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SOME STUDIES ON THE RESOLVING POWER OF AGAROSE-BASED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC MEDIA FOR THE SEPARATION OF MACROMOLECULES

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SUMMARY

Columns packed with small cross-linked agarose beads (a mixture of gel spheres with diameters in the range 3–10 μm) for the fractionation of biopolymers are characterized by high plate numbers and large ratios (up to 2.8) between inner volume (V_i) and void volume (V_0) and therefore compete favourably with other high-performance liquid chromatographic (HPLC) packings in resolving power. Interestingly, some compressibility of the packing material decreases the void volume considerably, which means an enhancement in both the plate number and the V_i/V_0 ratio, and, therefore, also in resolution.

INTRODUCTION

Conventional low-pressure chromatography for the fractionation of macromolecules on columns of agarose (Sepharose®, Bio-Gel A®) and derivatives of agarose is a well-known separation technique and is a routine method in all laboratories where biopolymers are studied. This agarose-based chromatographic technique has recently been improved, in the sense that the fractionations can now be performed much faster and with higher resolution[1–5]. This was made possible by increasing the rigidity of the agarose gel spheres used as packing material and decreasing their diameter.

The introduction of this new type of agarose beads means that an additional high-performance liquid chromatographic (HPLC) matrix has become available. An advantage of this column material is that those who employ chromatographic methods based on matrices of Sepharose or Bio-Gel A can now easily benefit from all the advantages of an HPLC technique without changing the basic structure of the packing material.

In previous papers we have shown that this new type of agarose beads can be utilized to advantage for separations based on molecular sieving, bioaffinity, and electrostatic and hydrophobic interactions[1–5]. In this communication we shall discuss

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the resolving power of columns packed with these beads in terms of plate numbers and V_i/V_0 ratios (V_i, inner volume; V_0, void volume).

EXPERIMENTAL AND RESULTS

Materials

The agarose (A37, electroendoosmosis = −0.17) was from IBF (Villeneuve, La Garenne, France); thyroglobulin, ovalbumin and chymotrypsinogen A from Pharmacia (Uppsala, Sweden); β-fructosidase from Boehringer (Mannheim, F.R.G.); cytochrome *c* from Sigma (St. Louis, MO, U.S.A.); human serum albumin from KABI-Vitrum (Stockholm, Sweden).

Agarose beads were prepared as previously described[3]. The number of theoretical plates N was calculated from the formula $N = 5.54\ (t_R/w_{\frac{1}{2}})^2$, where t_R = the retention time, $w_{\frac{1}{2}}$ = the peak width at half the peak height (expressed in time units).

The UV-monitor (2158 Uvicord SD) and the HPLC pump (2150) were from LKB (Bromma, Sweden); the printer-plotter (C-R1A) was from Shimadzu (Kyoto, Japan).

The number of theoretical plates

Cross-linked 12% agarose beads with diameters of 3–10 μm were packed at a constant pressure (45 bar) in a 6-mm I.D. Plexiglas tube to a height of about 30 cm. The column was equilibrated with 0.2 *M* sodium phosphate, pH 6.8. A 2-μl volume (20 μg) of a mixture of thyroglobulin (mol.wt. 669,000), β-fructosidase (270,000), human serum albumin (67,000), ovalbumin (43,000), chymotrypsinogen A (25,000) and cytochrome *c* (12,400) was applied. Using the phosphate buffer the column was eluted at a flow-rate of 0.05 ml/min. The chromatogram is presented in Fig. 1a. From this chromatogram the plate numbers per metre (N/m) were calculated for all the sample proteins except thyroglobulin and β-fructosidase because they were not sufficiently well separated. The experiment was then repeated at flow-rates of 0.1, 0.2 and 0.35 ml/min (Figs. 1b–d). From the chromatograms in Fig. 1 the plate numbers per metre (N/m) were calculated and plotted against the flow-rate (Fig. 2).

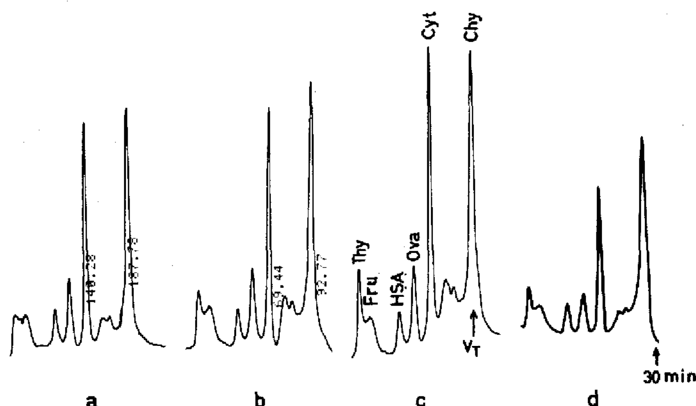

Fig. 1. High-performance molecular-sieve chromatography of model proteins on agarose. Thy = thyroglobulin (mol.wt. 669,000), Fru = β-fructosidase (270,000), HSA = human serum albumin (67,000), Ova = ovalbumin (43,000), Chy = chymotrypsinogen A (25,000), Cyt = cytochrome *c* (12,400). Flow-rate (ml/min): a, 0.05; b, 0.1; c, 0.2; d, 0.35.

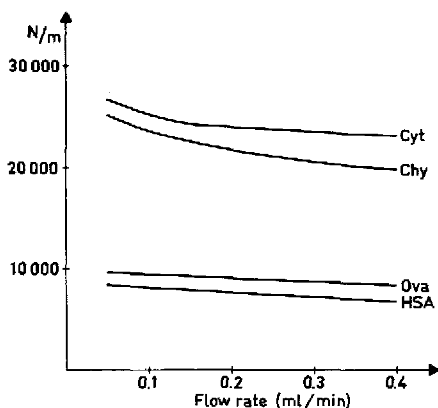

Fig. 2. Plate numbers per meter (N/m) for some proteins as a function of the flow-rate. The chromatograms in Fig. 1 were used for the calculations of N/m. For notations of the proteins, see legend to Fig. 1. The resolution is proportional to $\sqrt{N}$.

The ratio V_i/V_0 as a function of the packing pressure

As a marker for the void volume (V_0) we used thyroglobulin (mol.wt. 669,000) and for the total available volume (V_T) potassium chromate, which we have previously shown to give the same V_T values as ^2H_2O[4].

The same agarose beads and the same chromatographic tube were used as in the experiment shown in Fig. 1 for the determination of plate numbers. The packing was performed at a flow-rate of 0.05 ml/min which corresponded to a maximum pressure around 7 bars (the bed height was 31.7 cm). At this flow-rate and with the above markers the parameters V_0 and V_T were determined. From the relation $V_T = V_0 + V_i$, the inner volume (V_i) was calculated and then the ratio V_i/V_0.

The flow-rate was increased to 0.1 ml/min. The bed height then decreased to 31.2 cm and the pressure increased to 9 bars. The ratio V_i/V_0 was determined as above. Similarly, this ratio was also calculated for the flow-rates 0.2 ml/min (pressure: 13 bar; bed height: 29.3 cm), 0.3 ml/min (pressure: 30 bar; bed height: 27.8 cm), and 0.35 ml/min (pressure: 86 bar; bed height: 25.7 cm). The results are graphically presented in Fig. 3.

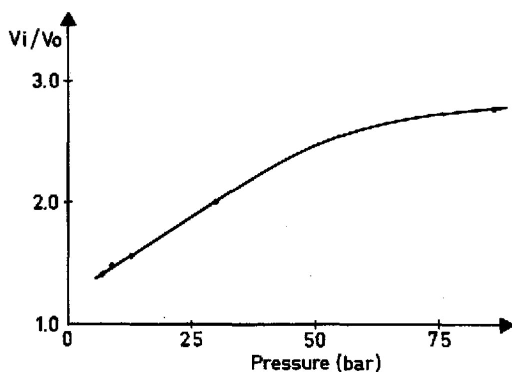

Fig. 3. The V_i/V_0 ratio as a function of the packing pressure. The resolution increases with an enhancement of the V_i/V_0 ratio.

DISCUSSION

The resolution R of a chromatographic column is determined by the expression[6]:

$$R = \frac{\sqrt{N}}{2} \cdot \frac{\frac{V_i}{V_0} \cdot K''\left(\frac{K''}{K'} - 1\right)}{2 \cdot \frac{K''}{K'} + \frac{V_i}{V_0} \cdot K''\left(\frac{K''}{K'} + 1\right)} \tag{1}$$

where N = the number of theoretical plates, K' and K'' = the distribution coefficients of two solutes.

The parameters N and V_i/V_0 determine, accordingly, the quality of a packing material. Therefore, it is of interest to know the actual values of these parameters for columns packed with the small, rigid agarose beads.

A plausible explanation of the large ratio V_i/V_0 (about 2.5) found in the experiment shown in Fig. 1 could be that the relatively high pressure used at the packing of the column (45 bar) caused the agarose beads to be somewhat deformed, which should decrease the void volume but hardly the inner volume. A microscopic examination of the beads immediately after they had been withdrawn from the column indead showed such a deformation (after some minutes on the microscope slide the beads resumed their regular, spherical shape). If the small void volumes obtained were caused by some deformation of the agarose beads then the void volumes should be larger at lower packing pressure. The experiments performed to test this hypothesis showed such a relationship (Fig. 3).

A decrease in void volume diminishes the distances over which a solute has to diffuse to enter a gel bead, which means that the distribution of a solute between the mobile and the stationary phases then more closely approaches the ideal equilibrium state, resulting in a narrower peak, *i.e.* an increase in plate number, which is equivalent to an increase in resolution according to eqn. 1. This view is supported by the fact that the plate numbers per metre in Fig. 2 are higher than those reported in ref. 4, where the packing was performed at a pressure much lower than that used in the experiments corresponding to Fig. 2. The effect of coming closer to an equilibrium state by compressing the beads is also reflected in less flow-rate dependence of the plate numbers in Fig. 2 in this paper than in Fig. 2 in ref. 4 (part of the differences between these figures must also be ascribed to a difference in the size of the beads used).

The conclusion may therefore be drawn that some compressibility (deformation) of a column material is desirable, since the void volume then decreases which is equivalent to an increase in both V_i/V_0 and N, and therefore in resolution (according to eqn. 1). It should be emphasized that the beads must not be made excessively compressible since the flow-rates will then become low.

A column for use in an HPLC experiment should not be packed at increasing pressure, as in the experiment corresponding to Fig. 3, but at a constant pressure equal to or somewhat higher than that used in the subsequent experiments[7]. This minimizes the risk of compression of the bed, (*i.e.* a decrease in its height) during an

experiment, which may give rise to a non-uniform packing and loss in resolution[6]. However, the homogeneity of the agarose-based HPLC columns is affected very little by compression of the bed, as evidenced by the fact that a column packed at constant pressure did not give significantly higher resolution than a column packed at constant flow-rate[4]. With the latter packing technique the pressure increased continuously, leading to compression of the bed (the final pressure at the termination of the packing was the same as that used in the packing at constant pressure).

The V_i/V_0 ratio for the agarose column used in the experiments presented in Fig. 1 was 2.5, while the maximum value in Fig. 3 is 2.8. For silica beds, ratios in the range 0.8–1.2 are common[8], although a value of 1.66 has been reported[9].

Chymotrypsinogen is eluted close to the total volume (V_T in Fig. 1). At higher salt concentrations (0.4 *M* phosphate buffer instead of 0.2 *M*) the retardation is greater, indicating a hydrophobic interaction. The other model proteins used have elution volumes (times) typical of a molecular-sieve chromatography experiment. This is evident from Fig. 4, which shows a plot of log $(t_R - t_0)$ against $M^{2/3}$ [M = the molecular weight of the proteins; t_R = the retention time; t_0 = the retention time of a protein (thyroglobulin) migrating at the void volume]. This method of plotting follows from a thermodynamic treatment of molecular sieving (see ref. 10 where, however, elution times (V_e, V_0) are used instead of retention times).

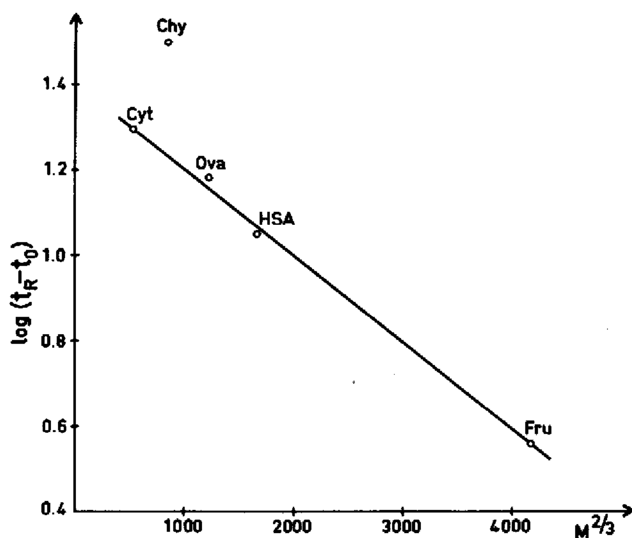

Fig. 4. A plot of log $(t_R - t_0)$ against $M^{2/3}$. All data were taken from the experiment shown in Fig. 1b. Details of the plotting technique are found in ref. 10.

ACKNOWLEDGEMENTS

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