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Biosep-SEC-S high-performance size-exclusion chromatographic columns for proteins and peptides

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ABSTRACT

Spherical silica particles (5 μm) of three different pore sizes, 145, 290 and 500 Å (Biosep-SEC-S2000, Biosep-SEC-S3000 and Biosep-SEC-S4000), were bonded with a hydrophilic coating and evaluated for the size-exclusion chromatography of proteins and peptides. The results of experiments with synthetic peptides and lysozyme under different mobile phase conditions indicated very nominal non-specific interaction of proteins with the stationary phases. The recovery of trypsin, protein mass and enzyme activity, determined at mobile phase pH values of 3 and 7, was excellent for all three column types. The new stationary phases showed superior stability towards common protein denaturants and organic modifiers.

INTRODUCTION

Size-exclusion chromatography (SEC) is one of the most widely used techniques for the characterization of proteins and peptides. This versatile procedure is often employed not only for protein purification but also in the determination of their molecular weights [1–3]. Over the past few years, new stationary phases for the SEC of biopolymers have been developed [4–6] and characterized [7]. Silica-based chromatographic supports provide great advantages such as rigidity, well defined pore-size distribution and stability towards a variety of mobile phase solvents. However, depending on the nature of the bonded phase and the presence of unreacted silanols, interaction of proteins or peptides with the stationary phase can occur, resulting in non-ideal size exclusion behavior [8–10]. Such interactions have been shown to be ionic or hydrophobic in nature [4,7,9]. These non-specific interactions can lead to errors in the determination of protein and peptide molecular weights and, in many instances lower recoveries of protein mass and diminished biological activity. We report here the development of a new silica-based support for the SEC of proteins and peptides. The new stationary phases were evaluated using synthetic peptides and natural proteins and were shown to operate essentially with a size-exclusion mechanism.

EXPERIMENTAL

Biosep-SEC-S series columns were prepared by bonding silica of pore sizes 145, 290 and 500 Å with a hydrophilic coating. Synthetic size-exclusion peptide standards were obtained from Synthetic Peptides (Edmonton, Canada). The sequences of five peptides are Ac–(Gly–Leu–Gly–Ala–Lys–Gly–Ala–Gly–Val–Gly)$_n$–amide (Ac = N-acetyl, amide = C-amide), where n = 1–5, giving rise to five peptides consisting of 10–50 residues. The peptides have increasing charge (+1 to +5) and hydrophobicity, with molecular weights of 826, 1595, 2362, 3129 and 3897 dalton, respectively.

All laboratory chemicals used in the preparation of mobile phase buffers were of HPLC grade and purchased from Fisher Scientific (Tustin, CA, USA), except sodium dodecyl sulfate (SDS), which was from Pierce (Rochford, IL, USA). The proteins used were either obtained from Boehringer Mannheim (San Diego, CA, USA) or Sigma (St. Louis, MO, USA). Some of the size-exclusion protein stan-

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dards were also purchased from Bio-Rad Labs (Richmond, CA, USA). Mobile phase buffers were filtered through 0.2-μm Anodisc 47, Anopore membrane disc filters (Phenomenex, Torrance, CA, USA) and degassed by helium sparging before use. Protein standards were prepared in the mobile phase buffer at a concentration of 2 mg/ml and were filtered through 0.2-μm Anotop 10-Plus inorganic membrane syringe filters (Phenomenex) prior to injection.

Chromatographic runs were performed on a Hewlett-Packard 1050 Series pumping system with an HP 1050 multiple wavelength detector. A Phenoflow flow meter (Phenomenex) installed at the outlet of the detector was used to monitor the flow and very stable flow was obtained with this pump.

The partition coefficient, K_D was calculated with the equation $V_e = V_0 + K_D V_i$ [7], where V_e is the elution volume of the analyte, V_0 is the column void volume and V_i is the pore volume of the support. As the total liquid volume, $V_T = V_0 + V_i$, substituting for V_i, then $K_D = (V_e - V_0)/(V_T - V_0)$. The total liquid volume (V_T) of the column was determined by chromatography of ethylene glycol with refractive index detection or dihydroxyacetone with UV detection at 300 nm, using water as mobile phase. The V_T values for the three types of columns (300 × 7.75 mm I.D.) with increasing pore size were calculated to be 11.54, 12.44 and 12.40 ml, respectively. The void volume, V_0, of the column was determined by injecting 10 μl of high-molecular-weight calf thymus DNA (2.5 mg/ml solution from Boehringer Mannheim), with 50 m*M* NaH_2PO_4 buffer (pH 6.8) as mobile phase and UV detection at 260 nm. The V_0 values for the three phases (300 × 7.75 mm I.D. columns) were 5.27, 5.45 and 5.80 ml, respectively.

RESULTS AND DISCUSSION

Recently, synthetic peptides have been introduced to diagnose the surface activities of silica-based chromatographic stationary phases, including reversed-phase, size-exclusion and ion-exchange matrices [11–13]. These peptides provide a very convenient method to test the performance of newly developed chromatographic supports. Two sets of peptide standards, with increasing hydrophobicity and cationic charge, were used to evaluate the behavior of Biosep-SEC-S2000 with a pore size of 145 Å. The sizes of the peptide standards are such that they ideally separate out on this stationary phase. As these peptides have increasing charge and hydrophobicity (see Experimental for the sequence), silica supports containing surface charge due to free silanols or hydrophobic character have been shown to retain the peptides by ionic or hydrophobic binding, resulting in reversal of the order of elution [11]. In extreme cases of such surface activity, the peptides have been shown to elute after the total permeation volume of the column, indicating in such instances that the column is behaving more like a cation exchanger than an SEC support.

Table I shows the effect of mobile phase composition on the retention times of three peptides (+5, +2 and +1) on Biosep-SEC-S2000. Under all the mobile phase conditions used, the peptides showed ideal SEC patterns, eluting in order of increasing molecular weight. The mobile phase composition had virtually no effect on the retention times. If there were unreacted silanols present on this stationary phase, the low ionic strenght of 100 m*M* phosphate buffer at pH 6.8 would have resulted in retention of the peptides due to ionic interactions. This would cause the largest peptide with the highest charge (+5) to elute much later than expected on the basis of size. Increasing the salt concentration in the buffer would suppress ionic interactions with the stationary phase, but could lead to hydrophobic binding and result in a non-ideal elution

TABLE I

EFFECT OF MOBILE PHASE COMPOSITION ON THE ELUTION BEHAVIOR OF SYNTHETIC PEPTIDES

A synthetic peptide mixture containing +5, +2 and +1 peptides was dissolved in water and 10 μl were subjected to SEC on a Biosep-SEC-S2000 column (300 × 7.75 mm I.D.) under the mobile phase conditions described, at a flow-rate of 1 ml/min. UV detection at 210 nm.

Mobile phase	t_R (min)		
	+5 Peptide	+2 Peptide	+1 Peptide
100 m*M* phosphate (pH 6.8)	8.85	9.58	10.04
100 m*M* phosphate (pH 6.8) containing 500 m*M* NaCl	8.91	9.66	10.25
0.1% aqueous TFA	8.87	9.68	10.10

pattern for the peptides. Thus, when the salt concentration in the mobile phase was increased by adding 500 m*M* NaCl to the 100 m*M* NaH_2PO_4 buffer, no effect on the retention time of the peptides was observed, indicating the absence of hydrophobic binding on this stationary phase.

In the separation of peptides, it is convenient to use trifluoroacetic acid (TFA) as an eluent, as it is easily removed by lyophilization. The acidic conditions of the mobile phase also suppress the ionic interaction with the stationary phase, similarly to the effect of increasing the salt content in mobile phase. The synthetic peptide mixture described above was subjected to SEC on the Biosep-SEC-S2000 column in 0.1% TFA. The retention times of the peptides remained unchanged with this mobile phase (Table I) compared with other mobile phases used. Some of the commercial columns require high concentrations of TFA (1%) to suppress completely the ionic activity, and such extreme conditions may have an adverse effect on the column lifetime [11]. The stationary phase described here shows ideal SEC behavior with 0.1% TFA as is normally used for such applications.

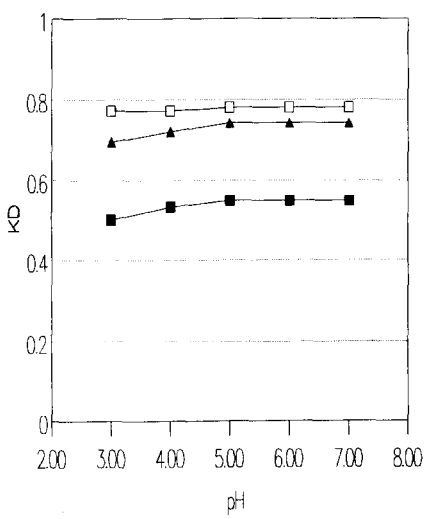

Fig. 1. Effect of mobile phase pH on the K_D of lysozyme. Biosep-SEC-S2000, -S3000 and S4000 columns (300 × 7.75 mm I.D.) were equilibrated with 0.1 *M* NaH_2PO_4 buffer at different pH values between 3 and 7. Lysozyme was dissolved in the same buffer as the mobile phase at 10 mg/ml and 20 μl of the solution were inhected. The flow-rate was 1 ml/min and detection was at 280 nm. Columns: ■ = Biosep-SEC-S2000; ▲ = Biosep-SEC-S3000; □ = Biosep-SEC-S4000.

The synthetic peptides were useful in the evaluation of the surface activity of Biosep-SEC-S2000 (pore size 145 Å) columns. However, because of the larger pore sizes of the supports, the peptide standards would not resolve on the Biosep-SEC-S3000 (pore size 290 Å) and Biosep-SEC-S4000 (pore size 500 Å) columns. Therefore, lysozyme was used as a probe for their evaluation. Biosep-SEC-S2000 was also included, to further confirm the results obtained with synthetic peptides. Lysozyme is a hydrophobic and very basic protein (p*I* = 11.0) and has been recommended as a probe for testing the surface activity of size-exclusion columns [7]. Some SEC supports show drastic changes in retention behavior of lysozyme under mobile phase conditions differing in ionic strength and pH, indicating ionic and hydrophobic interactions with the stationary phase [4,7]. The effect of changing the mobile phase pH on the SEC of lysozyme was tested on Biosep-SEC -S2000, -S3000 and -S4000 columns. The mobile phase pH values were varied between 3 and 7 with an ionic strength of the buffer (0.1 *M*) normally used for size-exclusion chromatography of proteins. Fig. 1 is a plot of mobile phase pH *vs.* K_D of lysozyme on the three types of columns. There was only a nominal increase in K_D of the enzyme with an

TABLE II

RECOVERY OF TRYPSIN FROM BIOSEP-SEC-S COLUMNS

The columns were equilibrated with 0.1 *M* NaH_2PO_4 buffer (pH 3.0 or 7.0). Trypsin was dissolved in NaH_2PO_4 buffer (pH 3.0) at 10 mg/ml. A volume of 20 μl of the solution was injected on to the columns and the peaks were collected. For each pH value, at least five runs were performed. The peaks were assayed for protein concentration and enzyme activity by the UV–VIS method. The enzyme activity was measured using N-benzoyl-L-arginine-*p*-nitroacetanilide as a substrate in Tris–HCl buffer (pH 8.0) containing $CaCl_2$. The hydrolysis was followed by the increase in absorption at 386 nm.

Column	pH	Protein (%)	Specific activity (%)
Biosep-SEC-S2000	3.0	85–100	86–100
	7.0	70–79	95–100
Biosep-SEC-S3000	3.0	98–101	96–100
	7.0	70–78	96–100
Biosep-SEC-S4000	3.0	92–100	95–101
	7.0	69–80	94–100

increase in pH of the mobile phase, suggesting a very low ionic-type interaction with the supports. For some commercial SEC supports, it has been observed that an increase in the ionic strength of the mobile phase above 0.2 *M* leads to binding of lysozyme by hydrophobic interaction [4]. However, when the buffer concentration was increased to 0.2 *M* at pH 7.0, the change in elution time was insignificant with the SEC phases described here (data not shown). These results complement and support earlier experiments with the synthetic peptides, and demonstrate essentially a size-exclusion mechanism operating under the normal mobile phase conditions, with other retention mechanisms having little or no influence.

It is important in any protein chromatographic

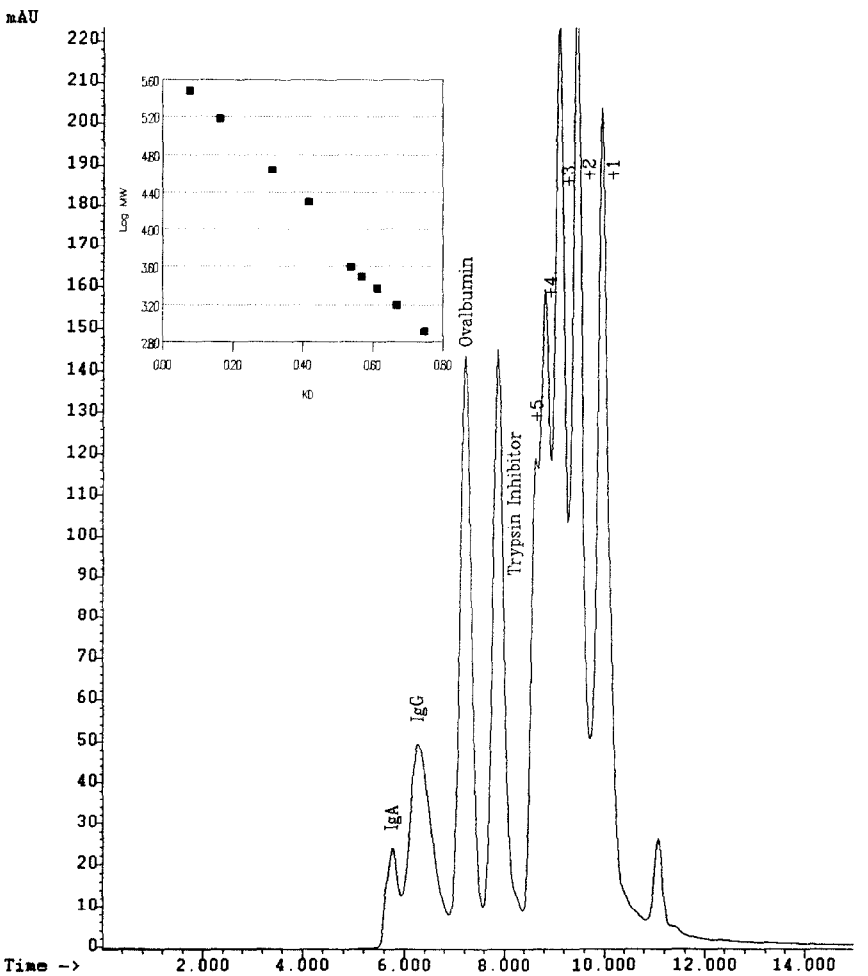

Fig. 2. SEC of proteins and peptides on Biosep-SEC-S2000. A 300 × 7.75 mm I.D. column was equilibrated with 50 m*M* NaH_2PO_4 buffer (pH 6.8) at a flow-rate of 1 ml/min. Protein standard was prepared by dissolving 4 mg each of γ-globulin (a mixture of immunoglobulins IgM, IgA and IgG), ovalbumin and trypsin inhibitor in 1 ml of mobile phase. About 4 μl of this mixture were added to 100 μl of a solution of synthetic peptides. A 10-μl volume of the sample was injected on to the column. The elution of proteins and peptides was followed by UV detection at 215 nm. Peaks identified: IgA (300 000 dalton), IgG (150 000 dalton), ovalbumin (44 000 dalton), trypsin inhibitor (20 100 dalton), peptide + 5 (3897 dalton), peptide + 4 (3129 dalton), peptide + 3 (2362 dalton), peptide + 2 (1595 dalton), and peptide + 1 (826 dalton). The inset is a plot of log molecular weight (MW) *vs.* K_D for this separation (r^2 = 0.9936). The K_D values were calculated using the equation $K_D = (V_e - V_0)/(V_T - V_0)$, where V_e is the elution volume and V_T and V_0 represent the total liquid volume and void volumes of the column, respectively. Time in min.

procedure to have good recoveries of protein mass from the column. It is equally important to retain the maximum amount of biological activity in the sample following the chromatographic step. In some instances, one may recover all the protein from a column, while losing much of the biological activity because of its interaction with the stationary phase [7]. Trypsin is another basic protein (p*I* 10.5) and is a suitable probe to study such interactions. Therefore, trypsin chromatography on Biosep-SEC-S phases was studied with respect to recovery of protein and enzyme activity, at mobile phase pH values of 3.0 and 7.0. The results are given in Table II. When 200 μg of the enzyme were injected on to the column, the typical recovery of the protein mass ranged from 85–100% at pH 3.0. However, at pH 7.0 the recovery was lower, ranging between 69 and 80% for all the phases. Earlier experiments with synthetic peptides and lysozyme have shown that non-specific interactions are virtually absent in these columns. The loss of trypsin mass may be due to some denaturation or possibly because of autodigestion. The recovery of enzyme activity, however, was excellent at both pH values for all the columns tested and ranged from 85 to 100%.

After ascertaining that there was no apparent surface reactivity of Biosep-SEC-S stationary phases, the separation range of these columns for proteins and peptides under native conditions was determined. First Biosep-SEC-S2000 was evaluated for its resolving capacity. A mixture containing five synthetic peptide standards (charge +1 to +5) and proteins (immunoglobulins, ovalbumin and trypsin inhibitor) was subjected to SEC on this column, equilibrated with 50 m*M* NaH_2PO_4 buffer (pH 6.8). The chromatographic profile is presented in Fig. 2. All the components of the mixture were adequately resolved. The plot of log (molecular weight) *vs.* K_D is given in the inset and shows excellent linearity (r^2 = 0.9936). The separation range for this phase for proteins and peptides can be conveniently set at 1000–300 000 dalton based on these results.

The resolving power of Biosep-SEC-S3000 was tested with a wide range of proteins. The column was equilibrated with 50 m*M* NaH_2PO_4 buffer (pH 6.8) and mixtures containing different proteins were subjected to SEC. For some proteins which co-eluted, such as trypsin inhibitor and carbonic anhydrase, runs were performed to determine and confirm the individual retention times. Fig. 3 is a plot of log (molecular weight) *vs.* K_D for all proteins separated using the Biosep-SEC-S-3000 column and shows linearity with r^2 = 0.977. It is well known that the size of a macromolecule depends not only on its molecular weight but also its conformation [14]. Different shapes, such as solid-sphere, random coil or rod-like structures, with the same molecular weight show different elution behaviors in SEC. Therefore, considering the diversity of the proteins used in this experiment, the correlation coefficient would appear to be acceptable. Thus, when insulin and cyanocobalamin were excluded from the calcu-

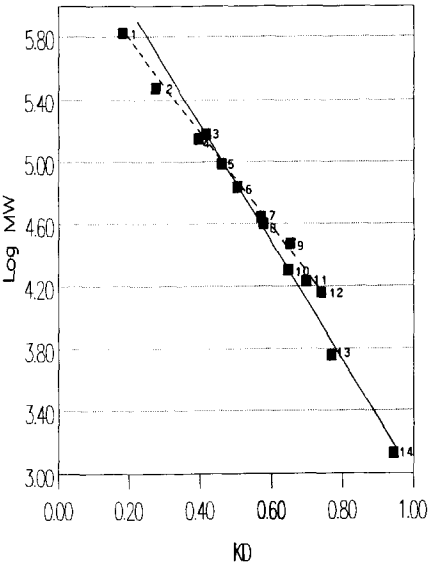

Fig. 3. Cromatography of proteins on Biosep-SEC-S3000. A mixture of different proteins was prepared by dissolving 2 mg each in the mobile phase buffer 50 m*M* NaH_2PO_4 (pH 6.8). A 10-μl volume of the sample was injected into a 300 × 7.75 mm I.D. column which was equilibrated with the buffer at 1 ml/min. UV detection at 280 nm was applied. Three different runs with mixtures of proteins were performed and the log MW *vs.* K_D plotted (solid line, for all proteins, r^2 = 0.977; dashed line, excluding insulin and cyanocobalamin, r^2 = 0.9905). Proteins: 1 = thyroglobulin (670 000 dalton); 2 = IgA (300 000 dalton); 3 = IgG (150 000 dalton); 4 = lactate dehydrogenase (134 000 dalton); 5 = phosphorylase *b* (97 000 dalton); 6 = bovine serum albumin (68 000 dalton); 7 = ovalbumin (44 000 dalton); 8 = horse radish peroxidase (40 000 dalton); 9 = carbonic anhydrase (30 000 dalton); 10 = trypsin inhibitor (20 100 dalton); 11 = myoglobin (17 000 dalton); 12 = lysozyme (14 400 dalton); 13 = insulin (5700 dalton); 14 = cyanocobalamin (1350 dalton).

lations, the correlation coefficient improved to 0.9905 and only carbonic anhydrase seemed to deviate from the best-fit curve in as much as it co-eluted with trypsin inhibitor. Although cyanocobalamin is not a protein and is used as a marker, its elution behavior may not be consistent with proteins or peptides. Insulin, on the other hand, being smaller in size than all the other proteins used in this study, may deviate from the elution pattern of these proteins simply because of differences in the tertiary structure and shape. The deviation of carbonic anhydrase from normal elution is surprising, as it is an acidic protein (pI = 5.3). This may be because of its conformation or its interaction with the stationary phase by a mechanism other than ionic or hydrophobic binding.

When used under denaturing conditions, SEC can be effectively employed for the determination of protein molecular weights. Proteins attain an extended rod-like or random coil structure under de-

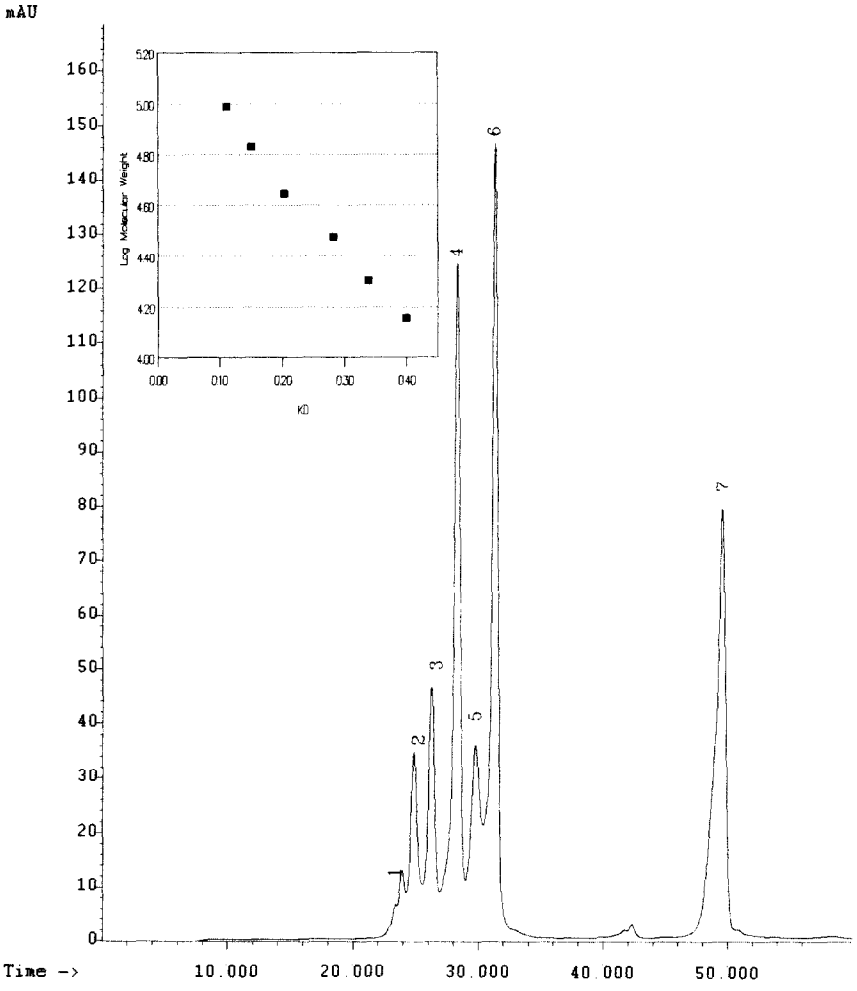

Fig. 4. SEC of proteins on Biosep-SEC-S3000 using 0.5% SDS. Protein mixture was prepared by dissolving 2 mg each of (1) phosphorylase *b* (97 000 dalton), (2) bovine serum albumin (68 000 dalton), (3) ovalbumin (44 000 dalton), (4) carbonic anhydrase (30 000 dalton), (5) trypsin inhibitor (20 100 dalton) and (6) lysozyme (14 400 dalton) in 0.5 ml of 20 mM NaH_2PO_4 buffer (pH 6.5) containing 1% SDS and 1% β-mercaptoethanol. The solution was boiled for 3 min and 20 μl were injected on to the column (600 × 7.75 mm I.D.), equilibrated with 20 mM NaH_2PO_4 buffer (pH 6.5) containing 0.5% SDS at a flow-rate of 1 ml/min. Peak 7 is BME. The inset is a plot of log MW *vs.* K_D with r^2 = 0.9908. Time in min.

naturing conditions, and because of uniformity in the conformation, the determination of molecular weight has been shown to be more accurate [3]. In fact, SEC separations of proteins are often found to be much better under denaturing conditions than when in a native state [15,16]. The Biosep-SEC-S3000 column was evaluated for its ability to separate proteins using 6 *M* guanidine hydrochloride (GnHCl) and SDS, and also for its stability toward these reagents.

The effects of buffer concentration on the retention times of proteins and peptides using SDS in SEC have been described [17]. The mode of separation was shown to be affected by the presence of

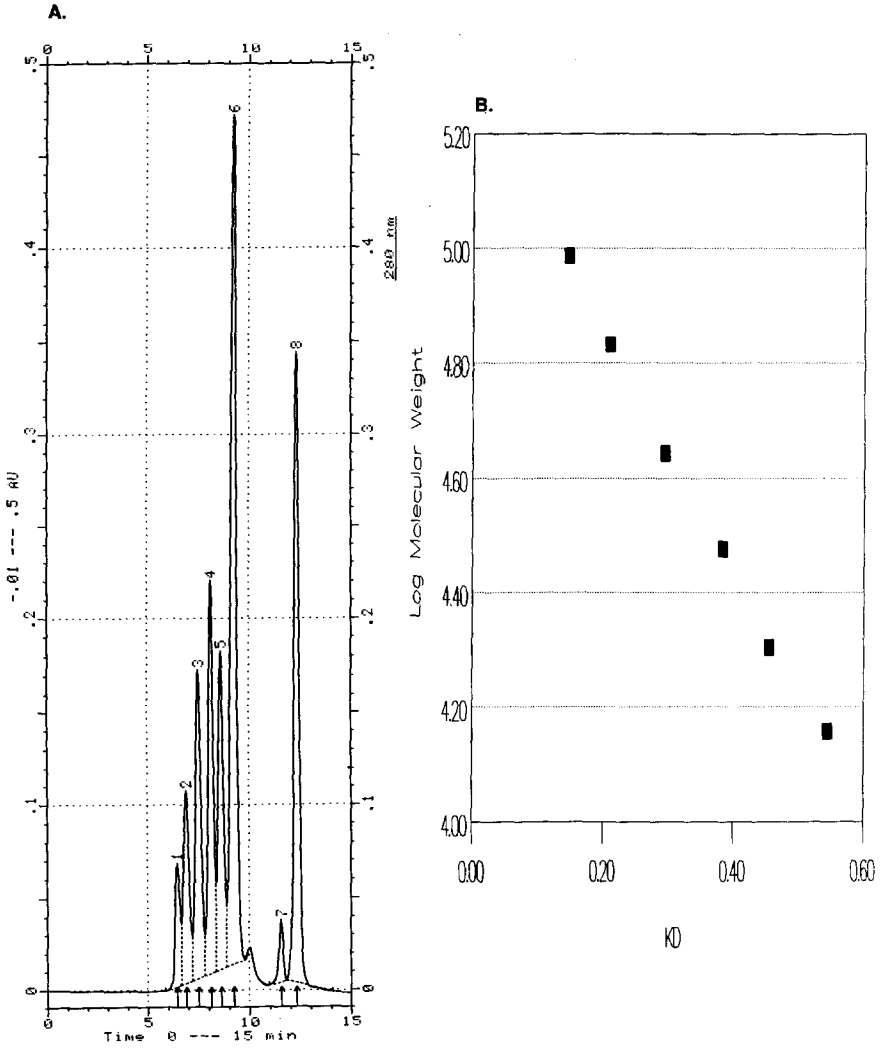

Fig. 5. (A) SEC of proteins on Biosep-SEC-S3000 using 6 *M* guanidine hydrochloride. The same set of proteins as in Fig. 3 was used. The proteins were dissolved in 0.5 ml of 20 m*M* NaH_2PO_4 buffer (pH 6.5) containing 8 *M* guanidine hydrochloride and 1% BME. The solution was boiled for 5 min and 20 μl were injected on to the column, equilibrated with 20 m*M* NaH_2PO_4 buffer (pH 6.5) containing 6 *M* GnHCl at a flow-rate of 1 ml/min. The separation profile was followed by UV detection at 280 nm. Peaks: 1 = phosphorylase *b* (97 000 dalton); 2 = bovine serum albumin (68 000 dalton); 3 = ovalbumin (44 000 dalton); 4 = carbonic anhydrase (30 000 dalton); 5 = trypsin inhibitor (20 100 dalton); 6 = lysozyme (14 400 dalton); 7 = a contaminant; 8 = β-mercaptoethanol. (B) Plot of log MW *vs.* K_D, r^2 = 0.9948.

excess of SDS. The concentration of this denaturant in the mobile phase was ideally kept at its critical micellar concentration, which depended on the buffer concentration. Therefore, the optimum conditions of buffer and SDS for the separation of a mixture of proteins on Biosep-SEC-S3000 was determined. To obtain consistent chromatographic profiles in repeated runs, 20 m*M* NaH_2PO_4 buffer (pH 6.5) containing 0.5% SDS was found to be essential for this support. When a lower concentration of SDS (0.1%) was used, the separation profile varied from run to run. Fig. 4 shows the separation of a mixture of proteins under the optimum, established conditions on a Biosep-SEC-S3000 column. There was a good linear relationship between log (molecular weight) and K_D (r^2 = 0.9908) for this separation, as shown in the inset. The last peak to elute was β-mercaptoethanol (BME) and showed a nearly ideal K_D of 1.09. Proteins are known to have an extended rod-like conformation in SDS, but they become increasingly spherical with decreasing molecular weight. Consequently, the lower limit of the molecular weight of proteins, for the calibration graph using SDS, is about 15 000 dalton [15].

Fig. 5A shows the separation of the same mixture of proteins on a Biosep-SEC-S3000 column using 6 *M* GnHCl in 20 m*M* NaH_2PO_4 buffer (pH 6.5). All proteins were well resolved and a plot of log (molecular weight) *vs.* K_D (Fig. 5B) showed excellent linearity (r^2 = 0.9948). The results conflict with those reported earlier [3], where phosphorylase *b* and serum albumin failed to resolve under similar conditions on a TSK3000SW column. These differences may be related to the pore size of the stationary phases. In this experiment, BME eluted a K_D of 0.98, which is ideal for a small, non-interacting molecule. The two proteins which failed to resolve under native conditions, carbonic anhydrase and trypsin inhibitor (Fig. 3), separated well using 6 *M* GnHCl and 0.5% SDS. These data show the in-

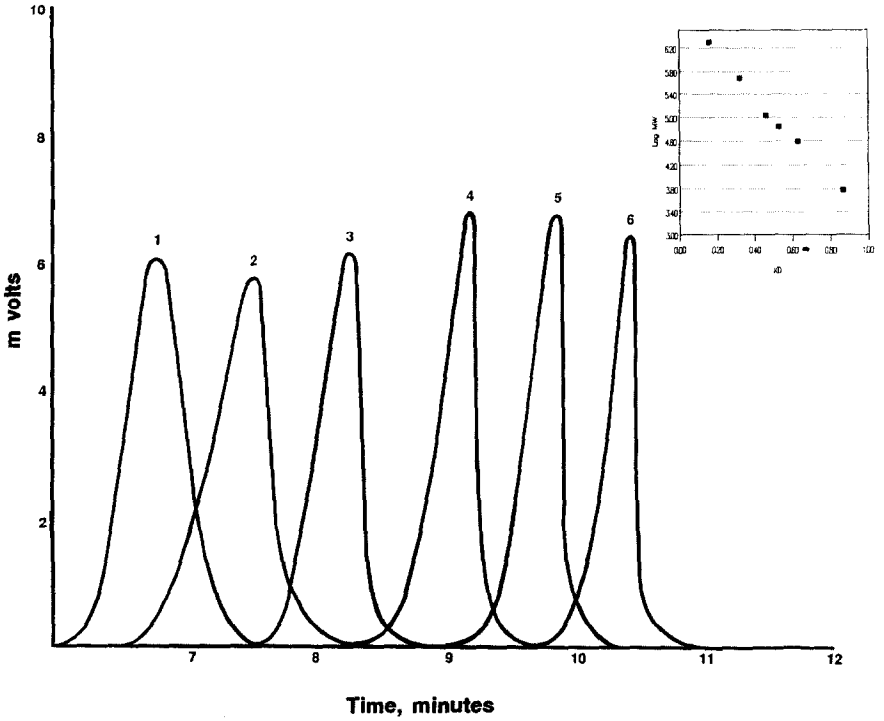

Fig. 6. Separation of dextrans on Biosep-SEC-S4000. Chromatographic runs were performed individually for each dextran. Dextran was dissolved in water at 25 mg/ml and 10 μl were injected on to the column, equilibrated with water at 1 ml/min. Peaks: 1 = blue dextran ($2 \cdot 10^6$ dalton); 2–6 = dextrans of molecular weight 500 000, 110 000, 70 000, 40 000 and 6000 dalton, respectively. The inset is a plot of log MW *vs.* K_D, r^2 = 0.993.

creased resolving power of the stationary phase under denaturing conditions. Following exposure to denaturants, Biosep-SEC-S columns could be easily regenerated by washing them overnight with water at a flow-rate of 0.2 ml/minute. Such regenerated columns could be reused under native conditions for the SEC of proteins without any decrease in resolution.

The resolving power of Biosep-SEC-S4000 with 500 Å pore size was tested with dextrans ranging from 6000 to $2 \cdot 10^6$ dalton. Stationary phases with this pore size are considered to be ideal for the SEC of dextrans [6]. On the other hand, well characterized polymers such as dextrans have been recommended for the calibration of SEC columns [15]. Each dextran sample was run individually on the column after equilibration with water. The separation profile is shown in Fig. 6 and is a composite of individual runs. All the dextrans were resolved with an excellent linear correlation of the log (molecular weight) *vs.* K_D plot ($r^2 = 0.993$). This phase was not able to resolve proteins of molecular weight below 15 000 dalton (data not shown), which permeate into the pores, but is suitable for the separation very high-molecular-weight proteins (15 000–$2 \cdot 10^6$ dalton).

For all the calibration graphs [log (molecular weight) *vs.* K_D plots] obtained here, excellent linearities were observed. It should be pointed out, however, that variation in the properties such as shape and size of macromolecular standards may result in sigmoidal curves for such plots. For a support of given pore size, the plot is suggested to be linear between K_D values of 0.1 and 0.9 [15]. It is therefore important to evaluate critically all the experimental parameters before using such plots for the determination of molecular weights.

CONCLUSIONS

The silica-based supports developed here for the high-performance SEC of proteins and peptides were evaluated for surface activity with synthetic peptides and proteins under different mobile phase conditions. The stationary phases showed excellent SEC properties, such as very low non-specific interaction with proteins and peptides, good resolution and wide molecular weight separation ranges. The supports showed excellent chemical stability toward common buffers and protein denaturants and could be easily regenerated following exposure to these denaturants.

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