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

II. ANION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

Macroporous agarose beads were rendered impermeable to proteins by shrinkage and cross-linking in organic solvents. The chromatographic properties of compressed beds of these non-porous beads derivatized for high-performance ion-exchange chromatography were studied, *e.g.*, the resolution as a function of gradient time, flow-rate (at constant gradient volume) and loading capacity. The columns permit high flow-rates and the resolution is about the same at low and high flow-rates. The beads are stable up to pH 14.

INTRODUCTION

In a recent paper[1] we briefly described the preparation of non-porous agarose beads and their application in the high-performance ion-exchange and hydrophobic-interaction chromatography of proteins. Axially compressed columns packed with these beads have the very attractive feature of giving a resolution that is almost constant with increase in flow-rate (at constant gradient volume), even when the beads are large (*e.g.*, a mixture of beads with diameters from 10 to 50 μm). On compression of the column the beads become deformed, resulting in a decrease in the average distance between them which, in combination with the lack of permeability for proteins, has a favourable effect on the partition rate of solutes between the beads. These circumstances partly explain the unique flow-rate-independent resolution, but in some experiments we have even observed an enhancement of resolution with an increase in flow-rate[1–3] (see ref. 1 for possible but uncertain explanations).

In Part I[4] we described in some detail the high-performance hydrophobic-interaction chromatography of proteins on compressed, non-porous agarose beads. This paper deals with analogous experiments on an anion exchanger.

For ion-exchange, reversed-phase and hydrophobic-interaction chromatography on non-compressible, non-porous packing materials, see refs. 5–9, 10–14 and 15, respectively.

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EXPERIMENTAL AND RESULTS

Equipment

The chromatographic equipment was as described in Part I[4].

All experiments were performed on a 6.2 cm × 0.6 cm I.D. column of 3-dimethylamino-2-hydroxypropyl (DMAHP)-agarose beads (diameter *ca.* 15 μm), prepared as described below. If not stated otherwise, the column was equilibrated with 0.01 *M* Tris–HCl (pH 8.5) and eluted with a linear 10-ml gradient generated from this buffer and 0.01 *M* Tris–HCl (pH 8.5) containing 0.18 *M* sodium acetate.

The design of the Plexiglas columns was described in detail in ref. 2.

Materials

Horse skeletal muscle myoglobin, chicken egg albumin (ovalbumin) and bovine serum albumin were bought from Sigma (St. Louis, MO, U.S.A.). Human transferrin was a gift from Dr. L.-O. Andersson (KabiVitrum, Stockholm, Sweden). Human haemoglobin was prepared by haemolysis of outdated blood and phycoerythrin from *Ceramium rubrum* as described in ref. 16.

1,4-Butanediol diglycidyl ether and glycidol (2,3-epoxy-1-propanol) were obtained from Aldrich (Milwaukee, WI, U.S.A.), dimethylamine from EGA Chemie (Steinheim/Albuch, F.R.G.) and boron trifluoride diethyl etherate from Serva (Heidelberg, F.R.G.).

Preparation of non-porous beads

Macroporous 11% agarose beads were prepared essentially as described previously[17]. A fraction of the beads with diameters in the range 15–20 μm were collected by wet sieving and 5 ml of these beads (sedimented) were shrunk and cross-linked in organic solvents using 1,4-butanediol diglycidyl ether as cross-linker[1,4].

Glycidol was employed as an agent to enhance the hydrophilic character of the matrix, as described by Eriksson[18]. The detailed procedure, as adapted to the preparation of amphiphilic, non-porous agarose beads for high-performance hydrophobic-interaction chromatography, was given in Part I[4]. This procedure was also employed for glycidol treatment of the agarose beads used as a matrix for the ion-exchange chromatographic experiments described here. The only differences were (1) that (after the cross-linking with 1,4-butanediol diglycidyl ether) three treatments with glycidol instead of one was used to obtain the desired degree of hydrophilicity and (2) that 150 μl of the boron trifluoride diethyl etherate were diluted in 2 ml of dioxane before it was added slowly during 5 min, with stirring, to the suspension of the agarose beads. The latter modification slows down the reaction rate and thereby eliminates the risk of aggregation of the beads. The former modification increases the number of OH groups in the beads, which makes them more hydrophilic and also increases the chance of achieving a high ligand density of the ion-exchanging groups (which are coupled to the beads via OH groups).

Synthesis of the anion exchanger and packing of the column

Following the third treatment with glycidol, the agarose beads were washed by centrifugation at 1500 *g* with three 5-ml portions of dioxane and then suspended in 15

ml of dioxane. A 1-ml volume of 1,4-butanediol diglycidyl ether was added slowly, with stirring, followed by 0.1 ml of boron trifluoride dietyl etherate. After activation of the beads for 30 min, 1 ml of dimethylamine was added and the stirring was continued for 20 h at room temperature. The DMAHP-agarose beads thus synthesized were washed with six 5-ml portions of water. The reaction scheme is outlined in Fig. 1.

The beads were packed in deionized water into a Plexiglas column (0.6 mm I.D.) at a flow-rate of 2 ml/min. The bed was then compressed by about 3 mm to a height of 6.2 cm by increasing the flow-rate to 5 ml/min.

Porosity of the DMAHP-agarose beads

On replacing the water in the agarose beads with chloroform and subsequent cross-linking (see above), the volume of the sedimented macroporous beads was reduced from 5 to 1.5 ml. One treatment with glycidol gave no significant change in the volume of the sedimented beads. These moderately hydrophobic beads, which were used for high-performance hydrophobic-interaction chromatography, are impermeable to proteins, as shown in Part I[4]. To suppress strongly the hydrophobicity of the beads and make them more suitable for ion-exchange chromatography, two additional treatments with glycidol were required, as mentioned above.

After three derivatizations with glycidol the volume of the sedimented beads had increased to 3.5 ml. If this increase from 1.5 to 3.5 ml represented swelling of the beads, they could have partially reassumed the macroporous structure. To investigate this possibility, the ion-exchange chromatographic column used in the experiments shown in Figs. 2–7 was equilibrated with 0.01 *M* Tris–HCl (pH 8.5) containing 0.2 *M* sodium acetate. A sample consisting of the proteins ribonuclease (mol.wt. 13 600), ovalbumin (43 000) and albumin (67 000) was applied. The elution was performed with the same buffer (the proteins are not adsorbed in this buffer). The retention times of the proteins were plotted against their molecular weights (see Fig. 2) and it is evident that the beads are impermeable (non-porous) to proteins, at least to those with molecular weights above 12 000.

Pressure–flow-rate dependence

The experiment was conducted in 0.01 *M* Tris–HCl (pH 8.5) at flow-rates in the

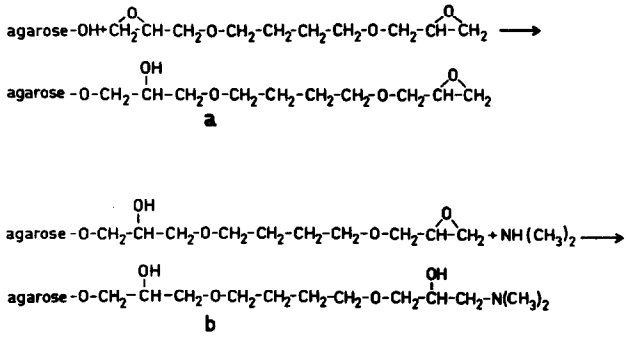

Fig. 1. Reaction scheme for the synthesis of 3-dimethylamino-2-hydroxypropyl (DMAHP)-agarose. (a) Activation of agarose with 1,4-butanediol diglycidyl ether. (b) Coupling of dimethylamine to activated agarose; a tertiary amino group, dimethylamino-2-hydroxypropyl (DMAHP), is formed.

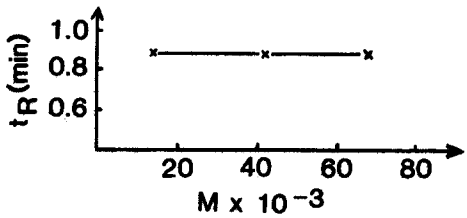

Fig. 2. Porosity of DMAHP-agarose beads. The column was equilibrated with 0.01 *M* Tris–HCl (pH 8.5) containing 0.2 *M* sodium chloride. The retention time (t_R) for three proteins of different molecular weights (*M*) was determined (these proteins were not adsorbed in this buffer). From the plot of retention time against molecular weight one can see that the beads are impermeable to proteins, as the retention time is independent of the molecular weight.

range 1–5 ml/min at increments of 0.5 ml/min and pressure was plotted against flow-rate (Fig. 3). The linear form of the curve indicates that the column can be operated at flow-rates above 5 ml/min.

Recovery

For determination of mass recovery we used ovalbumin, haemoglobin and transferrin. The proteins were adsorbed in the equilibration buffer [0.01 *M* Tris–HCl (pH 8.5)] and eluted with this buffer containing 0.2 *M* sodium acetate. Measurements of absorption at 280 nm of both the applied sample and the eluted fractions indicated recoveries of 96, 96 and 105%, respectively.

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

The sample [transferrin (20 μg) and ovalbumin (40 μg)] was dissolved in the equilibration buffer and elution was accomplished with a 2.5-min linear salt gradient (the composition of the equilibration buffer and the gradient is given under *Equipment*). The resolution (R_s) between the two proteins was determined using the equation

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

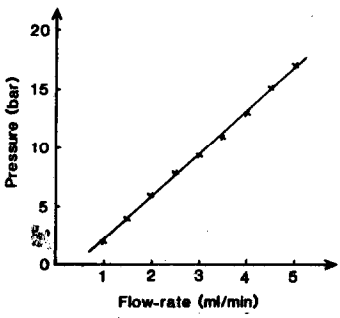

Fig 3. Pressure–flow-rate dependence. Bed dimensions: 6.2 cm × 0.6 cm I.D. Bead diameter: *ca.* 15 μm. Buffer; 0.01 *M* Tris–HCl (pH 8.5).

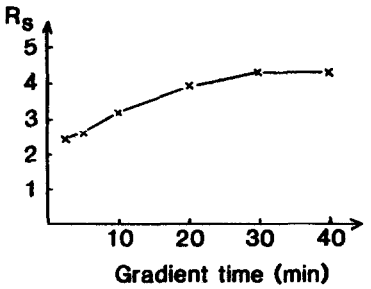

Fig. 4. Resolution (R_s) as a function of gradient time. Bed dimensions: 6.2 cm × 0.6 cm I.D. Bead diameter: 15 μm. Flow-rate: 1 ml/min. The diagram shows that there is no gain in resolution for gradient times exceeding 25–30 min.

where t_1 and t_2 are the retention times of transferrin and ovalbumin, respectively, and t_{w1} and t_{w2} are their peak widths at half-height. In an analogous way the resolution was calculated for gradient times of 5, 10, 20, 30 and 40 min. In Fig. 4 the resolution is plotted against gradient time. As this figure indicates, the highest resolution is obtained at gradient times of *ca.* 25–30 min.

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

For the composition of the equilibration buffer and the salt gradient, see *Equipment*. The sample [20–40 μg of each of the proteins myoglobin (1), haemoglobin (2), transferrin (3), ovalbumin (4), albumin (5) and phycoerythrin (6)] was dissolved in 20 μl of the equilibration buffer. A series of experiments were performed at flow-rates from 0.25 to 4.0 ml/min. The gradient volume was constant (10 ml). In each experiment the chart speed was proportional to the flow-rate to give chromatograms of the same width, which makes it easier to compare visually the protein patterns and the resolution at different flow-rates (Fig. 5). Fig. 5 shows that the protein pattern and the resolution are about the same at low and high flow-rates.

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

Peaks 2 and 3 in Fig. 5, corresponding to haemoglobin and transferrin, exhibit a relatively high degree of symmetry (and homogeneity). These peaks were therefore selected for determination of resolution as a function of flow-rate. Eqn. 1 was used for calculation of the resolution at different flow-rates (Fig. 6). Fig. 6 shows that the resolution varies only slightly with flow-rate.

Resolution and peak width on columns of compressed, non-porous agarose beads as a function of sample load

Most of the experimental conditions were similar to those described above under *Resolution on columns of compressed, non-porous agarose beads as a function of gradient time at constant flow-rate*. The flow-rate was 1 ml/min and the gradient time 10 min. Equal amounts of the model proteins transferrin and ovalbumin were dissolved in the equilibration buffer. The resolution between these two proteins was determined

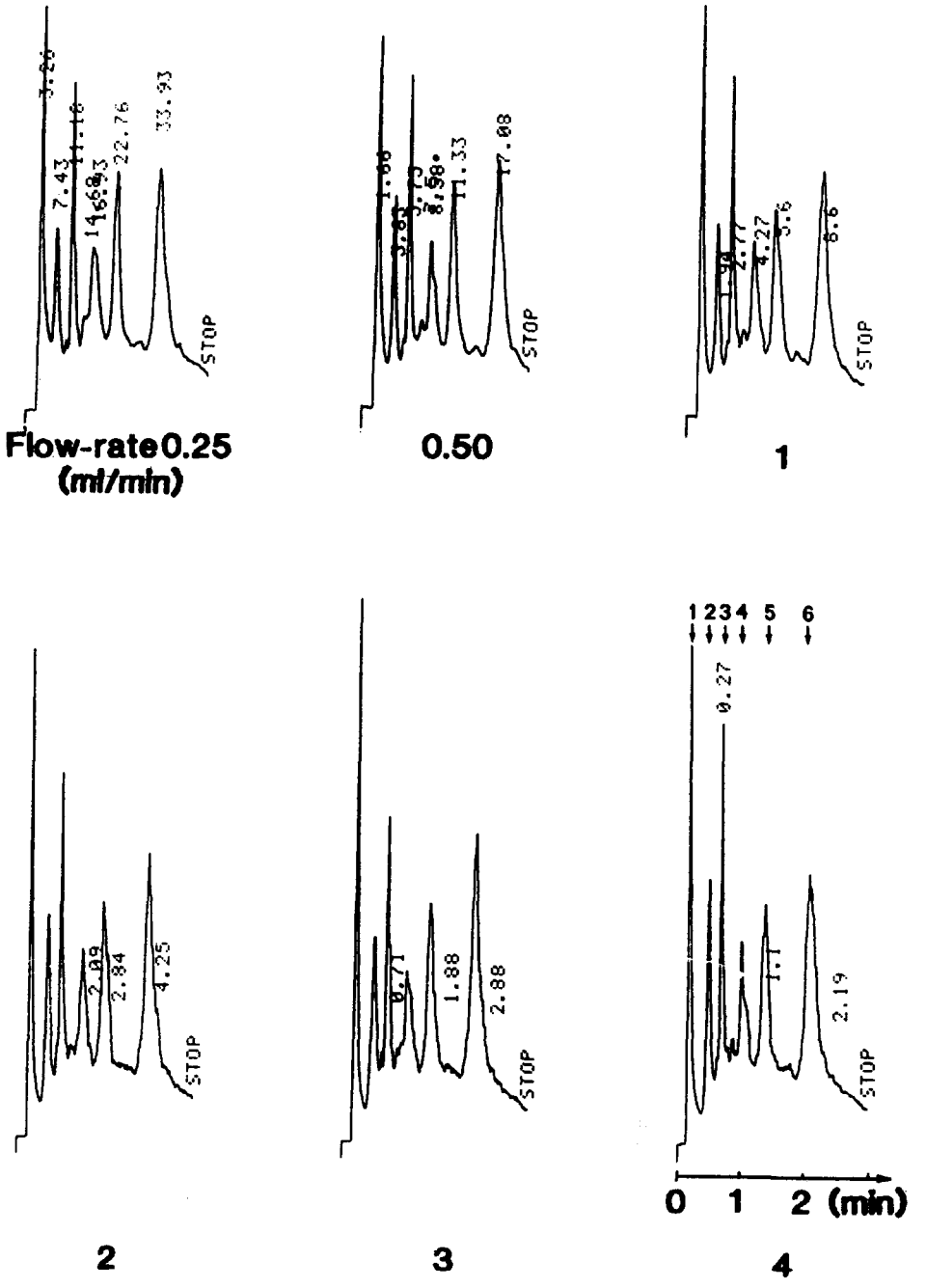

Fig. 5. Influence of flow-rate at constant gradient volume on the appearance of the chromatograms. Bed dimensions: 6.2 cm × 0.6 cm I.D. Bead diameter: 15 μm. Sample: 1 = myoglobin; 2 = haemoglobin; 3 = transferrin; 4 = ovalbumin; 5 = serum albumin; 6 = phycoerythrin. It is evident that the protein patterns and the resolution are virtually the same, *i.e.*, independent of the flow-rate. The gradient volume was 10 ml in all experiments. The recorder chart speed was proportional to the flow-rate.

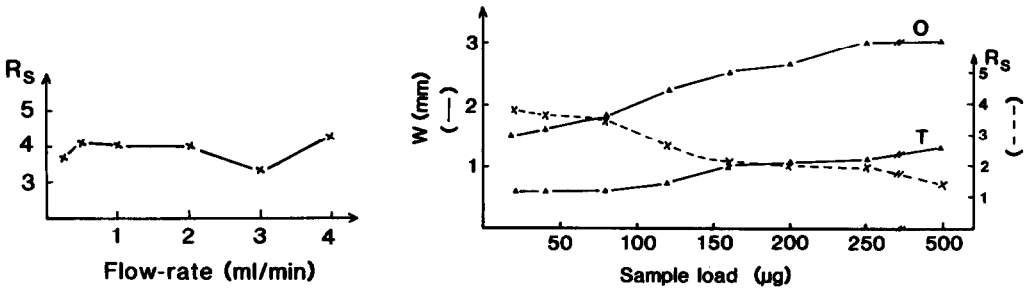

Fig. 6. Resolution (R_s) of haemoglobin and transferrin as a function of flow-rate at constant gradient volume. Bed dimensions: 6.2 cm × 0.6 cm I.D. Bead diameter: 15 µm. Gradient volume:10 ml.The recorder chart speed was proportional to the flow-rate.

Fig. 7. Peak width (w) and resolution (R_s) as a function of sample load. Bed dimensions: 6.2 cm × 0.6 cm I.D. Bead diameter: 15 µm. Gradient time: 10 min. Sample: T = transferrin; O = ovalbumin, each applied in the amount given on the abscissa. The peak widths and the resolution were affected only slightly by sample size at loads below 100 µg and unexpectedly little at even higher loads.

according to eqn. 1 for different amounts of protein applied and was plotted against amount (Fig. 7). The peak widths at half-height are also plotted in Fig. 7. It is evident that the resolution declines relatively slowly with increasing load, particularly when less than 100 µg of each protein are applied.

DISCUSSION

Shrinkage and cross-linking of the agarose

The strategy behind the shrinkage and cross-linking was discussed in Part I[4], where we employed in a one-step procedure 1,4-butanediol diglycidyl ether both as a cross-linker and as an agent to create non-polar groups in the agarose beads. The same cross-linker, which has the advantage of giving pH-stable ether bonds, was used for the preparation of the beads employed in this study. To suppress the hydrophobic nature of the beads, three treatments with glycidol were required. With glycidol one can therefore modify the degree of hydrophobicity of the non-porous beads in a simple way[18]. The reason for wishing to minimize hydrophobic interactions in ion-exchange chromatography is that these, in combination with electrostatic interactions, can cause irreversible adsorption of proteins, as it may be impossible to find a salt concentration at which both types of interactions are very small[19], which is a prerequisite for desorption (an increase in salt concentration to decrease the electrostatic interactions increases the hydrophobic interactions). To minimize hydrophobic side-reactions, the ion exchanger used is based on derivatization with dimethylamine (see Fig. 1) instead of the more commonly used diethylamine.

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

Fig. 2 shows that the ion-exchange beads are impermeable to proteins, which is one of the reasons why a high resolution is obtained also at very high flow-rates. Another reason is that the beads are deformed following the compression of the column bed, which means that the transport distances between the beads are smaller

than in a non-compressed bed, resulting in more rapid partition of the solutes. There are certainly also other reasons for the high resolution at high flow-rates (Fig. 5 and 6), although it is difficult to arrive at satisfactory explanations, particularly in a few instances where the resolution actually increased with an increase in flow-rate[1–3].

From Fig. 4 one can conclude that the resolution increases gradually with the gradient time (i.e., as the gradient becomes shallower) and finally becomes constant. Resolution is accordingly not impaired by the use of long gradient times. However, excessively shallow gradients give broad, diluted zones and increase the run time.

The resolution is affected relatively little by the sample load, as shown in Fig. 7: at a total load of 500 μg of protein the resolution is still about half of that at 80 μg. The recoveries were high for the three proteins tested (96, 96 and 105%).

This paper has been written with most section headings analogous to those used in Part I[4] to facilitate a general comparison between the two methods. Roughly one can state that the methods have similar features, taking into consideration that they are based on different separation parameters. For general information on agarose-based high-performance liquid chromatography, see ref. 2.

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