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EFFECTS OF SOLUTIONS USED FOR STORAGE OF SIZE-EXCLUSION COLUMNS ON SUBSEQUENT CHROMATOGRAPHY OF PEPTIDES AND PROTEINS

G. W. LINK, Jr.* and P. L. KELLER
Department of Surgery and Dental Research Center, University of North Carolina, Chapel Hill, NC 27514 (U.S.A.)
R. W. STOUT
E. I. du Pont-de Nemours & Co., Inc., Biomedical Products Department, Wilmington, DE (U.S.A.)
and
A. J. BANES
Department of Surgery and Dental Research Center, University of North Carolina, Chapel Hill, NC 27514 (U.S.A.)
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SUMMARY

The effects of storage of size-exclusion column packing materials in methanolic or azide-water solutions on subsequent separations were tested. Three commercially available columns were used in these studies; the Toyo-Soda Bio-Sil TSK 125, Bio-Sil TSK 250 and the DuPont Bio-Series GF-250. Upon initial chromatography, all three columns bound up to 760 μg of cytochrome *c* tryptic peptides. Sample binding to packing material is probably a function of the positively charged basic groups on peptides or proteins interacting with silanol groups. The larger the peptide, the less the opportunity for silanol-charged group interaction, hence, less binding. Initial samples introduced to a new column occupy the binding sites. Equilibration with neat methanol removes the bound protein revealing sites which bind sample. After absorption of peptides to binding sites on the packing material, storage in neat methanol regenerates the binding sites. Storage in 10% methanol diminished the binding phenomenon, but storage in azide–water reduced binding to a range below detection at the microgram level. Our recommendation to users of size-exclusion chromatographic columns is that one satisfy the absorption capacity of a new column by injecting a sufficient quantity of a basic peptide standard or other convenient sample to reduce available binding sites before using the column for important separations. Store columns in azide–water or 10% methanol to prevent the regeneration of exposed silanol groups.

INTRODUCTION

There is little published information on the effect of column storage media on

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size-exclusion chromatography (SEC) of peptides and proteins. Column manufacturers recommend using methanol, methanol–water or water–azide mixtures to protect against microbial growth, desiccation of the packing material or corrosion of column, tubing, fittings and pumps[1–3]. In addition, we have found that the selection of storage media must be made with regard to its effects on sample binding and recovery from the column. Users of SEC columns need to be aware of the influence of storage media on the packing material in order to optimize separations and recovery.

In the course of evaluating separations of peptides and proteins on several SEC columns[4], we observed a non-linear relationship between injected mass and area detected after column storage in methanol. New or used columns taken out of storage and equilibrated in various mobile phases, yielded inconsistent peak area to mass ratios among series injections. This phenomenon constituted an absorption of peptides of 1000 dalton average molecular weight (cytochrome *c* tryptic digest), or for certain basic proteins such as cytochrome *c* (11.5 kilodalton). This absorption was minimized when the columns were stored in azide–water. If the columns were equilibrated and stored in methanol, peptide absorption recurred. The binding phenomenon was detectable but not as great for proteins as for peptides. We report here on the effects of column storage in methanol (neat), 10% methanol, and azide–water on subsequent separations of cytochrome *c* tryptic peptides and a standard mixture of thyroglobulin, ovalbumin, and cytochrome *c*.

EXPERIMENTAL

Apparatus

SEC columns used were silica-based TSK 2000 (Bio-Sil TSK 125, Bio-Rad Labs., Richmond, CA, U.S.A.), 30 × 0.75 cm I.D.; TSK 3000 (Bio-Sil TSK 250), 30 × 0.75 cm I.D.; and zirconium-silica based GF-250 (Zorbax GF-250 Bio Series, DuPont, Wilmington, DE, U.S.A.), 25 × 0.94 cm I.D. (Table I). Other characteristics such as plate number and peak capacity of these SEC columns are described by Banes

TABLE I

CHARACTERISTICS OF GF-250, TSK-2000, AND TSK-3000 SIZE EXCLUSION COLUMNS

V_0, Elution time of unretained solute; V_t, elution time for total permeation.

Parameter	*Column*		
	GF-250 (Zorbax Bio Series)	*TSK-3000 (Bio-Sil TSK-250)*	*TSK-2000 (Bio-Sil TSK-125)*
Nominal molecular weight range (kD)	4–400	1–300	0.5–60
Nominal particle diameter (μm)	4	10	10
Nominal pore size (Å)	150	250	125
Length × I.D. (cm)	25 × 0.94	30 × 0.75	30 × 0.75
Internal volume (ml)	17.35	13.25	13.25
V_0 (min)	6.3	5.0	5.2
V_t (min)	13.2	11.9	10.2

et al.[4]. A 2 × 0.2 cm I.D. guard column, dry packed with the same packing material preceded the resolving column. An 8 × 0.62 cm I.D. pre-column (DuPont ODS, 18-μm particle diameter) was used between the pump (Waters M45, Waters Assoc., Milford, MA, U.S.A.) and the autosampler (Waters WISP). A Rheodyne 7060 column switching device (Rheodyne, Cotati, CA, U.S.A.) was used to select the appropriate column. Absorbance was monitored at 214 nm for peptides and 220 nm for proteins with a variable-wavelength UV spectrophotometer (Waters 450). Peptide and protein recoveries were quantitated by calculating the area under peaks using a chromatography integration system (Dynamic Solutions, Pasadena, CA, U.S.A.) with an Apple II+.

Chromatographic conditions

The flow-rate was 1 ml/min. All separations were made at room temperature (*ca.* 25°C). The mobile phase used for peptides was 0.1 *M* phosphate, pH 6.8 and for proteins, 0.1 *M* phosphate, 0.3 *M* sodium chloride, pH 7.0. Mobile phases were filtered through a sintered, fine glass filter and vacuum degassed prior to being used.

Sample preparation

Tryptic peptides were prepared from 50 mg of horse heart cytochrome *c* (Sigma C-2506) as described by Banes *et al.*[5] and dissolved in 0.02 *M* ammonium hydrogencarbonate. The protein was heat denatured at 60°C for 20 min then digested for 3 h at 37°C with 1% (w/w) TPCK trypsin (10 mg/ml in 0.01 *M* hydrochloric acid to inactivate chymotrypsin). The solution was then heat denatured at 60°C for 20 min and cooled to 37°C then digestion continued with the addition of 0.5% (w/w) of TPCK trypsin for 1 h at 37°C. The solution was lyophilized and reconstituted at 1 mg/ml in 0.1 *M* phosphate, pH 6.8.

A protein mixture containing thyroglobulin (Sigma T-1001), ovalbumin (Sigma A-7642), and cytochrome *c* (Sigma C-2506) at a concentration of 0.333 mg/ml each was prepared in 0.1 *M* phosphate–0.3 *M* sodium chloride, pH 7, with 9 mg/l sodium azide added as an antimicrobial compound during storage.

Storage conditions

Storage conditions were chosen to represent the typical use and storage of SEC columns in the laboratory. In the first experiment SEC of cytochrome *c* tryptic peptides were examined under four storage conditions, I–IV. (I) Pristine columns were equilibrated with 10 column volumes of methanol (neat) and stored for at least 12 h. (II) After use in phase I, columns were equilibrated with 10 column volumes of 0.14 m*M* sodium azide in water and stored for at least 12 h. (III) After use in phases I and II, columns were equilibrated with 10 column volumes of methanol (neat) and stored for at least 12 h. (IV) After use in phases I, II and III, columns were equilibrated with 10 column volumes of 10% methanol and stored for at least 12 h.

SEC of the protein mixture of thyroglobulin, ovalbumin, and cytochrome *c* was examined under two storage conditions, V and VI. (V) After use in phases I–IV, columns were equilibrated with 10 column volumes of 0.14 m*M* sodium azide and stored for at least 12 h. (VI) After use in phases I–V, columns were equilibrated with 10 column volumes of methanol (neat) and stored for at least 12 h.

Methods

All columns were equilibrated with 10 column volumes of deionized water and then equilibrated with 6–10 column volumes of the appropriate mobile phase prior to injection. An injection series of cytochrome *c* tryptic peptides increasing in concentration from 10 μg to 100 μg, in 20 μg increments (except under condition I where 10 μg increments were used), then decreasing in concentration from 90 μg to 10 μg, in 20 μg increments, was made into each column under conditions I–IV.Injection volumes ranged from 10 to 100 μl. At the end of the injection series, the columns were equilibrated with 10 column volumes of deionized water, then the appropriate storage medium and stored for at least 12 h.

In the second experiment (storage conditions V and VI), an injection series of the protein mixture was made into each column. The sample mass was increased from 3.33 to 33.3 μg (of each protein in the mixture; i.e. 100 μg total protein), then decreased from 29.97 to 3.33 μg (of each protein), in 3.33 μg increments. Injection volumes were 10 to 100 μl.

RESULTS

Condition I

Cytochrome *c* tryptic peptides were eluted in pristine SEC columns previously stored in methanol (neat). For each column, peak areas of initial injections (10–30

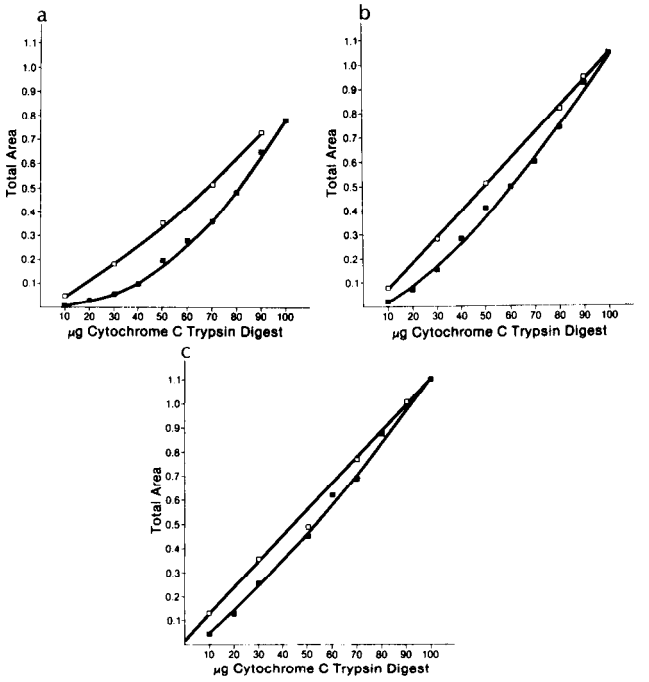


Fig. 1. Mass *versus* peak area for increasing (solid symbols) and decreasing concentrations (open symbols) of cytochrome *c* tryptic digest injected into pristine (a) GF-250, (b) TSK 3000 and (c) TSK 2000 SEC columns stored in neat methanol (I). Chromatographic conditions: flow-rate, 1 ml/min; mobile phase, 0.1 *M* phosphate, pH 6.8; detection, UV absorbance at 214 nm.

μg) were most affected. Peak areas of the increasing injection series were reduced when compared to corresponding areas of the decreasing series (30 to 10 μg of tryptic digest) (Fig. 1). Fig. 1 indicates that the relationship between peak area and injected mass was non-linear in each column tested. The percentage difference in recovery [(1 − total area increasing/total area decreasing) × 100] between increasing (10 to 90 μg) and decreasing concentrations (90 to 10 μg) were 13.6%, TSK 2000; 20.0%, TSK 3000; and 30.8%, GF-250 (Table II). Recovery became linear after 760 μg of cytochrome *c* tryptic peptides had been injected into the GF-250 column (Fig. 1a) and after 640 μg in the TSK columns (Fig. 1b and c).

Condition II

Cytochrome *c* tryptic peptides were chromatographed in columns stored in 0.14 m*M* sodium azide. There was a linear relationship between peak area and injected mass for each column (Fig. 2). Correlation coefficients (*r*) for least squares linear regressions of peak area and injected mass were $r = 0.9967$, GF-250; $r = 0.9992$, TSK 3000; and $r = 0.9989$, TSK 2000. The percentage differences in total area between increasing and decreasing concentrations were 3.88%, TSK 2000; 4.35%, TSK 3000; and 1.12%, GF-250 (Table II).

TABLE II

EFFECTS OF STORAGE CONDITIONS ON QUANTITATION OF TRYPTIC PEPTIDES

Chromatographic conditions, isocratic: flow-rate, 1 ml/min; UV absorbance monitored at 214 nm; buffer, 0.1 *M* phosphate, pH 6.8; ambient temperature (*ca.* 25°C).

Column	*Storage media*	*Area increasing* (Vmin)*	*Area decreasing** (Vmin)*	*Difference (%)****
Pristine				
GF-250	Methanol (neat)	1.2769	1.8461	30.83
TSK 3000		2.1175	2.6467	20.00
TSK 2000		2.4535	2.8387	13.57
GF-250	Azide	1.7362	1.7169	1.12
TSK 3000		1.8797	1.9653	4.35
TSK 2000		2.6178	2.7235	3.88
GF-250	Methanol (neat)	1.0869	1.5320	29.05
TSK 3000		0.5587	0.7597	26.46
TSK 2000		2.2323	2.6056	14.33
GF-250	Methanol (10%)	1.8401	1.9451	5.40
TSK 3000		2.4217	2.6672	9.21
TSK 2000		2.4706	2.6882	8.10

* Area increasing is the sum of the peak areas in a series of injections, increasing in concentration from 10 to 90 μg of cytochrome *c* tryptic peptides.

** Area decreasing is the sum of the peak areas in a series of injections, decreasing in concentration from 90 to 10 μg of cytochrome *c* tryptic peptides.

*** Percent difference = [(1 − area increasing/area decreasing) × 100]. Large differences indicate peptide absorption.

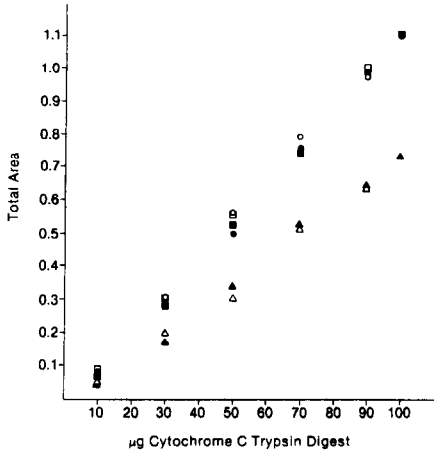


Fig. 2. Mass *versus* peak area for increasing (solid symbols) and decreasing concentrations (open symbols) of cytochrome *c* tryptic digest injected into SEC columns used under condition I, stored in 0.14 m*M* sodium azide then used under condition II. (○) TSK 2000, (□) TSK 3000, (△) GF-250. Chromatographic conditions as in Fig. 1.

Condition III

Columns which had been used under conditions I and II, and which yielded a linear relationship between injected mass and peak area were equilibrated and stored next in methanol (neat) for at least 12 h before chromatography of cytochrome *c* tryptic peptides. Peak areas of the initial injections (10, 30, and 50 μg) were reduced particularly in the GF-250 column (Fig. 3). The relationship between mass and area became linear after chromatography of 410 μg of cytochrome *c* tryptic peptides in

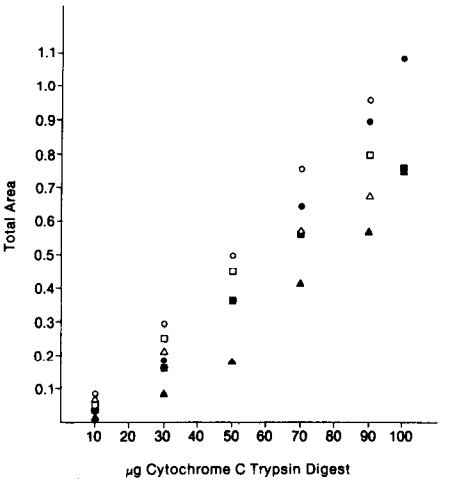


Fig. 3. Mass *versus* peak area for increasing (solid symbols) and decreasing concentrations (open symbols) of cytochrome *c* tryptic digest injected into SEC columns used under conditions I and II stored in neat methanol then used under condition III. Other conditions as in Fig. 2.

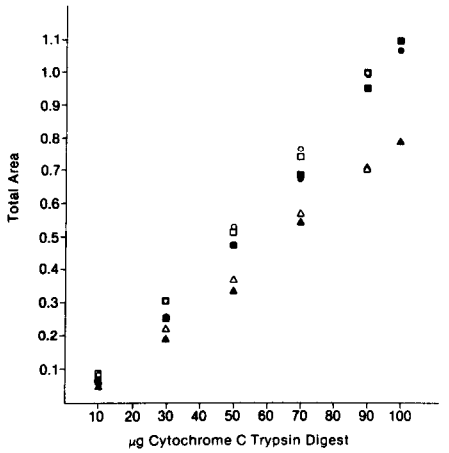


Fig. 4. Mass *versus* peak area for increasing (solid symbols) and decreasing concentrations (open symbols) of cytochrome *c* tryptic digest injected into SEC columns used under conditions I, II and III stored in 10% methanol then used under condition IV. Other conditions as in Fig. 2.

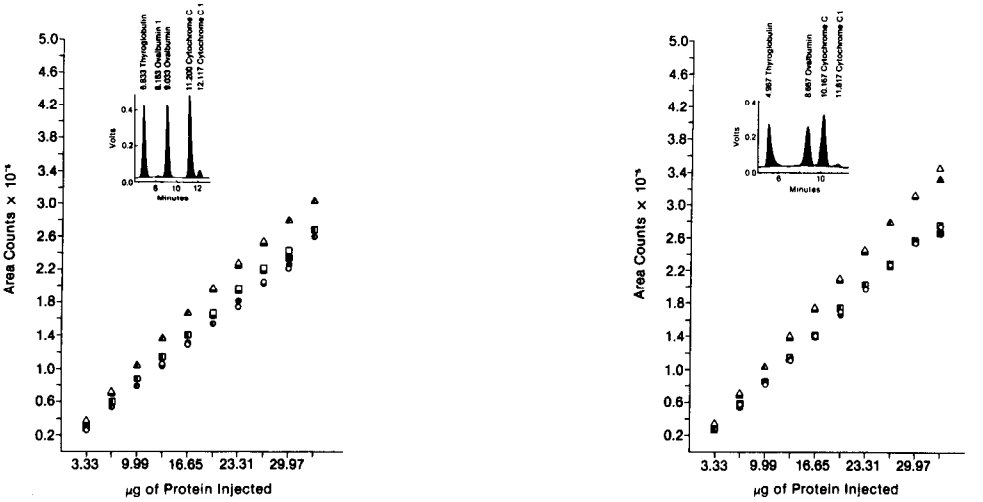


Fig. 5. Mass *versus* peak area for increasing (solid symbols) and decreasing concentrations (open symbols) of thyroglobulin, ovalbumin, and cytochrome *c* injected as a protein mixture into a GF-250 column previously stored in 0.14 m*M* sodium azide (V). A typical separation of the above protein mixture under these storage conditions is shown in the chromatogram (insert). Chromatographic conditions: flow-rate, 1 ml/mm; mobile phase 0.1 *M* phosphate–0.3 *M* sodium chloride, pH 7.0; detection, UV absorbance at 220 nm. (□) Ovalbumin, (○) thyroglobulin, (△) cytochrome *c*.

Fig. 6. Mass *versus* peak area for increasing (solid symbols) and decreasing concentrations (open symbols) of thyroglobulin, ovalbumin, and cytochrome *c* injected as a protein mixture into a TSK 3000 column previously stored in 0.14 m*M* sodium azide (V). A typical separation of the above protein mixture under these storage conditions is shown in the chromatogram (insert). Other conditions as in Fig. 5.

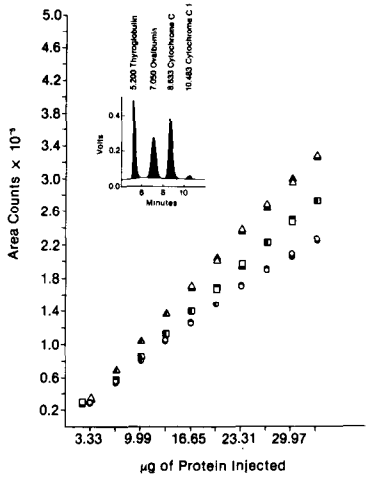
the GF-250 column, and after 340 μg in the TSK columns (Fig. 3). The percentage differences in total area between increasing (10 to 90 μg) and decreasing (90 to 10 μg) concentrations were 14.33%, TSK 2000; 26.46%, TSK 3000; and 29.05%, GF-250 (Table II).

Condition IV

Columns used in the previous work were stored in 10% methanol for at least 12 h prior to being tested. The relationship between mass and area was linear for each column (Fig. 4). Linear regression correlation coefficients (r) for the GF-250, TSK 3000 and TSK 2000 were $r = 0.9975$, $r = 0.9962$ and $r = 0.9965$, respectively. Percent differences in total area were 5.40%, GF-250; 8.10%, TSK 2000; and 9.21% TSK 3000 (Table II).

Condition V

Columns used in the previous phases were stored in 0.14 m*M* sodium azide before chromatography of a protein mixture of thyroglobulin, ovalbumin, and cytochrome *c*. The relationships between peak area and injected mass of each protein were linear in all three columns (Figs. 5–7). Linear regression correlation coefficients were high for all columns, ranging from 0.9978 to 0.9998. Differences in total areas between increasing and decreasing concentrations for proteins were less than 1.5% (Table III).



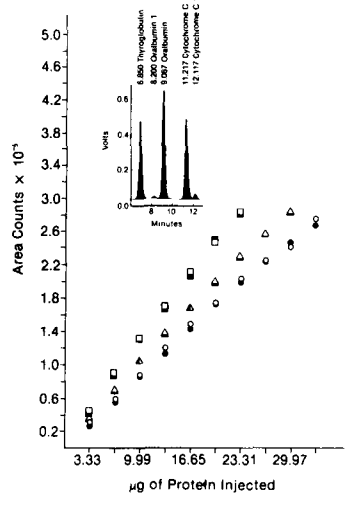


Fig. 7. Mass *versus* peak area for increasing (solid symbols) and decreasing concentrations (open symbols) of thyroglobulin, ovalbumin, and cytochrome *c* injected as a protein mixture into a TSK 2000 column previously stored in 0.14 m*M* sodium azide (V). A typical separation of the above protein mixture under these storage conditions is shown in the chromatogram (insert). Other conditions as in Fig. 5.

Fig. 8. Mass *versus* peak area for increasing (solid symbols) and decreasing concentrations (open symbols) of thyroglobulin, ovalbumin, and cytochrome *c* injected as a protein mixture into a GF-250 column previously stored in neat methanol (VI). A typical separation of the above protein mixture under these storage conditions is shown in the chromatogram (insert). Other conditions as in Fig. 5.

Conditions VI

The protein mixture was injected into columns that had been used under condition V and then stored in methanol (neat) for at least 12 h. The relationships between peak area and injected mass were linear for each column (Figs. 8–10), with correlation coefficients ranging from 0.9881 to 0.9997. Differences in total area of increasing versus decreasing injections for proteins were less than 5% (Table III).

DISCUSSION

Columns exposed to methanol absorbed cytochrome *c* tryptic peptides. The observed hysteresis between increasing and decreasing concentrations of cytochrome

TABLE III

EFFECTS OF STORAGE CONDITIONS ON QUANTITATION OF PROTEINS

Chromatographic conditions, isocratic: flow-rate, 1 ml/min; UV absorbance monitored at 220 nm; buffer, 0.1 *M* phosphate, 0.3 *M* sodium chloride, pH 7; ambient temperature (*ca* 25°C).

Storage media	*Protein*	*Column*	*Area increasing (Vmin)*	*Area decreasing (Vmin)*	*Difference (%)*
0.14 m*M* Azide	Thyroglobulin 660 kD	GF-250	14.296	14.202	0.66
		TSK 3000	15.362	15.501	0.90
		TSK 2000	13.462	13.509	0.35
	Ovalbumin 45 kD	GF-250	15.147	15.294	0.96
		TSK 3000	15.736	15.918	1.15
		TSK 2000	15.325	15.341	0.11
	Cytochrome *c* 11.5 kD	GF-250	17.689	17.803	0.64
		TSK 3000	18.921	19.192	1.41
		TSK 2000	18.498	18.549	0.27
Methanol (neat)	Thyroglobulin	GF-250	15.421	15.768	2.20
		TSK 3000	15.326	16.108	4.86
		TSK 2000	14.635	14.725	0.61
	Ovalbumin	GF-250	11.717	11.879	1.32
		TSK 3000	24.917	25.246	1.31
		TSK 2000	26.340	26.529	0.61
	Cytochrome *c*	GF-250	14.793	14.881	0.59
		TSK 3000	19.407	19.476	0.36
		TSK 2000	19.566	19.695	0.66

* Area increasing is the sum of the peak areas (of each protein) in a series of injections, increasing in concentration from 3.33 to 29.97 μg per protein of a mixture containing thyroglobulin, ovalbumin, and cytochrome *c*.

** Area decreasing is the sum of the peak areas (of each protein) in a series of injections, decreasing in concentration from 29.97 to 3.33 μg per protein of a mixture containing thyroglobulin, ovalbumin, and cytochrome *c*.

*** Percent difference = [(1 − area increasing/area decreasing) × 100]. Large differences indicate protein absorption.

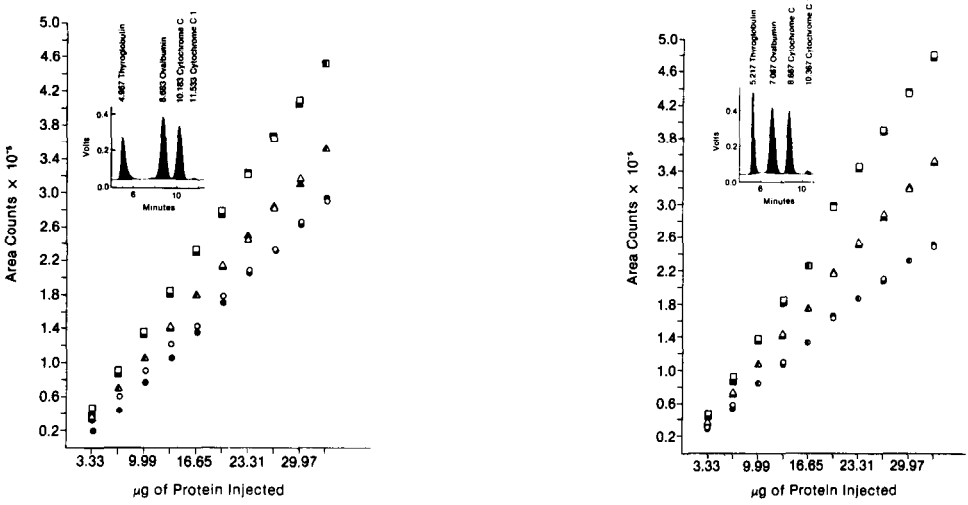


Fig. 9. Mass *versus* peak area for increasing (solid symbols) and decreasing concentrations (open symbols) of thyroglobulin, ovalbumin, and cytochrome *c* injected as a protein mixture into a TSK 3000 column previously stored in neat methanol (VI). A typical separation of the above protein mixture under these storage conditions is shown in the chromatogram (insert). Other conditions as in Fig. 5.

Fig. 10. Mass *versus* peak area for increasing (solid symbols) and decreasing concentrations (open symbols) of thyroglobulin, ovalbumin, and cytochrome *c* injected as a protein mixture into a TSK 2000 column previously stored in neat methanol (VI). A typical separation of the above protein mixture under these storage conditions is shown in the chromatogram (insert). Other conditions as in Fig. 5.

c tryptic digest was greatest for the columns stored in neat methanol (Fig. 1). As the total amount of tryptic digest chromatographed increased, the recovery curves became more linear. This decrease in column "appetite" for basic peptides has been noted by others[6,7].

Protein binding was less sensitive to storage conditions. Relationships between area and mass were linear for proteins chromatographed on columns stored in both 0.14 m*M* sodium azide and in neat methanol. Increases in slopes of the regression lines for proteins separated on columns stored in neat methanol resulted from degradation of the sample (Figs. 8–10). A fresh sample of the protein mixture was chromatographed in the columns; slopes were similar to those from columns stored in azide. Differences in area between increasing and decreasing concentrations were less than 5% (Table III), indicating minor binding of the proteins. These values were in agreement with the two manufacturer's advertised claims of 95% average recovery rates[8,9], as well as those reported (90 to 95% for TSK 2000 and 3000) by Pfannkock *et al.*[10].

The function of the bonded phase in size exclusion columns, is to create a hydrophilic layer that will deactivate the silica surface on the stationary phase[10]. Anhydrous methanol, by displacing water may reduce the hydrophilic layer in the bonded phase and increase accessibility to surface silanols on the packing material. When the columns are exposed to aqueous eluents, some of these silanols may be available as ionic binding sites. Hydrophobic interactions are also enhanced in polar solvents[11]. Ionic interactions between the amino groups (as NH_3^+) of the peptides

and exposed silanol groups on the packing material, as well as hydrophobic attractions, result in retention of peptides on the columns. As available active sites are filled by absorbed peptides, binding decreases or reaches equilibrium and sample recovery becomes linear.

Greater internal volume (17.35 ml in GF-250 *versus* 13.25 ml in TSK Bio-Sil columns plus greater density of the packing material (4 μm *versus* 10 μm particle diameter GF-250 and TSK; respectively) (Table I) results in increased surface area in the GF-250 column. The greater absorption of peptides in the GF-250 column (Fig. 1), is attributable to the greater surface area. However, absorption is overcome by column storage in 0.14 m*M* azide in water.

Column storage in 10% methanol did not result in as much binding as did storage in neat methanol. Subsequent separations of tryptic peptides were similar to those from columns stored in 0.14 m*M* azide in water. Relationships between area and mass were linear, although the correlation coefficients (r) were slightly lower for the TSK columns (see conditions II and IV). No significant hysteresis was observed after storage in 10% methanol but the differences between increasing and decreasing concentrations were higher than those of columns stored in azide (Table II). The high aqueous content of this storage medium may have retarded displacement of peptides previously bound to the columns and therefore any substantial increase in binding sites.

Columns whose binding sites had absorbed sample, then were equilibrated and stored in neat methanol regenerated binding sites for sample (condition III; Figs. 1 and 3). Hysteresis was observed between increasing and decreasing concentrations (Table II) indicating an increase in binding of peptides. Peptides apparently were displaced from the column by the anhydrous methanol equilibration procedure. The result was renewed column absorption upon subsequent chromatography of peptides.

None of the columns exposed to peptides, then stored in 0.14 m*M* azide in water showed hysteresis between increasing and decreasing concentrations of tryptic digest (Fig. 2, Table II). The three columns had linear relationships between peak area and injected mass. Differences in slopes between GF-250 and TSK columns may be due to an increase in binding because of the higher surface area and consequently more absorption sites. While the precise composition of the zirconium surface-stabilization compound of the GF-250 column is not known, the amphoteric nature of zirconium[12] may be responsible for ion-exchange reactions for either amino or carboxyl groups on peptides. However, once the ion-exchange and hydrophobic binding sites are occupied, the permeation mechanism predominates.

The availability of binding sites for proteins on the packing material appears to be reduced because of the larger molecular size and configuration of proteins. Although the SEC columns were designed for the separation of large molecules, these surfaces will interact with small molecules (K_D small molecules > 1; for adenine, $K_D = 2.69$)[10]. Therefore, SEC column packing materials act in the size-exclusion mode and an ion-exchange mode. The ion-exchange mode becomes important for small molecules or highly charged proteins (lysozyme).

Storage conditions did not seriously affect separations of proteins under the chromatographic conditions used. Other investigators have noted changes in retention times and abnormalities in SEC separations of proteins[13,14]. Mixed mechanisms allowing ion exchange, hydrophobic attractions, as well as steric exclusion occur.

The contribution of each can be manipulated by varying ionic strengths and mobile phase pH[3,10,11,13–16].

CONCLUSIONS

Binding of charged, low-molecular-weight peptides to SEC packing material is more dependent upon chemical characteristics of the storage medium than that of proteins with higher molecular weights. Our recommendations to users of SEC columns, in particular, those wanting to fractionate basic proteins for subsequent chromatography on reversed phase systems, are:

(1) Satisfy a new column's absorption capacity for samples by injecting a sufficient quantity of basic peptide standards or another convenient sample to reduce available binding sites before using the column for important separations.

(2) Store columns in azide–water or 10% methanol to prevent the renewal of binding capacity.

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