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EVALUATION OF ADVANCED SILICA PACKINGS FOR THE SEPARATION OF BIOPOLYMERS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

V. PERFORMANCE OF NON-POROUS MONODISPERSE 1.5-μm BONDED SILICAS IN THE SEPARATION OF PROTEINS BY HYDROPHOBIC-INTERACTION CHROMATOGRAPHY

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SUMMARY

Non-porous monodisperse 1.5-μm silicas were allowed to react with (A) and (B) N-acetylaminopropyltriethoxysilane to generate bonded phases useful in high-performance hydrophobic-interaction chromatography (HIC). Differences in the selectivity were observed between the amide and the ether phase. Peak capacities between 10 and 30 were achieved for several proteins with the amide and ether phase packed into columns of 36 × 8 mm I.D. and elution of the proteins under chromatographic conditions in which the gradient volume, V_G, was held constant by varying the gradient time between 20 and 2.5 min and the flow-rate between 0.5 and 4.0 ml/min. The S values derived from the dependences of log k' on the volume fraction of the low ionic strength buffer, φ_b, were of the same magnitude as reported for porous HIC silicas and showed a dependence on the molecular weight of the protein. Using these HIC stationary phases based on non-porous 1.5-μm supports, fast separations (< 5 min) could be carried out with high biological recoveries.

INTRODUCTION

Hydrophobic-interaction chromatography (HIC) is known as an effective means of resolving proteins under mild conditions by employing a descending salt gradient to control the elution strength. Conventional weakly hydrophobic carbohydrate gels, as pioneered by Hjertén[1] and Shaltiel[2], have now been replaced by microparticulate bonded silica packings, offering a higher performance for analytical

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and micropreparative applications. According to their surface structural properties, silica-based HIC packings can be divided into two categories: those having a hydrophilic monolayer linked via a hydrophobic spacer (*e.g.*, N-acetylamino[3,4] and ether groups[5]) and those coated with a highly hydrophilic layer with bonded hydrophobic groups at the top[6–16]. To gain a wider range of selectivities, mixed-mode bonded phases were recently synthesized with both weakly hydrophobic and ionic ligands to match the polytypic protein surface interactions[14,15].

Although high-performance HIC (HPHIC) of many globular proteins exhibits favourable features with regard to preservation of biological activity, as a separation technique it suffers from low peak capacities and long analysis times compared with reversed-phase mode chromatography (RPC). Gradient times in HPHIC, based on porous silica supports, typically range between 20 and 180 min[17]. With the introduction of non-porous monodisperse 1.5-μm silicas as packings for protein separation[18], further improvements in resolution, peak capacity and analysis time appear to be attainable.

This paper examines the properties of two types of HIC bonded phases, based on this novel type of packing material which has been specifically designed to simplify protein separation.

EXPERIMENTAL

Materials

Non-porous silica was manufactured in our laboratory. All chemicals were gifts from E. Merck (Darmstadt, F.R.G.), except dimethylchlorosilane, which was a gift from Wacker (Burghausen, F.R.G.). Column tubing (36 × 8 mm I.D.) was obtained from Bischoff (Leonberg, F.R.G.) and equipped with paper filters (No. 827 from Schleicher & Schüll, Dassel, F.R.G.). Proteins were purchased from Boehringer (Mannheim, F.R.G.). Serva (Heidelberg, F.R.G.) and Sigma (St. Louis, MO, U.S.A.). Eluents were prepared from redistilled water and analytical-reagent grade buffers.

Instrumentation

An LKB (Bromma, Sweden) solvent delivery system, consisting of a Model 2150 pump, a Model 2152 gradient controller and a low-pressure mixing unit, was used. Absorbance was monitored by means of a Biotronik (Maintal, F.R.G.) BT 3030 UV detector with a 1-μl flow-cell. For the recording and evaluation of chromatograms, we used a Shimadzu (Kyoto, Japan) C-R3A integrator and for enzymatic assays a Zeiss (Oberkochen, F.R.G.) DM 4 spectrophotometer.

Synthesis of bonded phases

N-Acetylaminopropyltriethoxysilane was synthesized from aminopropyltriethoxysilane and acetic anhydride and bonded to the silica surface according to a procedure described by Engelhardt and Mathes[3] to produce the amide-bonded phase. For the preparation of the ether-bonded phase, diethylene glycol monoallyl monomethyl ether was synthesized according to Miller *et al.*[5]. After distillation, the product was allowed to react with a 50% excess of dimethylchlorosilane under reflux for 12 h in the presence of H_2PtCl_6 as a catalyst. Fractional distillation yielded the

desired product (b.p. 358–364 K (70 Pa), which was identified by ^{13}C-NMR spectroscopy as the ether silane $ClSi(CH_3)_2$-$(CH_2)_3$-O-$(CH_2)_2$-O-$(CH_2)_2$)-O-CH_3. Bonding was achieved by refluxing 12 g of the non-porous silica with 1 g of silane and 1 g of 2.6-lutidine for 3 h in dry dichloromethane.

Both of the bonded phases were washed subsequently with, in succession, dichloromethane, ethanol, ethanol–water and water, and dried under vacuum. Columns were packed with a suspension of 5% of bonded silica in cyclohexanol–ethanol (60:40) at a pressure of 80 MPa.

Enzymatic assays

Enzymatic activity of catalase was assayed by immediate dilution of the eluate to 50 ml with 0.1 *M* phosphate buffer (pH 7.0), taking an aliquot of 2 ml (corresponding to 80 ng of enzyme), addition of 1 ml of 60 m*M* hydrogen peroxide solution and monitoring the decay of the hydrogen peroxide photometrically at 230 nm.

Lactate dehydrogenase (LDH) was diluted in the same manner, and its activity was measured by adding 0.1 ml of 23 m*M* sodium pyruvate solution and 0.05 ml of 14 m*M* NADPH and monitoring the absorption at 340 nm.

Calculations

The peak capacity (*PC*) was calculated according to the equation $PC = t_G/4\sigma$, where t_G is the gradient time and σ is the standard deviation of the peak.

RESULTS AND DISCUSSION

Synthesis and characteristics of amide- and ether-bonded silica

Synthesis of the amide-bonded silica was performed according to the procedure described by Engelhardt and Mathes[3]. The ether phase was synthesized by reaction of the silica with the monofunctional analogue of Miller *et al.*'s "ether-bonded phase II"[5]. Because of the low weight-% of the bonded phase, it was not possible to obtain accurate ligand densities from elemental analysis or modern surface analysis, *e.g.*, ^{13}C solid-state cross-polarization magic angle spinning NMR spectroscopy, with these non-porous 1.5-μm silicas, which have a specific surface area of about 2.0 mg/g. A theoretical 3.0 μmol/m^2 ligand density would have a weight fraction of 0.072% carbon for the ether and 0.03% carbon for the amide phase.

Under the chromatographic conditions employed, the ether-bonded column showed a decrease in performance after 50 runs, whereas the properties of the amide column remained unchanged throughout the investigations (*i.e.*, used for more than 6 months). This observation supports the view[16] that trifunctional alkoxysilanes give superior stability to the monofunctional derivatives.

Preservation of biological activity of proteins separated with both columns was monitored with catalase and LDH under various conditions. The biological recovery was found to be 97% for catalase and 92% for LDH on the ether column and 101% for catalase and 100% for LDH on the amide column.

Selectivity and peak capacity on the ether and amide columns

Table I lists the retention times and peak capacities of proteins obtained on (A) the ether and (B) the amide columns under identical conditions (f_v = 1.0 ml/min,

TABLE I

RETENTION AND PEAK CAPACITY OF PROTEINS ON (A) THE ETHER COLUMN AND (B) THE AMIDE COLUMN

Linear 10-min gradient from 2.5 to 0 *M* ammonium sulphate in 0.1 *M* phosphate buffer (pH 7.0); flow-rate, 1.0 ml/min; gradient dwell time, 2 min.

Protein	*Column A*		*Column B*	
	t_R *(min)*	*PC*	t_R *(min)*	*PC*
Ribonuclease	4.82	19.6	4.01	29.4
Cytochrome *c*	5.44	21	1.48	9.8
Lysozyme	7.87	15.5	5.36	28
Myoglobin	8.59	15.5	3.13	15.9
Ovalbumin	9.07	13.4	6.09	14
Lactate dehydrogenase	9.62	16.3	6.99	21.8
Catalase	10.67	14.1	8.56	29.4
Ferritin	11.32	14.7	9.33	19.6

t_G = 10 min). Comparison of the elution sequence of proteins on the laboratory-made ether column with the commercial ether column (HIC CAA, Beckman Instruments)[19] showed retention differences for cytochrome *c* and ribonuclease. This is probably due to the fact that different buffers were used in both studies. Further, the ligands bonded to the phases differed in the structure of their "anchor group".

The elution sequence of proteins on the ether column was identical with that obtained on a reversed-phase column eluted at pH 2.0 with a gradient of an organic solvent, with the exception of ovalbumin, which was eluted ahead of catalase[20]. Possibly the similarity between the monofunctional, long-chain ether ligand and the monofunctional C_8 or C_{18} ligand of the reversed phase was responsible for this behaviour. A different selectivity preference for basic proteins was observed with the amide phase. The elution order of cytochrome *c* and ribonuclease was reversed compared with the ether column. In addition, myoglobin was retained less on the amide column than on the ether column. These changes in selectivity are presumably associated in part with the weaker hydrophobic character of the amide phase. In addition, the selectivity reversals noted with cytochrome *c* and ribonuclease reflect the ability of the different bonded ligands to intercalate specifically[21–24] with hydrophobic binding sites (domains) at the surface of these proteins. The elution sequence of proteins on the non-porous amide phase corresponded to that obtained by Engelhardt and Schön[25] for a porous amide phase, except that they found almost equal retention times for ribonuclease and ovalbumin. The peak capacities calculated with the amide column were smaller by a factor of 2–3 than those observed with an *n*-octadecyl bonded silica of the same particle size and type and packed in columns of the same size, assessed under gradient elution conditions with an acidic aqueous–organic eluent[19]. This is explicable by the fact that resolution in HIC is a function of column length[25], whereas in RPC the influence of this parameter is small[26].

Effect of flow-rate on peak capacity

Examination of the effect of f_v on the peak capacity was carried out with the

TABLE II

PEAK CAPACITY OF PROTEINS ON THE AMIDE COLUMN

Conditions as in Table I.

Protein	*MW*	*PC**			
		A	*B*	*C*	*D*
Cytochrome *c*	12 500	9.4	10.9	9.8	10.9
Myoglobin	17 800	12.7	14.0	14.7	12.8
Ribonuclease	13 700	23.5	29.4	21.8	19.6
Lysozyme	14 300	19.6	21.0	19.6	15.5
Ovalbumin	45 000	13.4	14.7	13.4	14.3
Lactate	144 000	24.7	24.5	22.6	20.3
Catalase	240 000	23.5	26.7	21.0	19.6
Ferritin	450 000	21.4	23.5	21.0	17.3

* (A) f_v = 0.5 ml/min, u = 0.42 mm/s, t_G = 20 min; (B) f_v = 1.0 ml/min, u = 0.83 mm/s, t_G = 10 min; (C) f_v = 2.0 ml/min, u = 1.67 mm/s, t_G = 5 min; (D) f_v = 4.0 ml/min, u = 3.33 mm/s, t_G = 2.5 min.

amide column by varying the flow-rate between 0.5 and 4.0 ml/min and the gradient time between 20 and 2.5 min, *i.e.*, the gradient volume, being the product of flow-rate and gradient time, was maintained constant. As discussed for protein separation on ion-xchange columns by Stout *et al.*[27], the flow-rate of the eluent has little effect on peak capacity under otherwise constant conditions. The data obtained on the amide column show a slight maximum of the peak capacity at a flow-rate of 1 ml/min (Table II). Further, the pattern is similar for high- and low-molecular-weight proteins.

S values of proteins on the amide column

As in gradient elution reversed-phase chromatography, the retention of proteins in HIC can be described in terms of an empirical dependence of k' on φ_b and approximated by the equation

$$\log k' = \log k_a - S\,\varphi_b$$

where k_a is the solute capacity factor in pure solvent A, S is the strength of solvent B and φ_b is the volume fraction of the low ionic strength buffer B[27]. In this linearized form, plots of log k' *vs.* φ_b yield straight lines with a slope S proportional to the hydrophobic contact area established between the solute and the stationary phase surface.

Miller and Karger[17] and Fausnaugh and Regnier[28] have previously observed linear log k' *vs.* φ_b relationships for porous HIC packings. Although the higher molecular weight proteins showed essentially linear dependences of log k' on φ_b, the smaller proteins clearly diverged in their retention behaviour. The derived S values are listed in the caption of Fig. 1. For ribonuclease, the S value on the non-porous amide column was smaller by a factor of two than that reported for the porous ether column under similar chromatographic conditions[16]. The values for lysozyme (S

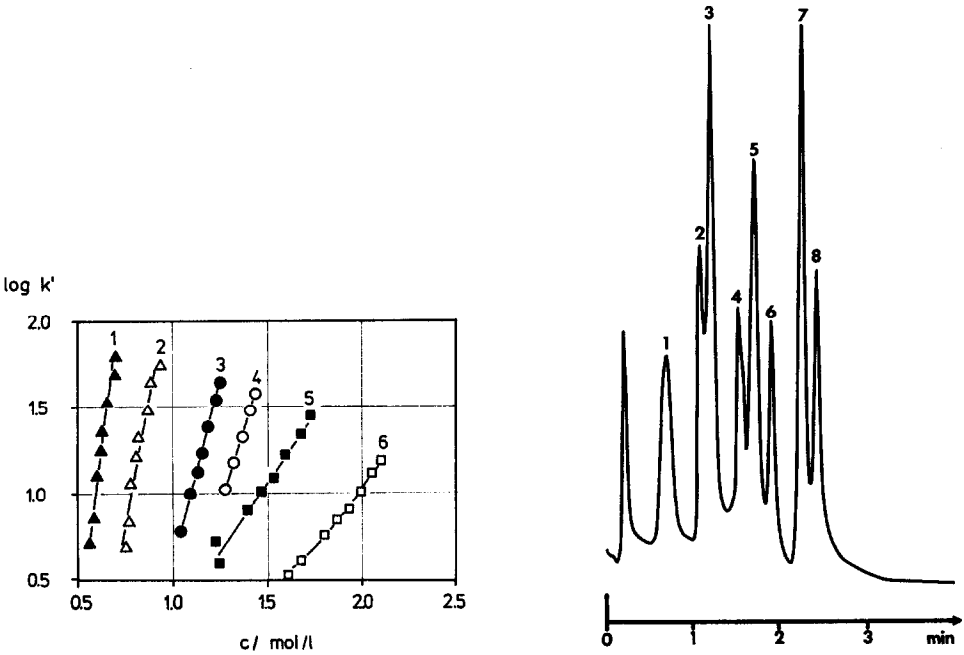

Fig. 1. Dependence of $\log k'$ on φ_b. Data obtained from elution with linear descending ammonium sulphate gradients (c = ammonium sulphate concentration). φ_b = 0, 0.1 *M* phosphate buffer (pH 7.0); φ_b = 1, 2.5 *M* $(NH_4)_2SO_4$ in 0.1 *M* phosphate buffer (pH 7.0). S values derived from linear regression:

Protein	*Curve No.*	*Mol.wt.*	S
Ribonuclease	6	13 700	3.3
Lysozyme	5	14 300	3.8
Ovalbumin	4	45 000	9.1
Lactate dehydrogenase	3	144 000	10.6
Catalase	2	240 000	14.2
Ferritin	1	450 000	18.5

Fig. 2. Fast HIC separation of eight standard proteins on the amide column (36 × 8 mm I.D.); 2.5-min descending gradient from 2.5 to 0 *M* ammonium sulphate in 0.1 *M* phosphate buffer (pH 7.0); flow-rate, 4.0 ml/min. Elution order: V_0 peak (breakthrough of part of the cytochrome *c* due to the low ionic strength of the injected solvent and the low peak dispersion), cytochrome *c* (1), myoglobin (2), ribonuclease (3), lysozyme (4), ovalbumin (5), lactate dehydrogenase (6), catalase (7), ferritin (8).

= 3.8) and ovalbumin (S = 9.1) can be compared with data obtained from isocratic measurements with porous amide silicas by Engelhardt and Schön[25]. Evaluation of the linear part of their k' *vs.* φ_b plots gives S values of 5.3 (lysozyme) and 8.7 (ovalbumin) for a 250 Å material and of 5.8 (lysozyme) and 10 (ovalbumin) for a 500 Å material. Further, there seems to be a significant dependence of S on the molecular weight of the protein.

CONCLUSION

The bonding chemistry of porous HIC silicas has been adapted to monodisperse, microparticulate, non-porous silica products. When these stationary phases are packed into columns of 36 × 8 mm I.D., proteins generate peak capacities from 10 to 30, even with very short gradient times. As a result, high-resolution separations can be performed on these columns within a few minutes (Fig. 2). The recovery is also high with these non-porous HIC supports.

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