of conditioning appears to be correlated with the hydrophobicity of the bonded phase, *i.e.*, increasing in the order of phases I → IV. In all cases, fewer than five injections of 5-μl volumes of protein

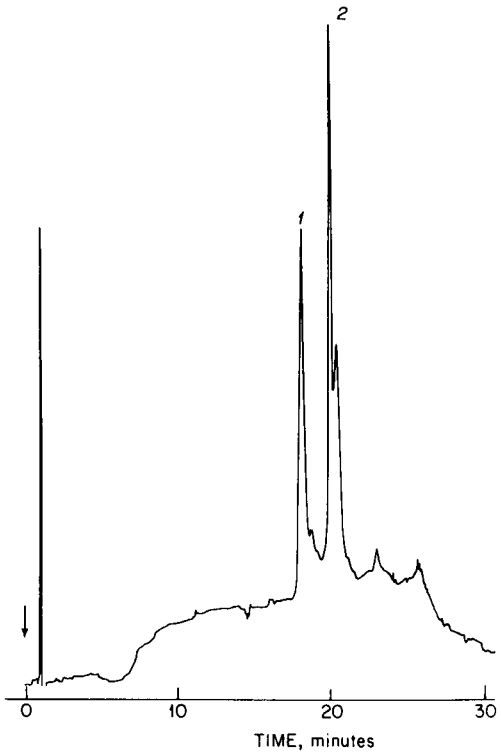

Fig. 3. Chromatography of transferrin (1) and soybean trypsin inhibitor (2) on ether phase II. All chromatographic conditions are as in Fig. 1. Injection volume, 5 μl; protein amounts, each 25 μg.

(*ca.* 5 mg/ml) are required to reach constant peak height. Upon conditioning, each column gives high and constant recovery of protein (see later).

Elution is found to follow the expected trends for HIC, as the salt and column temperature are changed (Tables III and IV). Using the same linear gradient from 2 *M* salt concentration, different salts produce retention on the basis of their lyotropic ability, according to the Hofmeister series[32]. Thus, retention is greatest for sodium sulfate, followed by ammonium sulfate and no retention with ammonium acetate (Table III). This behavior is also observed for HIC on agarose stationary phases[33]. Secondly, also in agreement with results on agarose[34], retention of proteins is found to increase with increase in column temperature (Table IV). This result can be contrasted with that found in RPLC, where higher temperatures result in shorter retention[35].

Mass and activity recovery. All experiments have revealed high mass recovery for the proteins tested. For example, a mass recovery greater than 90% was obtained for a 150 μg sample of lysozyme on the ether phase II column under HIC conditions. Secondly, calibration plots of peak area *vs.* sample amount for the four proteins, separated as in Fig. 1, had a zero intercept. Similar high mass recoveries were found on the other phases, except phase V. In contrast to RPLC, ghost peaks (from protein remaining on the column) have never been observed with blank gradient runs following the sample run.

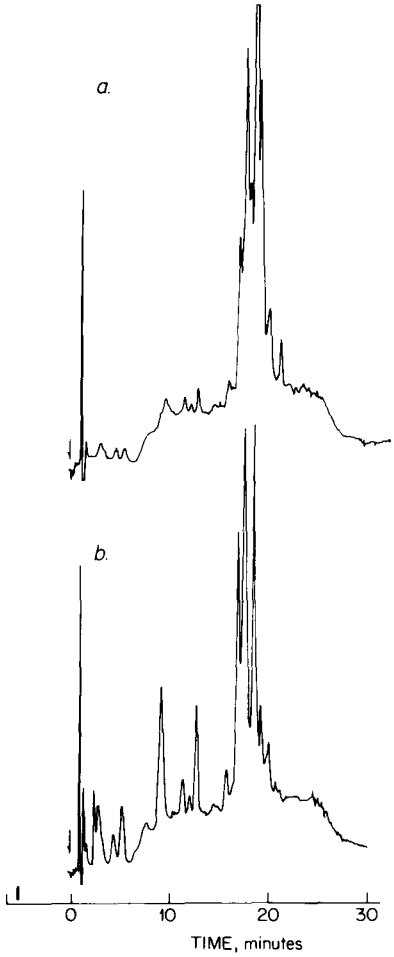

Fig. 4. Chromatography of trypsin and proteolytic products on ether phase II. All chromatographic conditions are as in Fig. 1: (a) Freshly prepared trypsin sample; injection volume, 5 μl; protein amount, 50 μg. (b) Sample of a, aged 1 week; injection volume, 5 μl.

The biological activity of a collected fraction of lysozyme was determined as described in the experiment section. In two separate runs, the specific activity of the chromatographic fraction was 170% over that of the original sample. In another example, collected fractions of papain and STI were found to be active, based on their RPLC chromatographic behavior[22,30]. Finally, HIC on phase II of fragile rat liver enzymes, such as carbamyl phosphate synthetase, resulted in an increase in specific activity of these enzymes[36]. Thus, in agreement with others[17,18,20], HIC yields, for the examples studied, proteins in an active state.

Column reproducibility and stability. Table V presents data for column-to-column reproducibility for ether phase II. 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 2.5% R.S.D. When compared to the average precision of carbon analysis for one phase (*ca.* 3% R.S.D.), it is seen that the phases are

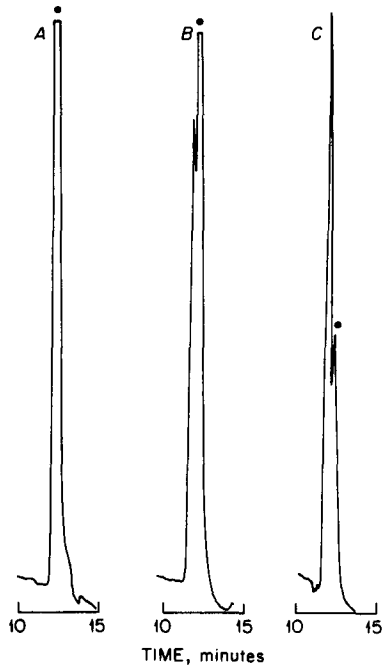

Fig. 5. Aging of ovalbumin as monitored by chromatography on ether phase II. All chromatographic conditions as in Fig. 1. (A) Freshly prepared sample of ovalbumin; injection volume, 5 μl; protein amount, 100 μg; (B) same solution injected after two days; (C) same sample injected after two weeks. The symbol ● indicates the peak declining in height with age of sample.

identical in terms of %C. The precision in chromatographic behavior under HIC conditions for proteins is also excellent. Retention is found to agree from column to column within *ca.* 5%. In addition, peak shape was found to be similar on all four columns.

Concern has been voiced in the literature on the hydrolytic stability of hydrophilic chemically bonded phases[2,3,15,29]. Accordingly, we carefully tested the reten-

TABLE III

EFFECT OF SALT TYPE ON HIC OF PROTEINS

Conditions: column: phase II; mobile phase; A = 2.0 *M* indicated salt, 0.5 *M* ammonium acetate, pH 6.0; B = 0.5 *M* ammonium acetate, pH 6.0; linear gradient from 0 to 100% B in 20 min; flow-rate 1.0 ml/min; temperature 25°C.

Gradient salt	V_g *(ml)***			
	*CYT**	*RNase**	*OVA**	*CHTG**
Ammonium acetate	0	0	0	0
Ammonium sulfate	0	1.8	5.4	12.5
Sodium sulfate	2.8	7.3	8.4	14.9

* The following abbreviations are used throughout this paper: CYT = cytochrome *c*; RNase = ribonuclease A; OVA = ovalbumin; CHTG = α-chymotrypsinogen.

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

TABLE IV

EFFECT OF TEMPERATURE ON HIC OF PROTEINS

Conditions: column: phase II; mobile phase: A = 2.0 *M* ammonium sulfate, 0.5 *M* ammonium acetate, pH 6.0; B = 0.5 *M* ammonium acetate, pH 6.0; linear gradient from 0 to 100% B in 20 min; flow-rate 1.0 ml/min.

Temperature (°C)	V_g *(ml)**			
	CYT	*RNase*	*OVA*	*CHTG*
10	0	0	3.8	10.6
25	0	1.8	5.4	12.5
40	0	3.1	6.3	14.0

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

TABLE V

COLUMN REPRODUCIBILITY OF PHASE II

Conditions as in Table II

*Phase**	*%C*	V_g *(ml)***			
		CYT	*RNase*	*OVA*	*CHTG*
II	4.05	5.0	8.6	10.2	14.4
IIA	4.02	4.7	8.4	9.6	14.2
IIB	3.83	4.4	8.2	9.3	14.1
IIC	3.99	4.6	8.5	9.6	14.4
Mean	3.97	4.7	8.4	9.7	14.3
R.S.D. (%)	2.5	5.3	2.0	3.9	1.1

* The four bonded phases were prepared separately using the same batch of silane and of silica.
** V_g = gradient elution volume corrected for gradient delay volume.

TABLE VI

STABILITY OF PHASE II FOR PROTEIN RETENTION

Conditions as in Table II.

Volume of mobile phase passed (l)	V_g *(ml)**		
	RNase	*OVA*	*CHTG*
0	8.5	9.8	14.4
10	8.8	10.5	14.8
30	8.5	10.1	14.1
50	8.8	11.0	14.7
Mean	8.7	10.4	14.5
R.S.D. (%)	2.0	5.0	2.2

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

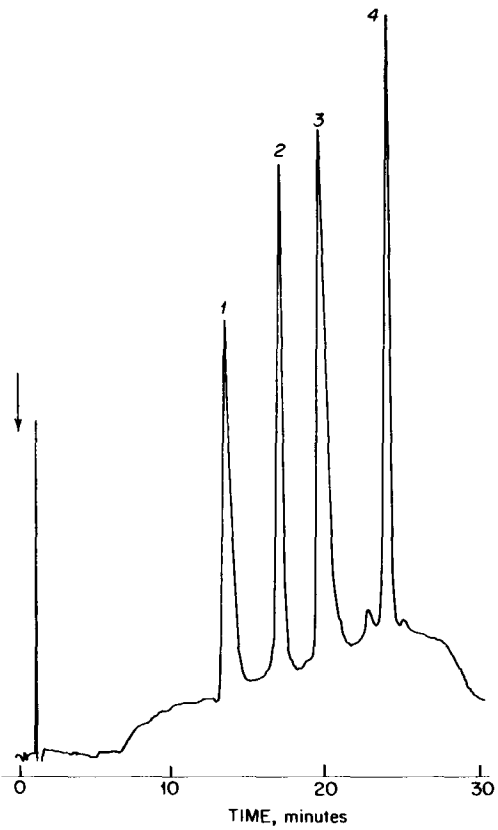

Fig. 6. Separation of standard protein mixture on ether phase II after 50 l mobile phase passed. All conditions same as in Fig. 1.

tion constancy of phase II as a function of usage. Table VI shows that retention does not vary by more than 5% with the use of 50 l of mobile phase, which represents a five-month period or over 1000 samples injected. Fig. 6 shows a chromatogram of the same proteins as in Fig. 1 with a fresh column after 50 l of mobile phase. It can be seen that the two chromatograms are similar. During the five-month period of column use, a wide variety of mobile phase and temperature conditions were employed. Periodically, especially after injecting crude samples, including serum, retention of the standard proteins would increase 5–10%. The column was then washed with a gradient of 10 m*M* orthophosphoric acid (pH 2.2) to 45% 1-propanol[35] and material would be eluted from the column. The retention of the standard proteins then returned to the previous values.

Effect of stationary phase. We next compared retention of the four standard proteins on the ether phases listed in Table I, and the results are presented in Table VII. Comparison of retention volumes for phases I–III reveals that V_g increases as the number of ethylene oxide groups decreases. Moreover, with a constant number of ethylene oxide groups, V_g also increases as the alkyl group at the end of the chain is varied from methyl to ethyl, phase II *vs.* IV. Phase IV is clearly more hydrophobic

TABLE VII

COMPARISON OF ETHER PHASES FOR HIC OF PROTEINS

Conditions: mobile phase: A = 3.0 *M* ammonium sulfate, 0.5 *M* ammonium acetate, pH 6.0; B = 0.5 *M* ammonium acetate, pH 6.0; linear gradient from 0 to 100% B in 20 min; flow-rate 1.0 ml/min; detection at 280 nm, 0.1 a.u.f.s.; temperature 25°C. *S* = Slope of log *k' versus* concentration fraction of ammonium sulfate.

Phase	*CYT*		RNase		*OVA*		*CHTG*	
	V_g *(ml)***	S	V_g *(ml)*	*S*	V_g *(ml)*	*S*	V_g *(ml)*	*S*
I	3.5	6.1	8.0	4.8	9.3	6.8	13.1	6.4
IIA	5.0	6.2	8.6	5.3	10.2	6.7	14.4	6.5
III	7.1	6.3	9.7	5.7	11.4	7.0	16.3	6.5
IV	9.9	7.4	10.5	4.6	12.4	7.0	17.4	5.4
V	—*	—	—	—	—	—	—	—

* No elution observed.

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

than phase II. Interestingly, selectivity does not appear to vary significantly from column to column.

When the alkyl group is changed to *n*-butyl (phase V), no elution is observed of any of the proteins. Evidently, the four-carbon chain is sufficiently long to create a strong hydrophobic surface for the stationary phase. Interestingly, elution of proteins may be accomplished with lower salt content at pH 6 when a gradient of increasing concentration of diethylene glycol monobutyl ether is applied to the column. Further work in this area is in progress. Finally, this phase acted as an RPLC phase, since use of a low-pH, 1-propanol gradient yielded an elution pattern similar to that with an *n*-butyl phase[35].

The silane of phase V can be used under the mobile phase conditions of Fig. 1, if it is co-bonded with the silane from phase II. With co-bonding of the two silanes, *i.e.*, with R = methyl and *n*-butyl, retention of the four standard proteins increased over that on phase II. Above a nominal bonding mole fraction of 0.20 for the *n*-butylsilane, recovery of ovalbumin (OVA) became problematical. It does appear, however, that ether silane II can be used as a diluent for HIC.

Returning to Table VII, the slopes of log *k' vs.* concentration fraction of ammonium sulfate, the *S* values[37], are reported for all proteins on each phase for which retention was observed. These *S* values were obtained either with the gradient or isocratically, and agreement was good between both approaches. It can be seen that the *S* values range between 5 and 7, resulting in protein *k'* values of roughly 2 when the gradient in Fig. 1 is used. These values can be nominally contrasted with the *S* values of approximately 50 obtained for proteins in RPLC with organic solvent gradients[38]. In the case of RPLC, the large *S* values require precise gradient control for retention reproducibility[39]. However, in HIC, since retention is a less sensitive function of the B solvent, the demands placed on the gradient system for retention reproducibility are less. This represents an advantage of HIC.

Size-exclusion chromatography

In the past, non-ionic weakly hydrophobic phases have been predominantly used for the SEC of biopolymers[2,12,40,41]. Indeed, it has been noted, as mentioned previously, that high salt concentration was to be avoided, since it would cause retention by hydrophobic interaction of the protein with the stationary phase[14,16]. Since the ether phases are weakly hydrophobic, we decided to examine whether these phases could be used for SEC at moderate to low salt concentrations. (It is also to be recalled that no hydrophobic retention of proteins was observed with a 2 *M* ammonium acetate salt concentration, Table III.) Besides the potential practical advantage of being able to operate a column in two separate chromatographic modes, this examination was also important from the point of view of the successful use of HIC. If at moderate to low salt concentrations electrostatic binding of proteins to the bonded phase surface were to occur, then some proteins would not be eluted from the column, since electrostatic binding would be expected to increase with decreasing mobile phase salt concentration. The need to minimize surface charge effects in HIC has been noted with alkylagarose gels as well[42].

A wide variety of proteins were chromatographed on phase II under mobile phase B conditions, *i.e.*, 0.5 *M* ammonium acetate, pH 6. Fig. 7 shows a calibration plot of log MW [η] *vs.* V_R, where MW = molecular weight, [η] = intrinsic viscosi-

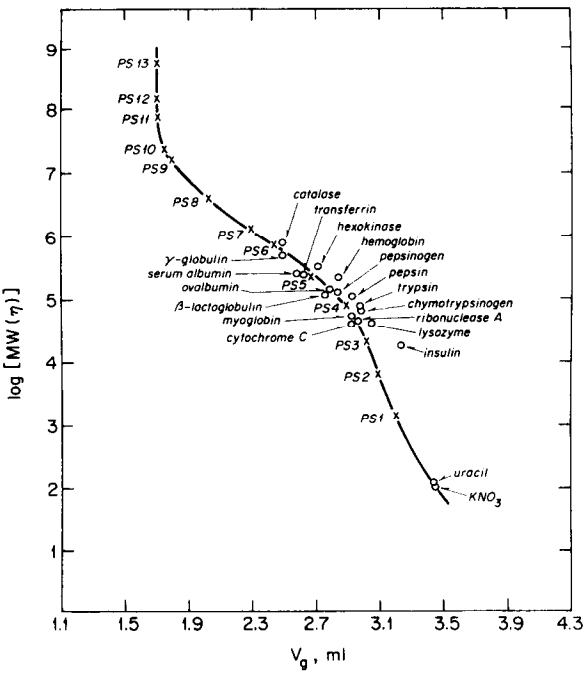

Fig. 7. Universal calibration curve for size-exclusion chromatography of proteins and polystyrene (PS) standards. The indicated proteins were chromatographed on three coupled columns totaling 300 × 4.6 mm of ether phase II at a flow-rate of 0.5 ml/min using a mobile phase of 0.5 *M* ammonium acetate, pH 6.0 at 25°C. Detector, 280 nm, 0.1 a.u.f.s. The indicated polystyrene molecular weight standards were chromatographed on the same set of columns under the same conditions but using a mobile phase of dichloromethane. Detector, 254 nm, 0.1 a.u.f.s.

ty[43,44], and V_R = retention volume. Included in this figure is a series of polystyrene standards, chromatographed with dichloromethane as solvent. As can be seen, a close correspondence exists between the SEC calibration plot for the polystyrenes and the proteins. Indeed, the results obtained are comparable to those on other size-exclusion columns[14,16].

Fig. 8 shows a chromatographic separation on a 30-cm column, in which the 10-cm columns of phases II, IIA and IIB were connected in series. It is worth noting that the silica used in this work was not optimized for SEC, as the pore volume was low (0.66 ml/g). However, the interstitial porosity, ε_0, was found to be 0.35, rather than the generally accepted value of 0.40. This favorable low value has also been observed on other spherical packing materials[45]. As a consequence of the ε_0 value, the ratio of internal porosity, ε_i to ε_0, was roughly 1, a value adequate for SEC[46].

In previous studies, our laboratory[16] and others[14] have shown electrostatic effects to be operative on SEC bonded phases below *ca.* 0.1 *M* salt concentration. In particular, because of the negative charge on the silica surface, retention was found to increase for basic proteins as salt concentration was reduced, whereas for acidic proteins the opposite trend was observed. Table VIII reveals that even at 0.05 *M* ammonium acetate, pH 6, no electrostatic effects were seen at the start of use of a

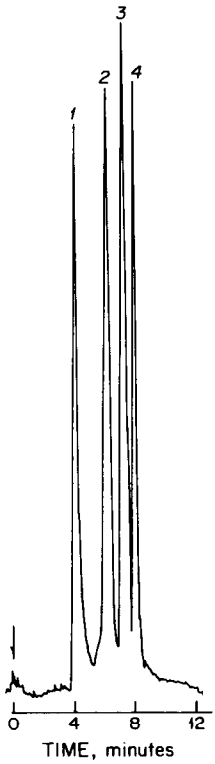

Fig. 8. Size-exclusion chromatography on ether phase II. Solutes (1 = Blue dextran, molecular weight, $2 \cdot 10^6$; 2 = human transferrin, $7.7 \cdot 10^4$; 3 = lysozyme, $1.4 \cdot 10^4$; 4 = uracil, $1 \cdot 10^2$) were chromatographed on three coupled columns of ether phase II totaling 300 × 4.6 mm at a flow-rate of 0.5 ml/min with a mobile phase of 0.5 *M* ammonium acetate, pH 6.0 at 25°C. Detection, 280 nm, 0.1 a.u.f.s.

TABLE VIII

COLUMN STABILITY AS MEASURED BY SEC RETENTION

Column: phase II; temperature, 25°C, V_0 (retention of uracil or KNO_3) = 1.2 ml.

Protein	*V_R (ml)*					
	0.50 M Ammonium acetate, pH 6.0			*0.050 M Ammonium acetate, pH 6.0*		
	0 l	*25 l*	*50 l*	*0 l*	*25 l*	*50 l*
BSA*	0.95	0.96	0.92	0.90	0.93	0.92
RNase	1.05	1.08	1.03	1.05	1.07	1.08
CHTG	1.10	1.12	1.05	1.05	1.09	1.15
LYS	1.10	1.12	1.15	1.07	1.16	2.07

* BSA = Bovine serum albumin.

column of phase II. Thus, silanol accessibility is minimized on the ether phase. As noted above, this is an important characteristic for HIC columns. Comparable results were also observed on phase I, in which three ethylene oxide groups were included in the ligand chain.

Table VIII also includes retention of the proteins after 25 and 50 l of use. These results extend the column stability data presented in Table VI, since the same column was used in both studies. In the case of 0.5 *M* ammonium acetate, little or no change in SEC retention is observed throughout the 50 l of use. On the other hand, for the mobile phase of 0.05 *M* ammonium acetate, the retention of chymotrypsinogen (CHTG) increased by 10% and of lysozyme (LYS) by a factor of 2 after 50 l. Clearly, these very sensitive tests of column stability with basic proteins reveal a slow deterioration of the column after five months of use. However, the change is minor, and the column can still be used for HIC and SEC (*cf.* Figs. 1 and 6).

CONCLUSIONS

The use of ether-based weakly hydrophobic phases for HIC and SEC has been demonstrated; a dual-purpose column has been achieved. The phases are neutral and nonionic and can be systematically varied to control the hydrophobicity of the stationary phase. Based on the SEC results, phases I and II have been shown to be non-interacting at moderate salt concentrations of *ca.* 0.5 *M* ammonium acetate, and thus these ligands can potentially be used as diluents with more hydrophobic ligands, such as phase V, *i.e.*, *n*-butyl at the end of the ether chain. Thus, it is possible to use mixed phases of methyl and *n*-butyl ethers for further control of hydrophobicity.

High mass recovery of proteins in an active state has been achieved for HIC. This is an advantage over the RPLC of proteins where denaturation is quite possible. Thus, given the high performance of these columns, it can be anticipated, in agreement with others[18], that HIC on weakly hydrophobic phases will be more widely used than RPLC when protein isolation is important. Of course, columns that are used in the HIC mode must be stable for long periods of time and must not permit electrostatic interactions with the proteins under the elution conditions. Work continuing in this area will be subsequently reported.

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REFERENCES

1 F. E. Regnier, *Anal. Chem.*, 55 (1983) 1298A.
2 K. K. Unger, *Trends Anal. Chem.*, 2 (1983) 271.
3 P.-O. Larsson, M. Glad, L. Hansson, M.-O. Mansson, S. Ohlson and K. Mosbach, *Advan. Chromatogr.*, 21 (1983) 41.
4 Z. Er-el, Y. Zaidenzaig and S. Shaltiel, *Biochem. Biophys. Res. Commun.*, 49 (1972) 383.
5 S. Hjerten, *Advan. Chromatogr.*, 19 (1981) 111.
6 B. H. J. Hofstee, *Pure Appl. Chem.*, 51 (1979) 1537.
7 J. Porath, L. Sundberg, N. Fornstedt and P. Ohlsson, *Nature (London)*, 245 (1973) 465.
8 G. Halperin, M. Breitenbach, M. Tauber-Finkelstein and S. Shaltiel, *J. Chromatogr.*, 215 (1981) 211.
9 W. Melander and Cs. Horváth, *Arch. Biochem. Biophys.*, 183 (1977) 200.
10 F. von der Haar, in P. V. Sundaram and F. Eckstein (Editors), *Theory and Practice in Affinity Techniques*, Academic Press, New York, 1978, p. 1.
11 R. P. Bywater and N. V. B. Marsden, in E. Heftmann (Editor), *Chromatography —Fundamentals and Applications of Chromatographic and Electrophoretic Methods*, Part A, Elsevier, Amsterdam, 1983, p. A257.
12 E. Pfannkoch, K. C. Lu, F. E. Regnier and H. G. Barth, *J. Chromatogr. Sci.*, 18 (1980) 430.
13 S. H. Chang, K. M. Gooding and F. E. Regnier, *J. Chromatogr.*, 120 (1976) 321.
14 H. Engelhardt and D. Mathes, *Chromatographia*, 14 (1981) 325.
15 H. Engelhardt and D. Mathes, *J. Chromatogr.*, 142 (1977) 311.
16 D. E. Schmidt, R. W. Giese, D. Conron and B. L. Karger, *Anal. Chem.*, 52 (1980) 177.
17 Y. Kato, T. Kitamura and T. Hashimoto, *J. Chromatogr.*, 266 (1983) 49.
18 J. L. Fausnaugh, E. Pfannkoch, S. Gupta and F. E. Regnier, *Anal. Biochem.*, 137 (1984) 464.
19 H. Engelhardt and H. Muller, *Eighth International Symposium on Column Liquid Chromatography, New York, 1984*, abstract No. 4p-B4.
20 Y. Kato, T. Kitamura and T. Hashimoto, *Third International Symposium on HPLC of Proteins, Peptides and Polynucleotides, Monte Carlo, 1983*, abstract No. 106.
21 M. T. W. Hearn, *Advan. Chromatogr.*, 20 (1982) 1.
22 S. A. Cohen, K. P. Benedek, S. Dong, Y. Tapuhi and B. L. Karger, *Anal. Chem.*, 56 (1984) 217.
23 O. Schou and P. Larsen, *Acta Chem. Scand., B*, 35 (1981) 337.
24 R. V. Vivilecchia, B. G. Lightbody, N. Z. Thimot, and H. M. Quinn, *J. Chromatogr. Sci.*, 15 (1977) 424.
25 E. sz. Kovats, German patent number 29-30-516, Dec. 2, 1981.
26 B. Feibush, M. J. Cohen and B. L. Karger, *J. Chromatogr.*, 282 (1983) 3.
27 J. L. Speier, J. A. Webster and G. H. Barnes, *J. Amer. Chem. Soc.*, 79 (1957) 974.
28 D. W. Sindorf and G. E. Maciel, *J. Amer. Chem. Soc.*, 105 (1983) 3767.
29 T. G. Waddell, D. E. Leyden and M. T. DeBello, *J. Amer. Chem. Soc.*, 103 (1981) 5303.
30 S. A. Cohen, S. Dong, K. Benedek and B. L. Karger, in I. Chaiken, M. Wilchek and S. Parikh (Editors), *Symposium Proceedings, Fifth International Symposium on Affinity Chromatography and Biological Recognition*, Academic Press, New York, 1983, p. 479.
31 M. E. Co-Sarno, M. A. Tapang and D. G. Lockhurst, *J. Chromatogr.*, 266 (1983) 105.
32 P. H. von Hippel and T. Schleich, in S. N. Timasheff and G. D. Fasman (Editors), *Structure and Stability of Biological Macromolecules*, Marcel Dekker, New York, 1969, p. 417.
33 S. Pahlman, J. Rosengren and S. Hjerten, *J. Chromatogr.*, 131 (1977) 99.
34 S. Hjerten, J. Rosengren and S. Pahlman, *J. Chromatogr.*, 101 (1974) 281.
35 K. A. Cohen, K. Schellenberg, K. Benedek, B. L. Karger, B. Grego and M. T. W. Hearn, *Anal. Biochem.*, 140 (1984) 223.

36 N. T. Miller, S. Powers-Lee, B. Feibush and B. L. Karger, in preparation.
37 J. P. Larmann, J. J. DeStefano, A. P. Goldberg, R. W. Stout, L. R. Snyder and M. A. Stadalius, *J. Chromatogr.*, 255 (1983) 163.
38 M. A. Stadalius, H. S. Gold and L. R. Snyder, *J. Chromatogr.*, 296 (1984) 31.
39 J. M. DiBussolo, J. R. Gant and C. E. Schmid, *Eighth International Symposium on Column Liquid Chromatography, New York, 1984*, abstract No. 4p-B1.
40 H. Engelhardt and D. Mathes, *J. Chromatogr.*, 185 (1979) 305.
41 K. Fukano, K. Komiya, H. Sasaki and T. Hashimoto, *J. Chromatogr.*, 166 (1978) 47.
42 L. Hammar, S. Pahlman and S. Hjerten, *Biochim. Biophys. Acta*, 403 (1975) 554.
43 C. Tanford, *Advan. Protein Chem.*, 23 (1968) 121.
44 J. Brandrup and E. H. Immergut (Editors), *The Polymer Handbook*, Wiley, New York, 1975, p. IV-17.
45 R. Ohmacht and I. Halasz, *Chromatographia*, 14 (1981) 155.
46 H. Engelhardt and G. Ahr, *J. Chromatogr.*, 282 (1983) 385.
47 P. G. Righetti, G. Tudor and K. Ek, *J. Chromatogr.*, 220 (1981) 113.