Journal of Chromatography, 457 (1988) 165–174
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 881

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS ON COMPRESSED, NON-POROUS AGAROSE BEADS

I. HYDROPHOBIC-INTERACTION CHROMATOGRAPHY

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(First received March 11th, 1988; revised manuscript received July 21st, 1988)

SUMMARY

Macroporous agarose beads were converted into non-porous beads by shrinkage and cross-linking in organic solvents. These beads could be used for high-performance hydrophobic-interaction chromatography without derivatization with non-polar ligands, because the 1,4-butanediol diglycidyl ether, used as cross-linker, gives relatively hydrophobic bridges. The resolution for compressed columns packed with these beads was determined as a function of gradient time at constant flow-rate, flow-rate at constant gradient volume and flow-rate at constant gradient time and as a function of loading capacity. Interestingly, the resolution is virtually independent of flow-rate at constant gradient volume even when the column is packed with relatively large beads (diameter 30 μm). The beads have the advantage of being stable up to pH 14.

INTRODUCTION

In a previous paper[1] we showed that peak widths for proteins, in terms of plate numbers, were constant or decreased very little with increasing flow-rate for a column packed with compressed, macroporous, cross-linked 12% agarose beads. Unfortunately, compression of these beads (diameter 3–10 μm) increased the flow resistance considerably, preventing the use of linear flow velocities higher than 3 cm/min for a 30-cm molecular-sieve chromatographic column. However, we have recently shown that columns of compressed, cross-linked, non-porous agarose beads for ion-exchange and hydrophobic-interaction chromatography (HIC) permit higher flow-rates and that the peak widths (and resolution) have the same attractive flow-velocity dependence (at constant gradient volume), even at extremely high flow-rates and for five-fold larger bead diameters; sometimes the resolution even increased with increasing flow-rate[2,3]. In this paper we give a more extensive description of the chromatographic properties of these non-porous, deformed agarose beads when used for the high-performance HIC of proteins. Ion-exchange chromatography on non-porous, deformed beads is treated in Part II[4].

0021-9673/88/$03.50

Janzen *et al.*[5] have recently described the high-resolution HIC of proteins on non-porous and non-compressible 1.5-μm silica beads. For reversed-phase chromatography and ion-exchange chromatography of proteins on non-porous, non-compressed packing materials, see refs. 6–10 and 11–15, respectively.

EXPERIMENTAL AND RESULTS

Equipment

The chromatographic system consisted of the following units: a Model 2150 high-performance liquid chromatographic (HPLC) pump, a Model 5152 LC controller and a Model 2151 variable-wavelength monitor from LKB (Bromma, Sweden), a Model C-RIA integrator from Shimadzu (Kyoto, Japan) and a Model 7125 injection valve from Rheodyne (Cotati, CA, U.S.A.). The column tubes were made of Plexiglas (their design is described in ref. 3).

Materials

Phycoerythrin was prepared as described in ref. 16. Blue Dextran, bovine serum albumin, bovine pancreas ribonuclease A, hen egg ovalbumin, bovine liver catalase and horse spleen ferritin were purchased from Pharmacia (Uppsala, Sweden) and bovine α-chymotrypsinogen A and horse heart cytochrome *c* from Sigma (St. Louis, MO, U.S.A.). Human transferrin was a gift from Dr. L.-O. Andersson (KabiVitrum, Stockholm, Sweden).

Macroporous 11% agarose beads were prepared essentially as described in ref. 17. By wet sieving, beads of a diameter *ca.* 30 μm were collected. The diameter was reduced to about 20 μm by shrinkage and cross-linking (described below). These beads were used in all the experiments. In most runs, the column bed was equilibrated with 0.01 *M* sodium phosphate (pH 6.8) containing 2.1 *M* ammonium sulphate and eluted with a linear, negative salt gradient generated from this buffer and 0.01 *M* sodium phosphate (pH 6.8) containing 0.25 *M* ammonium sulphate.

Reagents were obtained as listed in Part II[4].

Shrinkage of agarose beads

A 5-g amount (about 5 ml) of sedimented 11% agarose beads was washed by centrifugation at 1500 *g* for 1–2 min successively with 5 ml of deionized water, 5 ml of dioxane–water (1:1) and two 5-ml portions of dioxane.

The sedimented agarose beads were suspended in 2.5 ml of dioxane–chloroform (1:1). Dioxane was added dropwise with stirring until the opalescent suspension became transparent (if the opalescence did not disappear after addition of 1 ml of dioxane, chloroform was added until the suspension became transparent). An additional 2.5 ml of dioxane were then added. Following stirring for 1 min and centrifugation, the supernatant was removed and the beads were washed with five 5-ml portions of dioxane–chloroform (1:1). The shrunken beads were then washed with three 5-ml portions of chloroform and suspended in 20 ml of chloroform. By these washing procedures the volume of the sedimented agarose beads was reduced five-fold.

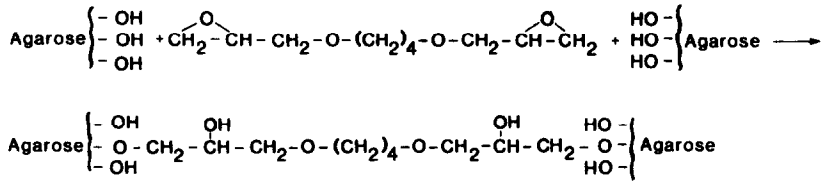

Fig. 1. Simplified reaction scheme for cross-linking of agarose with 1,4-butanediol diglycidyl ether.

Cross-linking of shrunken agarose beads with 1,4-butanediol diglycidyl ether

A simplified reaction scheme is shown in Fig. 1[18]. A 3.2-ml portion of 1,4-butanediol diglycidyl ether was added with stirring to the above suspension of shrunken agarose beads in chloroform, followed by dropwise addition during 10 min of 0.3 ml of boron trifluoride diethyl etherate diluted in 12 ml of chloroform. After stirring for 30 min in a covered beaker the beads were washed several times, first with dioxane and then with water. To increase their rigidity further the agarose beads were cross-linked again after transfer into chloroform using five 5-ml portions of dioxane, 5 ml of dioxane–chloroform (1:1), two 5-ml portions of carbon tetrachloride (this solvent has a high density and therefore facilitates flotation of the beads) and 5 ml of chloroform. The beads were suspended in 20 ml of chloroform and then cross-linked as described above.

When these beads were transferred into water and packed into a column for HIC, they shrank on exposure to high salt concentrations, resulting in a void at the top of the bed. To avoid this shrinkage the beads were treated with glycidol in a solution of dioxane, essentially as described in ref. 19. For this treatment the cross-linked shrunken agarose beads (which had a volume of about 1.5 ml) were washed several times with dioxane by centrifugation at 1500 *g* and then suspended in 10 ml of dioxane.

Glycidol (1.5 ml) was added with stirring followed by 200 μl of boron trifluoride diethyl etherate. After stirring for 1 h the gel was washed by centrifugation at 1500 *g* for 2 min with six 5-ml portions of water. The volume of the sedimented beads was still about 1.5 ml.

As 1,4-butanediol diglycidyl ether is relatively hydrophobic, no time-consuming derivatization with non-polar ligands is required in order to obtain amphiphilic beads suitable for HIC. (In a control expriment where the latter cross-linker was replaced with the more hydrophilic γ-glycidoxypropyltrimethoxysilane[2], the hydrophobic interaction with proteins was greatly reduced.) Two additional treatments of the agarose beads with glycidol makes them so hydrophilic that they cannot be used for HIC without derivatization with non-polar ligands.

Porosity of the shrunken, cross-linked beads

The porosity was studied by molecular-sieve chromatography on a 7.2 cm × 0.6 cm I.D. column of the shrunken, cross-linked 20-μm beads. After packing in water the column was equilibrated with 0.01 *M* sodium phosphate (pH 6.8). The sample consisted of Blue Dextran (mol.wt. 2 000 000), phycoerythrin (290 000), human transferrin (80 000), bovine serum albumin (67 000), hen egg ovalbumin (43 000),

horse heart cytochrome *c* (12 000) and potassium chromate (194). The retention times were plotted against the molecular weights (Fig. 2).

Pressure–flow-rate dependence

The experiment was conducted in 0.01 *M* sodium phosphate (pH 6.8) containing 2.1 *M* ammonium sulphate at flow-rates of 0.25, 0.50, 1.0, 2.0, 3.0 and 4.0 ml/min. The pressures were read and plotted against flow-rate. The linear form of the curve (Fig. 3) indicates that the column can be operated at flow-rates above 4 ml/min.

Recovery

The column was equilibrated with 0.01 *M* sodium phosphate (pH 6.8) containing 2.1 *M* ammonium sulphate. Ribonuclease (100 μg) dissolved in 50 μl of the equilibration buffer was applied. Desorption was achieved with 0.01 *M* sodium phosphate (pH 6.8).

Measurements of the absorption at 280 nm of the applied sample and the collected fractions indicated a recovery of 100%. For ovalbumin and catalase the recoveries were 99 and 98%, respectively.

Resolution on columns of compressed, non-porous agarose beads as a function of gradient time at constant flow-rate

A Plexiglas tube with a diameter of 0.6 cm was packed at a flow-rate of 2 ml/min with the cross-linked, shrunken beads with diameters of *ca.* 20 μm. The bed was then compressed to a height of 5.5 cm by increasing the flow-rate to 5 ml/min.

The sample consisted of ovalbumin (9 μg) and bovine α-chymotrypsinogen A (3 μg), dissolved in 20 μl of the equilibration buffer (0.01 *M* sodium phosphate, pH 6.8, containing 2.1 *M* ammonium sulphate). The elution was performed at a flow-rate of 1 ml/min with a linear, negative salt gradient formed from the buffer used for equilibration and 0.01 *M* sodium phosphate (pH 6.8) containing 0.25 *M* ammonium

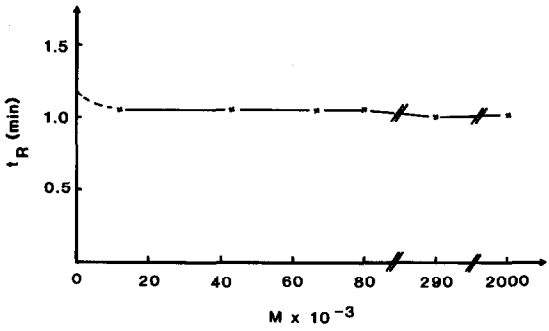

Fig. 2. Porosity of shrunken, cross-linked agarose beads. A column was packed with such beads (diameters *ca.* 20 μm) and the retention times (t_R) for proteins of different molecular weights (*M*) were determined in a molecular-sieve chromatography experiment. From the plot one can conclude that proteins (at least those with molecular weights above 12 000) cannot penetrate the beads.

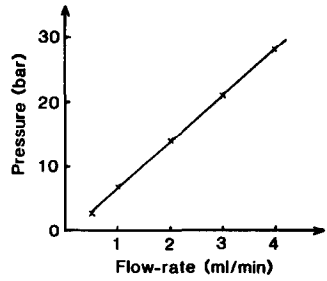

Fig. 3. Pressure–flow-rate dependence. Bed dimensions: 5.5 cm × 0.6 cm I.D. Bead diameter: *ca.* 20 μm. Buffer: 0.01 *M* sodium phosphate (pH 6.8) containing 2.1 *M* ammonium sulphate.

sulphate. Gradient times of 1.0, 3.0, 6.5, 13, 25 and 50 min were used. The recorder chart speed was inversely proportional to the gradient time to give all the chromatograms the same width, thereby facilitating direct visual comparison. The resolution, R_s, between ovalbumin and α-chymotrypsinogen A was calculated from the following equation for each run:

$$R_s = \frac{t_2 - t_1}{0.5(t_{w2} + t_{w1})} \quad (1)$$

where t_1 and t_2 are the retention times for ovalbumin and α-chymotrypsinogen A, respectively, and t_{w1} and t_{w2} are their peak widths at half-height. Fig. 4 shows a plot of resolution against gradient time.

Fig. 5a and b give a visual impression of the variation in resolution with gradient time. The experimental conditions were the same as those described above.

Influence of flow-rate at constant gradient volume on the appearance of the chromatograms

The bed was equilibrated with 0.01 *M* sodium phosphate (pH 6.8) containing 2.1 *M* ammonium sulphate. The sample (3–6 μg of each of the proteins ribonuclease, ovalbumin, α-chymotrypsinogen A, catalase and ferritin) dissolved in 40 μl of the equilibration buffer was applied. At a flow-rate of 0.06 ml/min elution was achieved with a linear negative salt gradient formed from the equilibration buffer and 0.01 *M* sodium phosphate (pH 6.8) containing 0.25 *M* ammonium sulphate (pH 6.8). The experiment was repeated at the flow-rates of 0.13, 0.25, 0.5, 1, 2 and 4 ml/min (Fig. 6). The gradient volume was 3.2 ml in all expriments. The recorder chart speed was increased parallel to the increase in flow-rate for the reasons mentioned above under *Resolution on columns of compressed, non-porous agarose beads as a function of gradient time at constant flow-rate.*

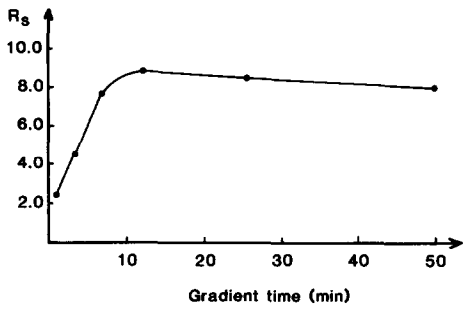

Fig. 4. Resolution (R_s) of ovalbumin and α-chymotrypsinogen A as a function of gradient time. Bed dimensions: 5.5 cm × 0.6 cm I.D. Bead diameter: 20 μm. Flow-rate: 1 ml/min. No gain in resolution is obtained for gradient times exceeding 12 min.

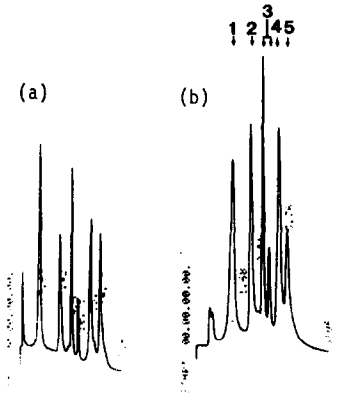

Fig. 5. Influence of gradient time at constant flow-rate on the appearance of the chromatograms. Bed dimensions: 5.5 cm × 0.6 cm I.D. Flow-rate: 1 ml/min. Bead diameter: 20 μm. Gradient time: (a) 15; (b) 3.2 min. For proteins 1–5 see legend to Fig. 6.

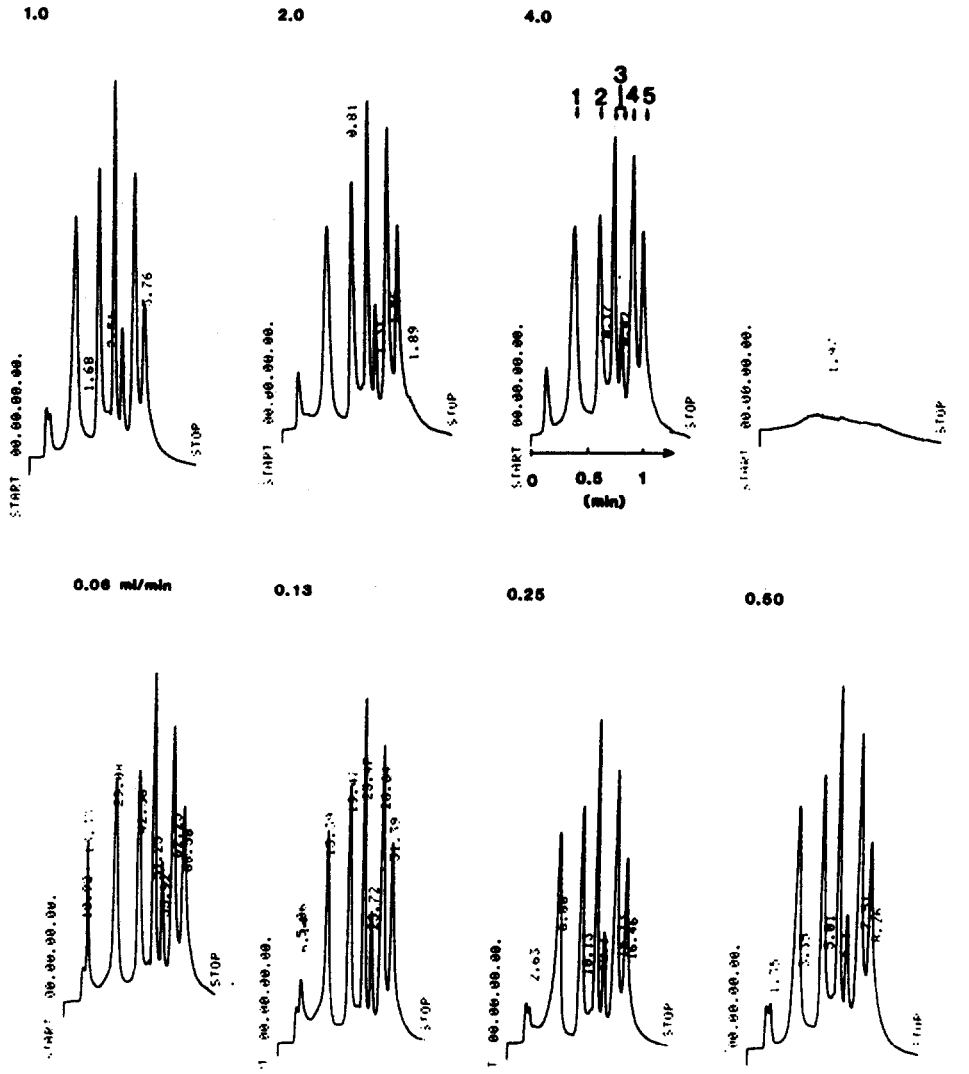

Fig. 6. Influence of flow-rate at constant gradient volume on the appearance of the chromatograms. Bed dimensions: 5.5 cm × 0.6 cm I.D. Bead diameter: 20 μm. Sample: 1 = ribonuclease; 2 = ovalbumin; 3 = α-chymotrypsinogen A; 4 = catalase; 5 = ferritin. The flow-rates are given at the top of each chromatogram. It is evident that the protein patterns are virtually identical, *i.e.*, independent of the flow-rate. The gradient volume was 3.2 ml in all experiments. The recorder chart speed was proportional to the flow-rate.

Influence of flow-rate at constant gradient time on the appearance of the chromatograms

The column was equilibrated with 0.01 *M* sodium phosphate (pH 6.8) containing 2.0 *M* ammonium sulphate. The sample consisted of about 7 μg of each of the proteins ribonuclease, ovalbumin, α-chymotrypsinogen A, catalase and ferritin and was dissolved in 20 μl of the equilibration buffer. The proteins were eluted with a negative

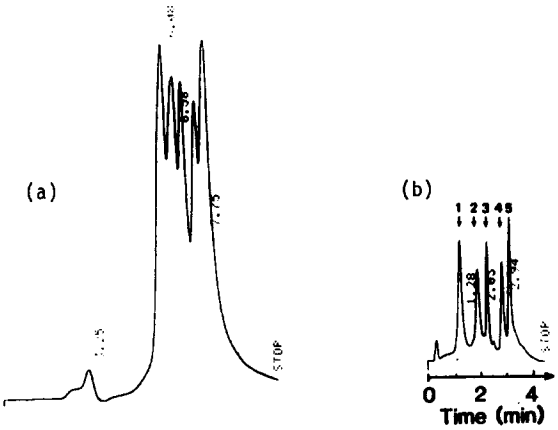

Fig. 7. Influence of flow-rate at constant gradient time on the appearance of the chromatograms. Bed dimensions, bead diameter and sample as in Fig. 6. Flow-rate: (a) 0.25; (b) 2.0 ml/min. The gradient time was 3.2 min in both experiments, whereas the recorder chart speed was constant. Linear gradient from 0.01 *M* sodium phosphate (pH 6.8) + 2.0 *M* ammonium sulphate to 0.01 *M* sodium phosphate (pH 6.8).

3.2-min linear salt gradient from the equilibration buffer to 0.01 *M* sodium phosphate (pH 6.8). The experiments were performed at flow-rates of 0.25 and 2.0 ml/min. The recorder chart speed was constant at 0.8 cm/min. At the higher flow-rate the gradient volume was larger and therefore the concentration gradient with respect to volume (dc/dV) is shallower which causes the peak heights to be lower in Fig. 7b than in Fig. 7a.

Resolution on columns of compressed, non-porous agarose beads as a function of flow-rate at constant gradient volume

The experimental conditions were similar to those described above under *Influence of flow-rate at constant gradient volume on the appearance of the chromatograms*, except for the bed height, which was 2.5 cm. The sample consisted of ovalbumin and α-chymotrypsinogen A. The resolution between these two proteins was calculated for different flow-rates from eqn. 1. Fig. 8 shows a plot of resolution against flow-rate.

Resolution and peak width on columns of compressed, non-porous agarose beads as a function of sample load at constant flow-rate and gradient time

The column and the experimental conditions were the same as those mentioned

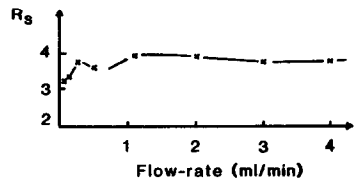

Fig. 8. Resolution (R_s) of ovalbumin and α-chymotrypsinogen A as a function of flow-rate at constant gradient volume. Bed dimensions: 2.5 cm × 0.6 cm I.D. Bead diameter: 20 μm. Gradient volume: 15 ml.

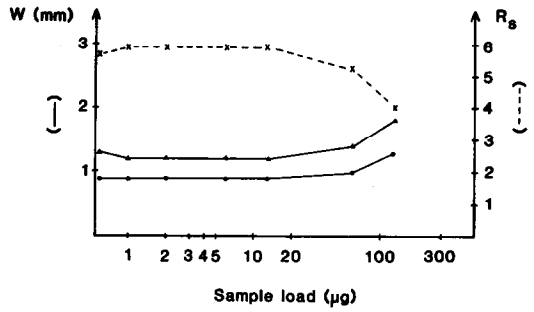

Fig. 9. Peak width (W)and resolution (R_s) as a function of sample load. Bed dimensions: 5.5 cm × 0.6 cm I.D. Bead diameter: 20 μm. Gradient time: 3.2 min. Sample: (▲) ovalbumin and (●) α-chymotrypsinogen A, each applied in the amounts given on the abscissa.

above under *Resolution on columns of compressed, non-porous agarose beads as a function of gradient time at constant flow-rate*, with the exceptions that the gradient time was fixed at 3.2 min and the amounts of each of the two proteins were varied from 0.5 to 120 μg (flow-rate 1 ml/min). Both the peak width and the resolution were determined in each run and were plotted against the amount of each protein applied (Fig. 9).

DISCUSSION

Shrinkage and cross-linking of agarose

Agarose is a polysaccharide built up of alternate residues of 3,6-anhydro-L-galactose and D-galactose[20]. In gels of agarose these polysaccharide chains form double helices collected in bundles, which confers a very porous structure on the gel[21]. When the water in the gel beads is replaced with a non-polar solvent, such as chloroform or carbon tetrachloride, one expects most of the hydrogen bonds to be broken, leading to collapse of the porous gel structure into non-porous beads. If these beads are cross-linked in the organic solvent the agarose retains its collapsed structure when the organic solvent is replaced with water. The shrunken, cross-linked beads will therefore swell only slightly when transferred to an aqueous medium, *i.e.*, their non-porous structure is preserved.

The preparation of amphiphilic beads for HIC often involves a derivatization with non-polar ligands. However, derivatization and cross-linking can be performed in one step if a cross-linker of an appropriate hydrophobicity is selected, as shown in this paper. The cross-linker chosen (1,4-butanediol diglycidyl ether) reacts with the OH groups in the agarose and gives ether bonds, which have the advantage of being stable up to pH 14.

That the agarose beads after shrinkage and cross-linking have lost their macroporous structure is evident from Fig. 2, which clearly shows that proteins do not penetrate the beads.

Resolution as a function of gradient time, flow-rate and sample load

Fig. 4 shows that the resolution (at constant flow-rate) first increases as the

gradient time increases (*i.e.*, when the slope of the salt gradient decreases) and then becomes constant. For each experiment (at constant flow-rate) there is accordingly an optimal run time. Shallower gradients will increase the duration of the experiment without any gain in resolution. Fig. 4 shows that maximal resolution of proteins on the column used in the experiment described herein was obtained at a gradient time of 12 min, *i.e.*, at a gradient volume of 12 ml, as the flow-rate was 1 ml/min. It is also evident that the resolution decreases rapidly with a decrease in gradient time (gradient volume), which is in agreement with the fact that the resolution is higher in the chromatogram in Fig. 5a (gradient volume 15 ml) than in Fig. 5b (gradient volume 3.2 ml).

Flow-rate and gradient time strongly affect the resolution in any chromatographic experiment on macroporous gels. Columns packed with compressed, non-porous agarose beads have the advantage that only one of these parameters needs to be considered, namely gradient time, as the resolution on these columns is virtually independent of the flow-rate as shown in Figs. 6 and 8. In some instances we have even observed an increase in resolution with an increase in flow-rate[2,3]. We have no satisfactory explanation for this phenomenon, although some hypotheses have been put forward[2].

When studying the resolution as a function of flow-rate, it is important to choose the experimental conditions such that it is meaningful to compare the chromatograms obtained. From the relationship

$$\frac{dc}{dV} \cdot \frac{dV}{dt} = \frac{dc}{dt} \tag{2}$$

where dV/dt is the flow-rate, one can see that one has to maintain either dc/dV (the concentration gradient with respect to the gradient volume) or dc/dt (the concentration gradient with respect to the gradient time) constant when the flow-rate is varied in a set of experiments. It is obvious that one should maintain dc/dV constant, because this is the gradient the proteins primarily "sense". If dc/dt is kept constant and the flow-rate is increased, then the steepness of the gradient dc/dV decreases according to eqn. 2, which contributes to changes (often increases) in the resolution. Accordingly, by keeping dc/dt constant one can get a false impression that the resolution increases with increasing flow-rate, when, in fact, it may increase instead as a consequence of the shallower dc/dV gradient, as is exemplified in Fig. 7.

There seem to be no other reports on the separation of macromolecules in which it is stated that the resolution is independent of, or increases with, the flow-rate, except for a study by Blanquet *et al.*[22]. However, they used a constant gradient time, which is equivalent to constant dc/dt for a linear gradient. The chromatograms therefore do not permit a correct comparison as mentioned above.

From diagrams in a paper by Duncan *et al.*[13] one can see that the plate height for proteins that are not adsorbed on a non-porous ion exchanger can be independent of or decrease or increase with the flow-rate. They did not comment on this and did not show any separations of proteins in which one could see the influence of flow-rate.

Fig. 9 shows that the resolution is independent of the amount of protein applied up to about 50 μg of each of the model proteins. For macroporous agarose beads the capacity is, of course, larger.

Recovery and time for regeneration

One of the advantages of the non-porous beads is that they give a high recovery[13]. In our experiments the recoveries were calculated to be 100, 99 and 98%. Another important advantage is that only a few bed volumes are required for regeneration which, together with the high flow-rates, contributes to a shorter total time for an experiment on a non-porous bed in comparison with the same experiment performed on a macroporous bed.

ACKNOWLEDGEMENTS

This work was supported by the Swedish Natural Science Research Council and the Knut and Alice Wallenberg and the Carl Trygger Foundations.

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