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PREPARATIVE SEPARATION OF PROTEINS AND ENZYMES IN THE MEAN MOLECULAR-WEIGHT RANGE OF 10,000–100,000 ON LiCHROSORB DIOL® PACKING BY HIGH-PERFORMANCE SIZE-EXCLUSION CHROMATOGRAPHY

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SUMMARY

LiChrosorb Diol® packing has been shown to be well-suited for the separation of proteins and enzymes according to a size-exclusion mechanism in a mean molecular-weight (MW) range between 10,000 and 100,000. Loadability of a small-bore column of 6 mm I.D. (A) and a large-bore column of 23.5 mm I.D. (B), both of 250 mm in length, were examined. Defining a 20% decrease of the number of theoretical plates as a loadability limit the volume load at constant mass of chymotrypsinogen as representative test solute was $\approx$ 100 μl for column (A) and 1500 μl for column B at $0.78 \cdot 10^{-5}$ g/g of packing for column A and $0.78 \cdot 10^{-6}$ g/g of packing for column B, respectively. Mass load at constant injection volume (20 μl for column A and 200 μl for column B, respectively) was $\approx$ 30 mg/ml or $\approx 1 \cdot 10^{-4}$ g/g of packing for both columns. As demonstrated in one instance, 75 mg of a mixture of 3 proteins which differ in MW by a factor of 2, could be separated in one run.

INTRODUCTION

A variety of well-established methods such as electrophoresis, radioimmunoassay, ultrafiltration, ultracentrifugation and classical chromatography (in size-exclusion, ion-exchange and affinity modes) is commonly applied for the analytical and preparative separation of proteins and enzymes in clinical and research laboratories[1,2]. The above methods have the disadvantage that they involve rather tedious and lengthy procedures which seldom allow any automation. For this reason, thorough efforts were made to apply modern high performance-column liquid chromatography (HPLC) to the separation of biopolymers, because of its rapidity and ability to perform both in an analytical as well as in a preparative mode. The degradation and denaturation of sensitive biopolymers, which are frequently observed during elution on native silica packings, can be circumvented by using surface modified derivatives with grafted terminating polar groups[3–6]. In a recent study[7], we found a silica with bonded 1,2-dihydroxy-3-propoxy-propyl groups to be extremely

suitable for the separation of biopolymers although Engelhardt and Mathes[8] claimed an amide-bonded type to be superior. As a 1,2-dihydroxy-3-propoxypropyl modified silica is commercially available as LiChrosorb Diol®, our examinations were carried out on this packing. The purpose of this work was to study the loadability behaviour of LiChrosorb Diol columns for some standard proteins for two reasons. Firstly, a separation of high- from low-molecular-weight species is often required as a primary clean-up step. Secondly, large amounts of materials are charged on the column to obtain sufficient material for further investigtions. Up to now there have been no preparative separations reported on inorganic packings in the literature. Another aspect was to perform high-speed preparative separations of proteins. Traditionally, such preparative separations are carried out on large-bore Sephadex columns. Sephadex, a swollen non-rigid gel, however, does not permit the application of high pressure and hence operation at high flow-rates, *i.e.* preparative separations require long periods of time.

EXPERIMENTAL

The bonded phase packing was LiChrosorb Diol, particle diameter 5 μm, purchased from E. Merck (Darmstadt, G.F.R.) carrying at the surface the functional grouping:

$$\equiv Si\text{–}(CH_2)_3\text{–}O\text{–}CH_2\text{–}\underset{\displaystyle OH}{CH}\text{–}\underset{\displaystyle OH}{CH_2}$$

The specific surface of the batch determined by us according to BET was 271 m^2/g. The mean pore diameter calculated by means of the Kelvin equation and the Pierce method see Gregg and Sing[9] was 10.4 nm. Two columns, A and B, purchased from DuPont de Nemours (Bad Nauheim, G.F.R.), were used with the following dimensions: A, 250 × 6 mm I.D., B, 250 × 23.5 mm I.D. Both columns were packed according to the slurry technique. Column A contained 5 g, column B 50 g of packing material. The eluent was phosphate buffer comprising 0.008 *M* K_2HPO_4, 0.042 *M* Na_2HPO_4 and 0.1 *M* NaCl of pH 7.5. Standard proteins and enzymes of Boehringer (Ingelheim, G.F.R.) listed in Table I were used as solutes. Solutions were always freshly prepared and stored at 277° K. The chromatograph was a DuPont Model 830 instrument fitted either with a septum injection system or with a Rheodyne injection system and a UV photometer of 254 nm wavelength. In the case of loop injection, loops of 20, 50, 100, 200, 500 and 1200 μl, respectively, were employed. Columns were used at ambient temperature.

RESULTS AND DISCUSSION

Retention of Biopolymers on LiChrosorb Diol

Biopolymers eluted on LiChrosorb Diol, in a buffered eluent of pH 7.5 at a relatively high ionic strength, follow a size-exclusion mechanism similar to that observed on a swollen polysaccharide type of packing such as Sephadex[10]. Table I lists the properties of proteins and enzymes studied and their corresponding reten-

TABLE I

MEAN MOLECULAR WEIGHT (MW), ISOELECTRIC PH (p*I*) AND RETENTION VOLUME V_R ON COLUNN A OF PROTEINS AND ENZYMES

Designation	*MW*	*pI*	V_R *(ml)*
Cytochrome *c*	12,000	10.7	4.35
Lysozyme	14,300	10.5	4.20
Chymotrypsinogen A	25,000	9.5	3.60
Albumin (egg)	45,000	4.6	3.35
Albumin (bovine)	68,000	5.1	2.70
Aldolase (rabbit)	158,000	9.5	2.40
Catalase (beef liver)	240,000	8.0	2.40
Ferritin	450,000	—	2.40

tion volume, V_R, on column A. The plot of the logarithm of molecular weight (MW) *vs* the retention volume gives a straight line for MW = 10,000–100,000. Biopolymers of MW > 100,000 are excluded in terms of size exclusion. It may be expected that some residual silanol groups on the surface of the LiChrosorb Diol packing may give rise to adsorption effects which enlarge the retention volume. According to the pK_a value of surface silanols of 7.0 ± 0.5[11], half of the total amount present is deprotonated in the eluent of pH 7.5, forming negatively charged surface siloxanyl groups. The effect of these groups on retention of charged biopolymers can be minimized by using an eluent of relatively high ionic strength. A very sensitive test for the presence of undissociated weak acidic silanol groups on diol modified silica, is to elute the strongly basic lysozyme and to subsequently measure its retention volume and peak shape. At complete coverage of the surface by diol groups, lysozyme should be eluted before the totally permeating solute according to its expected position in the linear part of the calibration plot, log MW *vs.* V_R. It also should exhibit a symmetrical peak shape. On LiChrosorb Diol columns, lysozyme has a retention volume close to that of cytochrome *c* (see Table I) and the peak shows no tailing as is demonstrated in Fig. 1 b.

Since the separation range of size-exclusion columns is limited, a reasonable resolution is only obtained for highly efficient columns. In our case, both columns were packed with the material of the same batch under identical conditions. The injection system was a common Rheodyne valve with exchangeable sample loops. Swagelok column inlet and outlet fittings were used with porous metal plugs of $< 2\ \mu m$ porosity at both column ends. As indicated in Table II the large bore column generated twice as many theoretical plates as the small bore column at the same column length. On the large bore column, almost 10,000 theoretical plates could be achieved for chymotrypsinogen as solute under analytical conditions, surprisingly high, particularly for a buffered eluent. The higher number of theoretical plates, *N*, at the large-bore column, can be understood by the lack of any wall effects, which contribute to peak broadening.

Loadability of LiChrosorb Diol columns

In discussing the term "loadability of columns", two major cases can be distinguished, according to Scott and Kucera[12]:

TABLE II

VOLUME AND MASS LOAD ON COLUMNS A AND B, RESPECTIVELY

Sample: Chymotrypsinogen A (Ch). Column A contains 5 g; column B contains 50 g of packing.

Column A *Ch = 0.3906 mg*			*Column B* *Ch = 0.3906 mg*			*Column A* *Injection vol. = 20 µl*			*Column B* *Injection vol. = 200 µl*		
Volume injected (µl)	*N*	*S (g/g)*	*Volume injected (µl)*	*N*	*S (g/g)*	*Conc. of Ch (mg/ml)*	*N*	*S (g/g)*	*Conc. of Ch (mg/ml)*	*N*	*S (g/g)*
5	4350	$7.8 \cdot 10^{-5}$	—	—	—	0.78	5140	$3.1 \cdot 10^{-6}$	0.78	8550	$3.13 \cdot 10^{-6}$
20	4200	$7.8 \cdot 10^{-5}$	20	9360	$7.8 \cdot 10^{-6}$	1.56	5080	$6.3 \cdot 10^{-6}$	1.56	8510	$6.25 \cdot 10^{-6}$
50	3790	$7.8 \cdot 10^{-5}$	50	9020	$7.8 \cdot 10^{-6}$	3.13	4780	$1.25 \cdot 10^{-5}$	3.12	8180	$1.25 \cdot 10^{-5}$
100	3260	$7.8 \cdot 10^{-5}$	100	8800	$7.8 \cdot 10^{-6}$	6.25	4540	$2.5 \cdot 10^{-5}$	6.25	7900	$2.50 \cdot 10^{-5}$
200	2030	$7.8 \cdot 10^{-5}$	200	8710	$7.8 \cdot 10^{-6}$	12.5	4300	$5.0 \cdot 10^{-5}$	13.81	7490	$5.25 \cdot 10^{-5}$
500	800	$7.8 \cdot 10^{-5}$	500	8630	$7.8 \cdot 10^{-6}$	25.0	4150	$1.0 \cdot 10^{-4}$	19.52	7040	$7.81 \cdot 10^{-5}$
			1200	7400	$7.8 \cdot 10^{-5}$	—	—	—	31.25	6900	$1.25 \cdot 10^{-4}$
						—	—	—	62.5	5750	$2.5 \cdot 10^{-4}$

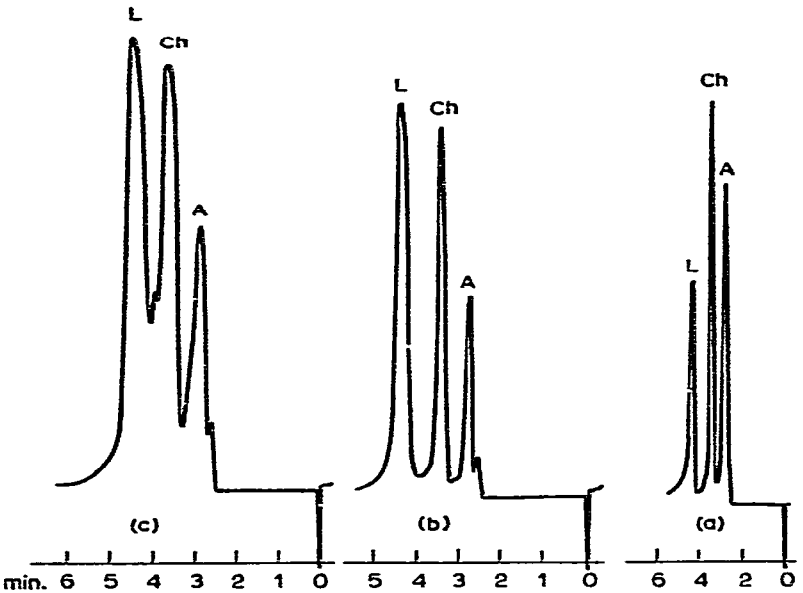

Fig. 1. Separation of albumin (bovine), A (MW = 65,000); chymotrypsinogen A, Ch (MW = 25,000); and lysozyme, L (MW = 14,300) on columns (A) and (B), respectively (a) Conditions: column dimensions, 250 × 6 mm I.D.; packing, LiChrosorb Diol, particle diameter 5 μm; eluent, phosphate buffer pH 7.5; flow-rate, 2.0 ml/min; pressure drop, 1350 p.s.i.; detector, UV 254 nm; attenuation, 0.016 a.u.f.s. (10 mV); injection volume, 5 μl; sample amount, 0.1 mg of A, 0.1 mg of Ch, 0.05 mg of L; chart speed of recorder, 300 mm/h. (b) Conditions: column dimensions, 250 × 23.4 mm I.D.; packing and eluent as in (a); flow-rate, 21.0 ml/min; pressure drop, 2230 p.s.i.; detector as in (a); attenuation, 0.04 a.u.f.s. (100 mV); injection volume, 500 μl; sample amount, 25 mg of A, Ch and L, respectively; chart speed of recorder, 600 mm/h. (c) Conditions as in (b), except sample amount, 50 mg of A, Ch and L, respectively.

(i) "... when samples that are relatively insoluble in the eluent are being separated, the column may be overloaded with respect to sample feed volume".

(ii) "... for samples which are considerably soluble in the eluent, column overload can arise from excess of mass".

Furthermore, in deriving any loadability data of HPLC columns one should strictly discriminate between:

(i) volume load, which is measured at increasing injection volume at constant mass of solute and is expressed in volume units injected to a column of given dimensions, and

(ii) mass load, which is measured at increasing concentration of solute, maintaining the injection volume constant. Mass load is expressed in g solute per g packing.

The special features relating to volume and mass overload were recently discussed by Coq *et al.*[13]. Originally ,loadability was expressed in terms of linear sample capacity $\Theta_{0.1}$ as g solute/g adsorbent which is observed as a 10% decrease of the capacity factor of solute compared to that at infinite dilution[14].

The solutes here under investigation are very soluble in the eluent. Moreover, as the mechanism in our instance is size exclusion governed by a bulk effect, the

solute concentration can be much higher than that used for adsorption columns, at which retention is based on surface phenomena. Sample concentration in size-exclusion chromatography usually varies by about 0.1%[15]. The limiting factor for sample size is the viscosity of solution to be injected. The viscosity should generally be no greater than twice that of the eluent[16].

Since the capacity in size-exclusion chromatography loses its original meaning, the criteria for loadability of LiChrosorb Diol columns was taken to be a 20% decrease of N, referred to a standard value of N under essential analytical conditions.

The results of volume and mass loadability studies are listed in Table II. On column A, sample volume was varied from 5–500 μl at constant mass of chymotrypsinogen, while on column B, the largest injection volume was 1200 μl. Taking a 20% decrease of N, compared to the value at the smallest injection volume, the volume load of column A approximates 100 μl and that of column B about 1500 μl, the latter value being extrapolated. In an examination of the column loadability of adsorption columns, Beck and Halász[17] reported that a 20% decrease of N is observed at an injection volume which corresponds to about 1–2% of total volume of the empty column. This is in satisfying agreement with our data. For chymotrypsinogen, the volume load amounts to about 3% of the internal volume accessible to the solute on both columns.

The observed decrease of N at increasing injection volume can also be discussed in terms of the peak variances[18]. The measured total variance of an eluted peak, σ^2_{total}, can be written as the sum of several contributions:

$$\sigma^2_{\text{total}} = \sigma^2_{\text{column}} + \sigma^2_{\text{detector}} + \sigma^2_{\text{connection}} + \sigma^2_{\text{injection}} \qquad (1)$$

σ^2_{column} corresponds to the variance of the column, whereas the other three represent extra-column variances originating in the detector, connecting lines and injection system, respectively. $\sigma^2_{v(\text{total})}$ being the variance of solute in volume units is given by the equation:

$$\sigma^2_{v_i(\text{total})} = \frac{V^2_{R(i)}}{N_i} \qquad (2)$$

where $V_{R(i)}$ is the retention volume and N_i the number of theoretical plates of solute i. The variance from sample injection calculated on a volume basis is:

$$\sigma^2_{v(\text{injection})} = \frac{V^2_{\text{injection}}}{12} \qquad (3)$$

where $V_{\text{injection}}$ is the injection volume[18]. Both $\sigma^2_{v(\text{total})}$ and $\sigma^2_{v(\text{injection})}$ can be calculated from our experimental results as shown in Table III.

For a given column at a constant eluent velocity, the relative contribution of $\sigma_{v(\text{column})}$, $\sigma^2_{v(\text{detector})}$ and $\sigma^2_{v(\text{connection})}$, respectively, to $\sigma^2_{v(\text{total})}$ can be regarded as constant at first approximation, and any change in $\sigma^2_{v(\text{total})}$ should result from variations of the injection volume. Moreover, at low injection volumes (*e.g.* 5 μl), the contribution of $\sigma^2_{v(\text{injection})}$ to $\sigma^2_{v(\text{total})}$ should be negligibly small. As can be seen in Table III, the contributions of the injection volumes (5 μl for column A; 20 μl

TABLE III

TOTAL VARIANCE OF CHYMOTRYPSINOGEN PEAK, $\sigma^2_{v_t(\text{total})}$ ON COLUMNS A AND B, RESPECTIVELY AND VARIANCE $\sigma^2_{v(\text{injection})}$ AT VARIOUS INJECTION VOLUMES

Injection volume (μl)	$\sigma^2_{v(\text{injection})}$ *(μl)² according to eqn. 3*	$\sigma^2_{v_t(\text{total})}$ *(μl)² according to eqn. 2*	
		Column A	*Column B*
5	2	3.000	—
20	30	3.100	267.500
50	210	3.400	277.500
100	830	3.900	285.000
200	3.300	6.400	287.500
500	20 800	16 200	290 000
1200	120 000	—	337 000

for column B) to the total variance are small. It is assumed that at these conditions $\sigma^2_{v(\text{total})}$ originate mainly from the sum of the three terms $\sigma^2_{v(\text{detector})}$, $\sigma^2_{v(\text{column})}$ and $\sigma^2_{v(\text{connection})}$, respectively. Then $\sigma^2_{v_t(\text{total})}$ at a given injection volume, (corrected by subtracting $\sigma^2_{v_t(\text{total})}$ at 5 μl for column A and 20 μl for column B) should correspond approximately to $\sigma^2_{v(\text{injection})}$ also for the given injection volume. An inspection of the data on the analytical column A reveals that this assumption holds only for an injection volume of up to 200 μl. At $>$ 500 μl, $\sigma^2_{v(\text{injection})}$ becomes much larger than the corresponding $\sigma^2_{v_t(\text{total})}$ corrected by $\sigma^2_{v(\text{injection})}$ at 5 μl. On the preparative column B, deviation is observed even for an injection volume of 1200 μl. This disagreement at injection volumes larger than 200 μl on column (*A*) and 1200 μl on column B, respectively, can be explained by the fact that eqn. 3 does not describe accurately the contribution of the injection volume at high volumes to the total variance of the peak.

Returning once more to a 20% decrease of N for the assessment of the mass loaded, the limiting sample capacity for both columns is about $1 \cdot 10^{-4}$ g solute/g packing, which is in agreement with Kirkland and Antle[19] regarding polystyrene with a MW = 4800 on a silica size-exclusion column. Fig. 1 demonstrates the loadability of LiChrosorb Diol columns. The first chromatogram shows a separation of three proteins under analytical conditions on column A within 5 min. The same mixture of proteins was run on the large bore column. In Fig. 1b, the total amount injected was 75 mg, *e.g.* 25 mg of protein are eluted within one given peak. Resolution is much better as compared to column A due to higher efficiency. Analysis time in this case is about 6 min. The chromatogram of Fig. 1c corresponds to an overload, a total of 150 mg of proteins being injected. In this case, the peaks are resolved but mutually interfere, and moreover, the upper limit of the linear range of the UV detector is achieved.

CONCLUSION

LiChrosorb Diol can be packed very efficiently into columns of 1 in. I.D. generating about 10,000 theoretical plates per 250 mm column length, with a buffered eluent. Biopolymers are fractionated on these columns according to a size exclusion mechanism within a mean molecular weight of 10,000–100,000. At a flow-

rate of about 20 ml/min, solutes are eluted within 6 min. Up to 1.5 ml of solution can be injected with a loss of only 20% of the total number of theoretical plates at $1 \cdot 10^{-5}$ g solute/g packing. Linear sample capacity at 0.2 ml injection volume amounts to $1 \cdot 10^{-4}$ g, taking a 20% decrease of N as the standard. As the large bore LiChrosorb Diol column contains about 50 g of packing, 30 mg of a protein can be chromatographed without overloading the column. It is of interest to compare our results on LiChrosorb Diol columns with that on Sephacryl® S-200 superfine columns, which are presently used as polysaccharide-base organic packing for high-performance gel filtration[20]. The smallest average particle size of Sephacryl available is about 70 μm. Maximum pressure allowable at 30 cm of column length is reported to be about 10 bar[10]. In contrast, LiChrosorb Diol columns can be operated at pressures of up to 300 bar. Under optimal conditions, 30 mg of protein can be separated on a large bore LiChrosorb Diol column at a flow-rate of 20 ml/min within 6 min whereas the same separation on Sephacryl requires several hours. Moreover, the 30 mg of separated solute in our case are eluted in about 20 ml, while comparable separations on Sephacryl require large volumes.

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