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COMPARATIVE STUDY OF ZORBAX BIO SERIES GF 250 AND GF 450 AND TSK-GEL 3000 SW AND SWXL COLUMNS IN SIZE-EXCLUSION CHROMATOGRAPHY OF PROTEINS

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SUMMARY

A reduction of the mean particle diameter of silica-based packings in the size-exclusion chromatography (SEC) of proteins to about 5 μm generates the expected increase in column plate number over the traditional 10 μm SEC columns, as demonstrated for the Zorbax Bio Series GF 250 and GF 450 and TSK-Gel 3000 SWXL columns. The slightly lower column efficiency of the TSK-Gel 3000 SWXL compared with the GF 250 column is compensated by the fact that the phase ratio of the 3000 SWXL column is higher by a factor of two. Hence both columns show nearly the same peak capacity of about 20–30. When the ionic strength of the eluent was changed by varying the salt concentration, the elution volume of proteins was found to be affected differently on the two columns. These alterations are attributed to the differences in the surface compositions of the two types of packings.

INTRODUCTION

Bonded silicas of adjusted pore size have become very popular for the size-exclusion chromatography (SEC) of proteins[1]. Three major aspects must be considered in the design of silica size-exclusion columns for protein separation:

(1) the mean pore size and the pore size distribution determining the molecular weight fractionation range and the linearity of the calibration graph for the column[2];

(2) the internal porosity of the column packing with regard to high resolution and sufficient mechanical stability[3]; and

(3) the bonding chemistry in grafting hydrophilic non-ionic polar functional groups or depositing coatings with appropriate functionalities[4].

The mean pore size of silica-based SEC packings typically ranges from 6 to 100 nm, matching a molecular weight fractionation range of 5 to 2000 kilodaltons for globular proteins[1]. The internal porosities, expressed in terms of the specific pore volume, vary from 0.5 to 2.0 ml/g, with corresponding packing densities between 0.7 and 0.3 g/ml (ref. 3). The bonding chemistry follows one of two routes: either monolayer types of polar non-ionic functional groups (*e.g.*, diol, acetamido, ether) or coat-

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ings of hydrophilic organic polymers are bonded or deposited on silicas.The bonding chemistry and related techniques have been reviewed in depth[1,4].

Whereas the first generation of silica size-exclusion columns was packed with particles of mean diameter 10–12 μm, the second generation, marketed during the last 2 years, has a particle diameter, d_p, $\leqslant$ 5 μm. Typical products are the Zorbax Bio Series GF 250 and GF 450 columns from DuPont (Wilmington, DE, U.S.A.)[5], the TSK-Gel SWXL columns from Toyo Soda (Tonda, Japan)[6] and the Si 300 Polyol column from Serva (Heidelberg, F.R.G.)[7]. A reduction of the mean particle size by a factor of 2 from d_p = 10 to 5 μm increases the column plate number by the same factor at a constant column length and packing quality. Moreover, the optimum flow-rate at which the column shows its highest performance is doubled, shortening the analysis time 4-fold. The theoretical framework of optimum particle size for the resolution of proteins by SEC was developed by Guiochon and Martin[8].

This study was intended to elucidate the properties of two types of columns for the SEC of proteins, namely Zorbax Bio Series GF 250 and GF 450 and TSK-Gel SW and SWXL. In two sets of experiments, the following dependences were examined: (1) the elution volume, V_e, of proteins as a function of the salt concentration of the buffered eluent at constant pH and (2) the plate height, H, of proteins as a function of the flow-rate of the eluent at constant mobile phase composition.

EXPERIMENTAL

Columns (Table I)

The following columns were investigated: the Zorbax Bio Series GF 250 and GF 450 from DuPont (250 × 9.4 mm I.D.) and the TSK-Gel SW and SWXL columns from Toyo Soda (300 × 7.8 mm I.D.).

Eluents

Phosphate buffer (50 m*M*) was adjusted to pH 7.0 with sodium hydroxide solution and 0–500 m*M* sodium chloride was added to the buffer. The columns were stored in 0.05% (w/v) sodium azide solution.

Chromatographic equipment

Two sets of HPLC equipment were employed: (A) DuPont Series 8800 and LKB 2151 (Pharmacia LKB Biotechnology, Bromma, Sweden); (B) LKB 2150 pump

TABLE I

CHARACTERISTICS OF THE SEC COLUMNS EMPLOYED

Type	*Mean particle size, d_p (μm)*	*Molecular weight fractionation range for globular proteins (kilodaltons)*
Zorbax GF 250	4–5	10–250
Zorbax GF 450	6	25–900
TSK G3000SWXL	5	1–300
TSK G3000SW	10	1–300

(Pharmacia LKB), Rheodyne 7125 injector (ERC, Aldeglofsheim, F.R.G.), Biotronic 3030 (Biotronic, Maintal, F.R.G.) and Uvicord 2158 (LKB) detector.

Set A was used to study the dependence of the elution volume of selected proteins on the salt concentration of the eluent (range 0–500 m*M* sodium chloride, pH 7.0). Set B was used to measure the dependence of the plate height of selected proteins on the flow-rate of the eluent. Flow-rates were varied for the TSK columns from 0.02 to 1.0 ml/min and for the Zorbax columns from 0.02 to 2.0 ml/min.

The absorbance was monitored at 220 nm. The injection volume was 20 μl and the sample concentration varied from 0.3 to 1.0 mg/ml, depending on the UV absorbance of the corresponding protein or peptide.

Samples

All proteins, peptides and Dextran Blue were purchased from Serva: thyroglobulin from pig (660 kilodaltons), ferritin from horse spleen (450 kilodaltons), catalase from bovine liver (240 kilodaltons), γ-globulin from bovine serum albumin (169 kilodaltons), aldolase from rabbit muscle (150 kilodaltons), bovine serum albumin (BSA) (68 kilodaltons), ovalbumin (45 kilodaltons) and chymotrypsinogen A from bovine pancreas (25 kilodaltons), myoglobin from horse muscle (17.8 kilodaltons), lysozyme from egg white (14 kilodaltons), ribonuclease A from bovine pancreas (13 kilodaltons), trypsin inhibitor from bovine lung (6.5 kilodaltons), Glu–Thr–Tyr–Ser–Lys (627 daltons).

Dextran Blue (2000 kilodaltons) and sodium azide (65 daltons) served as the V_0 and V_m markers, respectively.

Calculations

The distribution coefficient is given by

$$K_{SEC} = \frac{V_e - V_0}{V_m - V_0} \tag{1}$$

where V_e is the elution volume of the protein, V_0 the interstitial volume corresponding to the elution volume of the V_0 marker (Dextran Blue, 2000 kilodaltons) and V_m the total permeation volume, corresponding to the elution volume of the V_m marker (sodium azide, 65 daltons).

The plate number is given by

$$N = 5.54\left(\frac{V_e}{w_{1/2}}\right)^2 = \frac{L}{H} \tag{2}$$

where V_e is the elution volume of the solute, $w_{1/2}$ the peak width at the peak half-height in volume units, L the column length and H the plate height.

The peak capacity is given by

$$n = 1 + \frac{\bar{N}^{1/2}}{4} \ln\left(\frac{V_{e2}}{V_{e1}}\right) \tag{3}$$

where V_{e1} and V_{e2} are the elution volume of the first and the last eluted peak, respectively, and $\bar{N}$ the average column plate number.

RESULTS AND DISCUSSION

Prediction of performance of SEC columns

To compare the performances of columns containing packings of different particle size and operated with solutes of widely different molecular weight, dimensionless reduced parameters have commonly been employed, such as the reduced plate height, h, and the reduced linear velocity of the eluent, v. These are defined as follows:

$$h = \frac{H}{d_p} \tag{4}$$

$$v = \frac{ud_p}{D_{im}} \tag{5}$$

where d_p is the mean particle size of the packing, u the linear velocity of eluent and D_{im} the diffusion coefficient of the solute in the eluent.

Whereas in interactive high-performance liquid chromatography u corresponds to the average linear flow velocity of an unretained, totally permeating solute (denoted by u_m), in SEC one often refers to the linear flow velocity of a totally excluded solute (denoted by u_0)[8]. The numerical values of u_m and u_0 differ by a factor corresponding to the ratio of the total to the interstitial porosity of the SEC column. In this paper, u_m is used. It is calculated from the retention time of a totally permeating compound, divided by the column length.

The flow dependence of the reduced plate height has been shown to be approximated by the following equation:

$$h = \frac{b}{v} + av^{1/3} + cv \tag{6}$$

where a is a constant reflecting the packing quality of the column (a = 1–2), b is a constant related to the eddy diffusion (b = 1.2–1.5) and c a constant associated with the mass transfer kinetics (c = 0.02–0.1)[9–15]. A plot h against v gives a minimum at about h = 2 and v = 3. The general validity of this approach allows the computation of the optimum flow-rate of the eluent at which the smallest plate height of a SEC column of given dimensions is achieved for a solute by using the two equations:

$$u_m = \frac{3D_{im}}{d_p} \tag{7}$$

and

$$f_{v(opt)} = \frac{\pi d_c^2}{4} \cdot \varepsilon_t u_m \tag{8}$$

where d_c is the column diameter, ε_t the total porosity of the column and u_m the linear velocity; ε_t is calculated by dividing the elution volume of a total permeating solute (sodium azide) by the geometrical column volume.

Table II lists the flow-rates for solutes of molecular weight between 1000 and 1 000 000. The diffusion coefficients, D_{im}, were taken from ref. 8, calculated by using the Stokes–Einstein equation for globular molecules.

The calculated optimum volume flow-rate of both columns ranged between 0.1 and 0.5 ml/min, depending on the molecular weight of the solute to be resolved within the molecular weight fractionation range. The experimental data derived from the measured H *vs.* f_v curves in Figs. 2 and 4 were of the order of magnitude of the predicted values.

Curve fitting of the experimental data in Figs. 2 and 4 by eqn. 6 allows the calculation of a, b and c for both columns. For the Zorbax Bio Series GF 250 column the following values were obtained: Glu–Thr–Tyr–Ser–Lys (627 daltons), $a = 1.24$, $b = 4.40$; myoglobin (17.8 kilodaltons), $a = 1.24$, $b = 4.40$; bovine serum albumin (68 kilodaltons), $a = 0.88$, $b = 4.6$, $c = 0.02$. For TSK-Gel 3000 SWXL only the values for myoglobin (17.8 kilodaltons) were calculable as $a = 2.43$ and $b = 16.4$.

It can be seen that the values of a fall in the range observed for well packed columns. The constant b was found to be higher than reported. The c value of BSA on the Zorbax Bio Series GF 250 column reflects the excellent mass transfer, as expressed by the flat part of the H *vs.* f_v curve at higher flow-rates.

The peak capacities, n, calculated from eqn. 3 are *ca.* 20–30 for both columns (GF 250 and G 3000 SWXL) with an eluent flow-rate range between 0.15 and 0.30 ml/min and demonstrate their high column performance and resolving power.

TABLE II

CALCULATED OPTIMUM VOLUME FLOW-RATE, $f_{v(opt)}$, SETTING $v = 3$ FOR GLOBULAR PROTEINS ON THE ZORBAX BIO SERIES GF 250 COLUMN (I.D. = 9.4 mm, $\varepsilon_t = 0.71$) AND THE TOYO SODA TSK G 3000 SWXL COLUMN (I.D. = 7.8 mm, $\varepsilon_t = 0.89$)

The values in parentheses are the $f_{v(opt)}$ values taken from the minima of the measured H *vs.* f_v dependences for proteins in Figs. 2 and 4.

Solute mol. wt. (kilodaltons)	D_{im} *(10^{10} m²/s) (T = 308 K)*	$f_{v(opt)}$ *(ml/min)*	
		Zorbax GF 250 column	*TSL-Gel 3000 SWXL column*
1	3.54	0.71 (0.73)*	0.54
10	1.64	0.33 (0.23)**	0.25 (1.0)**
100	0.77	0.15 (0.12)***	0.11 (0.02)***
1000	0.35	0.07	0.06

* Peptide (627 daltons).
** Myoglobin (17.8 kilodaltons).
*** BSA (68 kilodaltons).

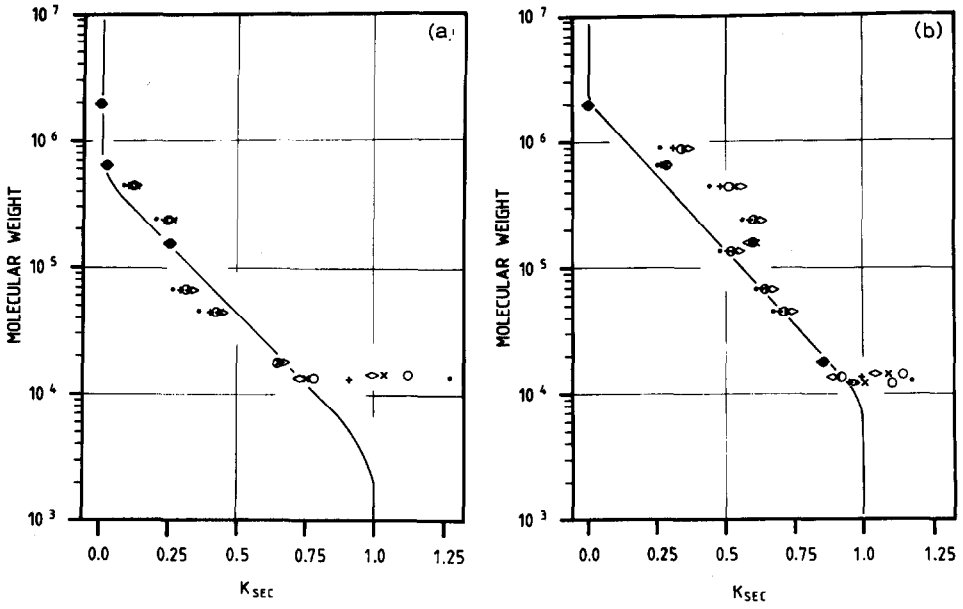

Fig. 1. Calibration graph for proteins on (a) Zorbax Bio Series GF 250 and (b) GF 450 columns (308 K). Eluent: 50 m*M* phosphate buffer (pH 7.0) with (□) 0 m*M* NaCl; (●) 100 m*M* NaCl; (+) 200 m*M* NaCl; (○) 300 m*M* NaCl; (×) 400 m*M* NaCl; (◇) 500 m*M* NaCl.

Zorbax Bio Series GF 250 and 450 columns

The Zorbax Bio Series GF columns contain a 15- and 30-nm pore-size silica, which is stabilized with zirconia and functionalized with diol groups[16]. Fig. 1a and b show the calibration graphs of the two columns under identical conditions. They also allow the effect of salt concentration on the elution volume of proteins to be determined. The observed alterations of V_e with the salt concentration enabled us to relate these shifts to certain types of interaction other than size exclusion. On both columns, V_e of proteins for the upper part of the calibration graph is seen to be slightly enhanced with increasing salt concentration, attributed (1) to additional hydrophobic interactions between the proteins and the surface of the packing and (2) to the diminution of electrostatic repulsion forces between the negatively charged sorbent surface and the proteins with a negative net charge. Similar observations were made by Stout and DeStefano[5]. These secondary effects produce an S-shaped form of the upper part of the calibration graph for the GF 450 column.

For basic proteins with an isoelectric point of pI > 9, such as lysozyme, the elution volume decreased with increasing salt concentration, reflecting the suppression of electrostatic interactions between the negatively charged sorbent surface and the positively charged proteins. Therefore, it is recommended that basic proteins be chromatographed on these columns at a salt concentration of about 0.5 *M*.

Inspection of the dependences of plate height on flow-rate derived for selected proteins on both columns revealed that H values between two and three times the particle size were achieved at the optimum flow-rates of 0.1–0.5 ml/min (Fig. 2a and

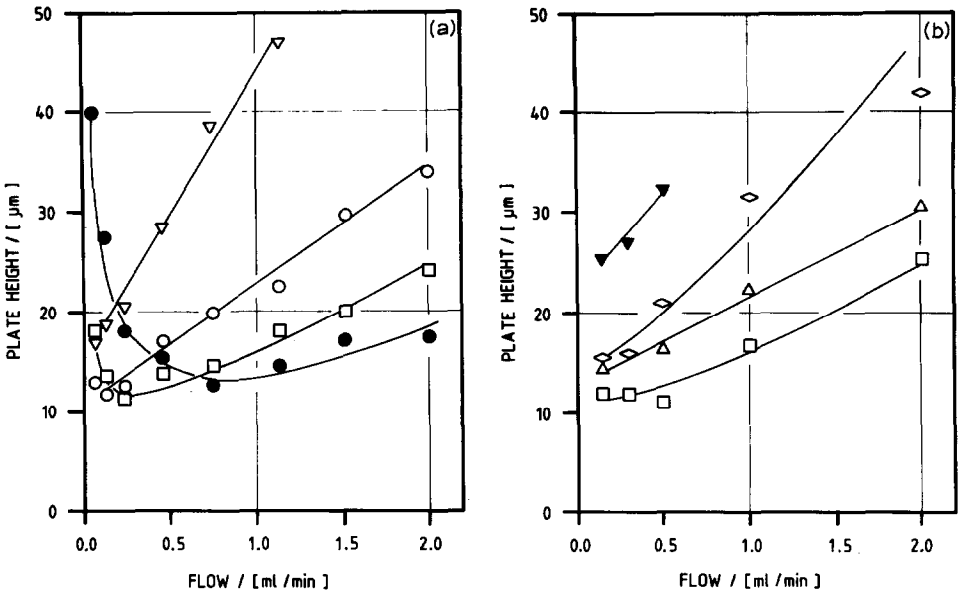

Fig. 2. Plate height, H, of proteins as a function of volume flow-rate, f_v, of selected proteins in Zorbax Bio Series (a) GF 250 and (b) GF 450 columns (308 K). Eluent: 50 mM phosphate buffer (pH 7.0) + 300 mM NaCl. Proteins: ▼, thyroglobulin (660 kilodaltons); ▽, ferritin (450 kilodaltons); ◇, catalase (240 kilodaltons); ○, BSA (68 kilodaltons); △, ovalbumin (45 kilodaltons); □, myoglobin (17.8 kilodaltons); ●, Glu–Thr–Tyr–Ser–Lys (627 daltons).

b). Higher flow-rates than the optimum can be used to shorten the analysis time with little decrease in resolution.

In conclusion, the Zorbax Bio Series GF columns offer a high flexibility of choice between optimum resolution and short analysis time.

TSK-Gel 3000 SW and SWXL columns

The TSK-Gel 3000 SW column is one of the most popular columns for the SEC of proteins. Calibration graphs for proteins on the TSK G 3000 SW columns have been thoroughly investigated[10]. As shown in Fig. 3, the elution volumes of proteins are scarcely affected by the salt concentration of the eluent. The TSK-Gel 3000 SWXL column exhibits a much wider scatter of V_e of a given protein as a function of the salt concentration (Fig. 3b), as observed for the 3000 SW column (Fig. 3a). For the TSK-Gel 3000 SWXL column, electrostatic exclusion effects appear to be dominant, causing an earlier elution of proteins than expected, in addition to hydrophobic interactions, generated at higher salt concentrations. Apparently, the degrees of homogeneity of the stationary surface are not identical for the two columns. Also, a slightly lower fractionation range of the 3000 SWXL column compared with the 3000 SW column is noted.

The improvement in column performance on going from the SW to the SWXL column is evident by comparing the H *vs.* f_v curves (Fig. 4a and b). The column plate height for BSA, for instance, decreases by a factor of about two, as expected from

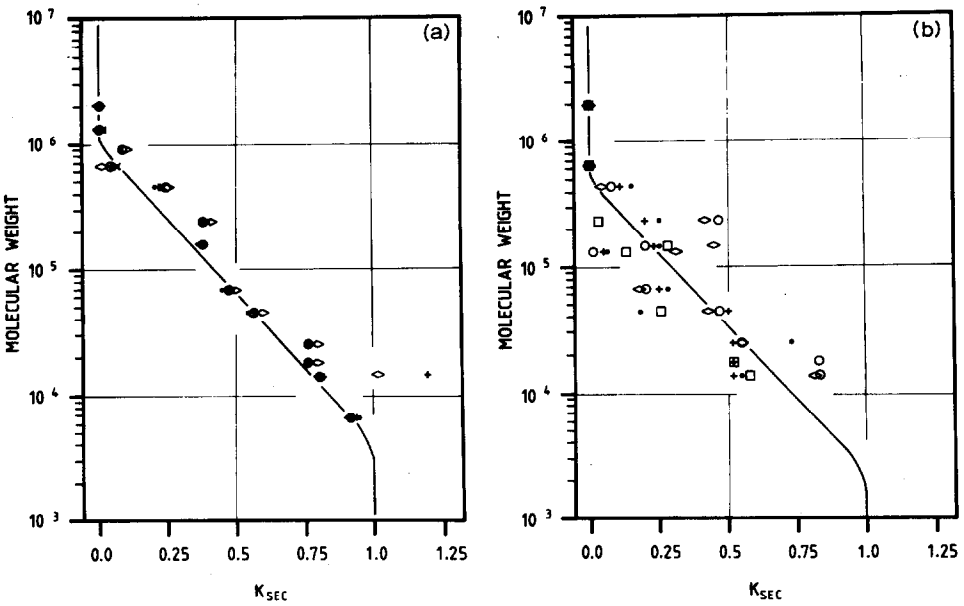

Fig. 3. Plate height, H, as a function of volume flow-rate, f_v, for selected proteins in (a) TSK Gel 3000 SW and (b) SWXL columns. Conditions as in Fig. 2.

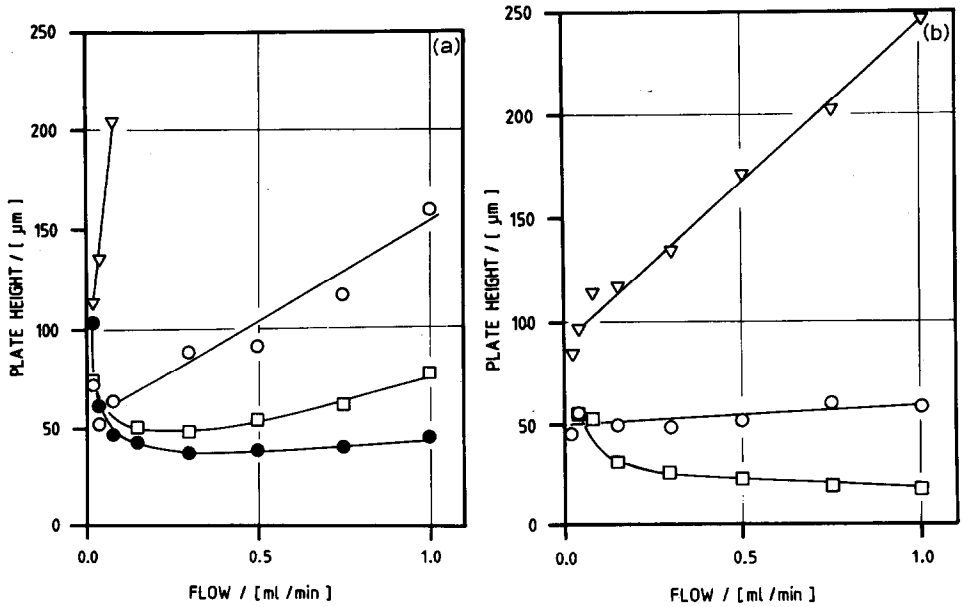

Fig. 4. Calibration graph for proteins on (a) TSK-Gel 3000 SW and (b) SWXL columns (308 K). Conditions as in Fig. 1. The data points at molecular weights of $9 \cdot 10^5$ and $1.4 \cdot 10^6$ refer to the dimer and trimer of ferritin, respectively.

the reduction in particle size from d_p = 10–12 to 5 μm. However, the plate heights at the optimum eluent flow-rates are much higher than twice the mean particle diameter ($H \approx 5d_p$). This is probably due to the fact that the highly porous particles of the TSK-Gel 3000 SW and SWXL limit the packing pressure required to achieve dense column beds. The flat part of the H *vs.* f_v curve of the 3000 SWXL column at $f_v > 0.5$ ml/min suggest the use of higher flow-rates, shortening the analysis time without a decrease in column efficiency. However, owing to the great fragility of the porous particles, the column cannot be operated at flow-rates above 1.0 ml/min.

TSK-Gel 3000 SWXL vs. Zorbax Bio Series GF 250 columns

Both columns resolve proteins according to a size-exclusion mechanism within about the same molecular weight range. In contrast to the GF 250 column, the TSK-Gel 3000 SWXL column exhibits a more hydrophobic stationary surface, and this results in a greater scatter of the elution volume of some proteins when the salt concentration is varied. Adsorption interactions were observed for proteins of pI > 9 on the GF 250 column. They can be suppressed by increasing the salt concentration to 0.5 M. Although the TSK-Gel 3000 SWXL column gave lower plate heights of $H \approx 5d_p$ at the optimum flow-rate compared with the GF 250 column ($H \approx 2$-$3d_p$), this disadvantage in terms of resolution is compensated for by the higher internal column porosity (0.53 to 0.32) and phase ratio (1.49 to 0.82) of the 3000 SWXL column. Apart from the deviations of V_e for certain proteins that are sensitive to the salt concentration, the resolutions of both columns are nearly identical. It is worth noting that SEC separations on the Zorbax Bio Series GF 250 column can be carried out at flow-rates higher than 1.0 ml/min without much decrease in column perrformance but with a gain in the speed of analysis.

CONCLUSION

The results confirm the theoretical predictions that the column performance in the SEC of proteins can be improved by reducing the particle diameter of the packing from 10 to 5 μm. A further reduction in the particle size of silica based silica packings to about 1 μm seems to be possible[16–18]. However, before the separation potential of such columns can be realized, a number of experimental and instrumental problems must be solved. First, the current manufacture and sizing technology yields 1–2-μm particles with a much wider particle size distribution than that in the 5-μm materials. Second, the packing of highly efficient columns, even with narrow-sized 1–2-μm particles, becomes a difficult task. Third, columns packed with 1–2-μm particles generate a high back-pressure, which is limited to *ca.* 40 MPa by the instrument design. Fourth, frictional heat effects, causing a radial temperature gradient within the column, can give rise to additional peak broadening.

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