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Note

Silica-bound polyethyleneglycol as stationary phase for separation of proteins by high-performance liquid chromatography

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Hydrophobic interaction chromatography (HIC) was originally introduced for the separation of proteins by using alkyl- or aryl-agarose of low ligate density as the stationary phase and decreasing salt gradient in the eluent[1–3]. Less hydrophobic stationary phases were also prepared by covalently bonding polyethylene glycol (PEG) rather than alkyl functions to agarose[4,5]. The soft gels generally employed as column material in this technique, however, are not suitable for use in high-performance liquid chromatography (HPLC). Therefore, polar bonded stationary phases with low density alkyl functions were recently developed also with silica support[6–9] for HIC by HPLC. Some of these stationary phases, however, contain fixed ionogenic groups and as a consequence separation does not always take place by hydrophobic interactions alone[10].

In this report we describe the use of a siliceous bonded stationary phase with covalently bound PEG siloxane bridges for HIC of proteins by HPLC. This column material exhibited retentive properties weaker than those commercially available for HIC. In comparison to "hard" silica bound hydrocarbonaceous stationary phases generally employed in reversed-phase chromatography and requiring organic modifier in the eluent the new stationary phase can be considered as "soft" because it allows elution of biopolymers with neat aqueous eluents although the separation in both cases is governed by solvophobic interactions[11]. As a result of the mild elution conditions protein denaturation on the column is minimum as demonstrated by the good recovery of enzymic activity of adenosine deaminase.

MATERIALS AND METHODS

The chromatographic system consisted of two Model 100A pumps, a Model 420 gradient controller, a magnetic mixer (Altex, Berkeley, CA, U.S.A.), a Model SF-770 Spectroflow detector (Kratos, Westwood, NJ, U.S.A.) and a Model 56 recorder (Perkin-Elmer, Norwalk, CT, U.S.A.). Column eluent was monitored at 230 nm. Columns (100 × 4.6 mm I.D.) were packed by using a slurry of the column material in 2-propanol–methanol (3:1). A Model 25 spectrophotometer (Beckman, Fullerton, CA, U.S.A.) was used in the measurement of enzyme activity.

All inorganic chemicals and organic solvents as well as Carbowax PEG 400 were of reagent grade or HPLC grade from Fisher (Fairlawn, NJ, U.S.A.). Hypersil

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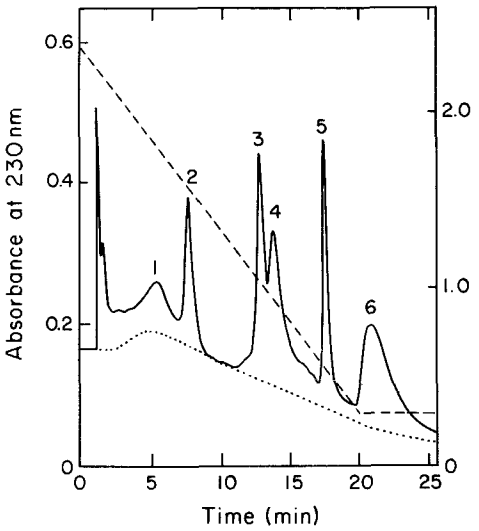

Fig. 1. Separation of proteins on siliceous PEG bonded phase. Column, 100 × 4.6 mm I.D.: flow-rate, 1.0 ml/min; temperature 25°C. Linear gradient elution with decreasing salt concentration: starting eluent, 3.0 *M* $(NH_4)_2SO_4$ in 0.02 *M* phosphate, pH 4.35; gradient former, 0.3 *M* $(NH_4)_2SO_4$ in the same phosphate solution. Sample components: 1 = cytochrome *c*; 2 = ribonuclease A; 3 = trypsinogen; 4 = α-chymotrypsin; 5 = chymotrypsinogen A; 6 = BSA. The ordinate on the right gives the molar concentration of $(NH_4)_2SO_4$.

wide pore silica having mean particle size 5-μm and pore diameter 300 Å was from Shandon Southern Instruments (Sewickley, PA, U.S.A.). 3-Glycidoxypropyltrimethoxysilane was obtained from Aldrich (Milwaukee, WI, U.S.A.). All proteins used in this work were purchased from Sigma (Cleveland, OH, U.S.A.). Water was purified by using a Milli-Q system (Millipore, Bedford, MA, U.S.A.).

RESULTS AND DISCUSSION

The silica support was first treated with 3-glycidoxypropyltrimethoxysilane and subsequently Carbowax PEG 400 was coupled to the alkyl moieties via the terminal hydroxyl groups. The stationary phase is believed to consist of a silica bound hydrocarbonaceous sublayer to which the long polyether chains are attached to form a soft, highly hydrated polar top layer. The carbon content of the material was 1.34% (w/w) as determined by elemental analysis.

A mixture of six proteins was chromatographed on this stationary phase by linear gradient elution with decreasing salt concentration and the result is shown in Fig. 1. It is seen that the proteins can be separated within 20 min with the exception of trypsinogen and α-chymotrypsin. The broad peak of bovine serum albumin (BSA) is typical for hydrophobic interaction chromatography and indicative for the heterogeneous nature of this biopolymer[12].

The effect of salt concentration in the eluent on the retention of proteins was investigated by using sodium sulfate. Upon increasing the concentration of sodium sulfate from 0 to 1.5 *M*, the capacity factors were found to increase very rapidly

TABLE I

COMPARISON OF THE EFFECT OF SALT CONCENTRATION ON THE RETENTION OF PROTEINS ON VARIOUS HIC COLUMNS.

The slopes and intercepts were obtained from linear regression of the relation between log k' and Na_2SO_4 concentration in 0.02 *M* phosphate buffer, pH 7.0, used as the eluent.

Proteins	*Silica–PEG**			*TSK-GEL butyl***			*TSK-GEL phenyl***		
	Slope	*Intercept*	*r*	*Slope*	*Intercept*	*r*	*Slope*	*Intercept*	*r*
Ribonuclease	2.226	−2.743	0.998	1.092	−1.647	0.999	0.879	−1.253	0.989
Cytochrome *c*	2.208	−1.889	0.994	1.090	−1.227	0.994	0.748	−0.694	0.993
α-Chymotrypsin	3.756	−3.656	0.996	1.518	−1.399	0.993	1.237	−0.315	0.993
BSA	1.446	−1.108	0.988	1.452	−1.396	0.989	1.490	−0.949	0.999

* Column described in this report.
** Calculated from data in ref. 6.

and the results indicated that proteins can be desorbed from the surface of the stationary phase under very mild conditions without risk of denaturation.

Plots of log k' *vs.* salt concentration were linear. The slopes and intercepts were compared to those calculated from retention data obtained on two types of commercially available column for HIC[6] and are given in Table I. Comparison of the slopes, which measure the increase in retention with salt concentration, suggests that the surface of our stationary phase is less hydrophobic than that of the commerical column materials. It is not surprising as the polarity of the PEG functions with terminal hydroxyl groups is believed to be higher than that of the surface of the reference stationary phases which have alkyl or aryl ligates. The effects of eluent pH on the separation of proteins has also been studied in gradient elution mode and no large variation in relative retentions was observed when the pH was changed from 4.35 to 7.00.

Selectivity and efficiency of the separation were significantly better when $(NH_4)_2SO_4$ was used instead of Na_2SO_4 in the mobile phase. This may be due to some specific changes caused by the relatively high molal surface tension increment of sodium sulfate[13], in the bonded organic moiety at the surface of the stationary phase.

In order to investigate the recovery of enzymic activity 5 μl of adenosine deaminase (5 mg/ml) in glycerol–0.01 *M* phosphate buffer pH 6.0 (6:1) was chromatographed with a 10-min linear gradient of descending $(NH_4)_2SO_4$ concentration from 2.4 to 0.3 *M* in 0.02 *M* phosphate buffer pH 7 at a flow-rate of 1 ml/min. The column effluent containing the adenosine deaminase peak was collected and the enzymic activity was determined by using adenosine as the substrate. The recovery of the enzymatic activity of adenosine deaminase upon chromatography with ammonium sulfate gradient in phosphate buffer, pH, was 90%. The recovery of all proteins investigated in this work was significantly better than 90% as measured by the Coomassie blue method[14].

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