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HIGH-PERFORMANCE CHROMATOFOCUSING OF PROTEINS ON AGAROSE COLUMNS

II. DEFORMED NON-POROUS 12–15 μm BEADS

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SUMMARY

By shrinkage and cross-linking in organic solvents, macroporous agarose beads were rendered impermeable to proteins. Beads derivatized with polyethyleneimine in an aqueous solution gave a relatively linear pH gradient upon elution with Polybuffer™. The titration capacity was 6–7 and 3–4 mequiv. per 100 ml agarose gel at basic and acidic pH, respectively. The columns were packed at pressures high enough to deform the beads, which increases the resolution owing to the decrease in the distance between the beads. The resolution has been determined as a function of the flow-rate, concentration of eluting buffer (Polybuffer) and sample load. Interestingly, the resolution increased with an increase in flow-rate. The importance of choosing an optimum concentration of Polybuffer was also demonstrated in a series of experiments with human growth hormone, which was fractionated into four components when the Polybuffer was diluted 1:80, but not at a dilution of 1:40. The mass recovery was 96% for human serum albumin and 93% for ovalbumin, whereas the recovery of the activity of β-galactosidase was 90%. In comparison with macroporous beads, the deformed (compressed) non-porous beads have the advantage of permitting a more rapid separation and regeneration and to give a resolution which is independent of or even increases with the flow-rate. The protein capacity of the latter beads is not much lower than that of the macroporous beads.

INTRODUCTION

Recently we have described the preparation of chromatographic columns of deformed non-porous agarose beads and their application to the separation of proteins by high-performance hydrophobic interaction chromatography[1,2], ion-exchange chromatography[1,3] and adsorption chromatography based on interaction

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with metal (hydr)oxides[4,5]. These beads have the attractive property that the resolution is almost independent of or increases with an increase in flow-rate.

The main aim of the present work was to investigate whether the latter feature also applied for chromatofocusing on such beads. The paper is thus an extension of the studies presented in the preceding paper[6], which deals with chromatofocusing on macroporous agarose beads. For a rapid comparison of the two agarose beads we have used the same subtitles, when suitable, in these two papers.

MATERIALS AND METHODS

12% Macroporous agarose beads (dimensions: 15–20 μm) were a gift from Mr. Sten Porrvik, Casco Nobel, Sundsvall, Sweden. The beads were prepared essentially according to a method described in ref. 7. Polyethyleneimine (molecular weight 30 000) and boron trifluoride diethyletherate (BF_3) were from Serva (Heidelberg, F.R.G.). Polybuffer™ was from Pharmacia (Uppsala, Sweden). β-Galactosidase, *o*-nitrophenyl-D-galactopyranoside, Bis-Tris[bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane], 1,4-butanediol diglycidyl ether, conalbumin, ovalbumin and a baker's yeast extract were from Sigma (St. Louis, MO, U.S.A.). Dioxane was from Fisons (Loughborough, U.K.). Sodium borohydride and chloroform were from E. Merck (Darmstadt, F.R.G.) and dimethylformamide from BDH Chemicals (Poole, U.K.). γ-Glycidoxypropyltrimethoxysilane was a gift from Mr. H. Gustafsson, Sikema, Stockholm, Sweden; human growth hormone from Professor P. Roos (this institute) and human serum albumin and transferrin from Dr. L.-O. Andersson (KabiVitrum, Stockholm, Sweden). Alcohol dehydrogenase activity was determined essentially as described in ref. 8, with the main modifications that the substrate was ethanol and that the assay was performed at pH 8.5 (this reference deals with the activity measurement of glucose-6-phosphate dehydrogenase).

All solutions were prepared with Milli Q pure water and degassed before use.

INSTRUMENTATION

The chromatographic equipment was as described in Part I[6].

The column tubes were made of Plexiglas with an inner diameter of 0.6 cm (for design details, see ref. 9). They were packed in water at a flow-rate higher than that used in the subsequent experiments and sufficiently high to deform the beads strongly. The movable piston was then pressed down to make contact with the upper surface of the gel bed to prevent the compressed bed from expanding upon reduction of the flow-rate for chromatographic experiments.

EXPERIMENTAL AND RESULTS

Preparation of non-porous agarose beads

The macroporous agarose beads were shrunk and cross-linked in organic solvents[1,2]. Briefly, 5 g of agarose beads were washed with distilled water by centrifugation at 1000 *g* for 2 min (these centrifugation conditions were used in all subsequent washings) and then transferred to dioxane by washing three times with water–dioxane (1:1) and three times with dioxane. Finally, 5 ml of a mixture of dioxane–chloroform

(1:1) was added. This washing procedure was repeated twice. The volume of the beads was then about 1/3 of that originally. The shrunken beads were suspended in 25 ml of chloroform. With stirring, 4 ml of the cross-linker, γ-glycidoxypropyltrimethoxysilane, was added followed by 0.9 ml of BF_3 in 15 ml of chloroform as a catalyst for activation of the epoxide groups in the cross-linker. After 30 min the beads were transferred back to water by centrifugation and washing with dioxane, dioxane–water (1:1) and water. The beads, thus prepared, were impenetrable to proteins[1–3]. The diameters of the shrunken, cross-linked beads were 12–15 μm.

Coupling of polyethyleneimine to non-porous beads and determination of the titration capacity

This coupling was performed essentially as described in refs. 10 and 11 with some modifications. A 1-g amount of the cross-linked non-porous agarose beads was suspended in 1.0 ml of 0.5 *M* sodium bicarbonate (pH 8.0) containing 6 mg of sodium borohydride, and 1.0 ml of polyethyleneimine was then added. This imine has been used previously for the preparation of ion exchangers suitable for chromatofocusing[12]. The mixture was stirred at room temperature for 4 h. The beads were then transferred to chloroform and cross-linked once more with γ-glycidoxypropyltrimethoxysilane according to the procedure described in the preceding section.

The titration capacity was determined in the presence of 0.5 *M* sodium chloride as described in Part I[6]. The result is presented in Fig. 1. Curve A was obtained for beads prior to the second cross-linking and curve B afterwards. Beads corresponding to curve B were used in this study, because they permitted higher flow-rates, even though the capacity was lower than that of the beads cross-linked only once (curve A). Higher titration capacity was accomplished by prolonging the time of reaction with polyethyleneimine, but at the expense of the rigidity of the beads.

Coupling in an organic solvent (dimethylformamide) was carried out as follows: 1 g of cross-linked non-porous agarose beads was transferred to dimethylformamide by repeated centrifugation and washing steps. After the last washing, 10 ml of dimethylformamide and then 1.0 ml of polyethyleneimine were added, followed by 30 mg of $SnCl_4$ as a catalyst[13]. The mixture was stirred at room temperature for 1 h. The gel had an higher total titration capacity but the capacity within different pH intervals

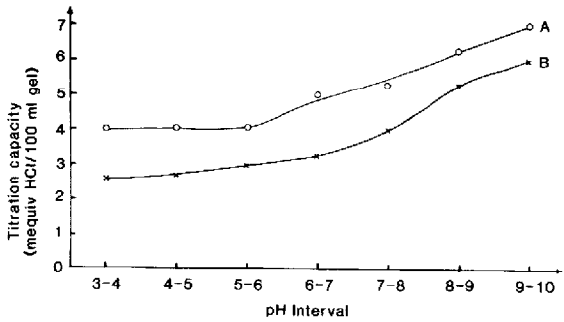

Fig. 1. The titration capacity within different pH intervals. A 3-mg amount of gel in 10 ml of 0.5 *M* NaCl solution was titrated with 0.1 *M* HCl. (A) Cross-linked gel coupled with 1.0 ml of polyethyleneimine per gram gel for 4 h; (B) gel (A) cross-linked again.

varied in a non-regular way and the resolution on these beads was not satisfactory. Therefore they were not used further.

Estimation of protein recovery

Human serum albumin (HSA) and ovalbumin were employed as samples for estimation of mass recovery. HSA (0.2 mg) dissolved in 100 μl of the starting buffer (0.025 *M* Bis-Tris–HCl, pH 5.5) was applied to the column (5.0 cm × 0.6 cm I.D.) and eluted with Polybuffer 74 (diluted 1:40)–HCl, pH 4.0, at a flow-rate of 2 ml/min. Ovalbumin was treated in the same way, but the amount of protein was 1 mg. The volumes of the eluted fractions were measured along with their absorption at 280 nm. From these data, the mass recovery of HSA was estimated at 96% and that of ovalbumin at 93%.

A sample of β-galactosidase (500 μg in 100 μl of the starting buffer) was subjected to chromatofocusing under the same experimental conditions, although the bed height was 2.5 cm and the flow-rate 1 ml/min. The recovery of the enzyme activity was 90%.

Resolution as a function of flow-rate and Polybuffer concentration

A Plexiglas column with an inner diameter of 0.6 cm was packed in water at a flow-rate of 5.0 ml/min to a height of 3.0 cm with the 12% shrunken non-porous agarose beads (see Instrumentation). The bed was equilibrated with 0.025 *M* Bis-Tris–HCl, pH 6.5.

Human transferrin (0.25 mg) was dissolved in 50 μl of the equilibration buffer (starting buffer) and applied to the column. The sample was desorbed at a flow-rate of 4.8 ml/min with Polybuffer 74 (diluted 1:40)–HCl, pH 5.0. Similar experiments were performed at the flow-rates 2.4, 1.2 and 0.6 ml/min. Two transferrin peaks were obtained. The resolution, R_s, between them was calculated at different flow-rates from eqn. 1 in Part I[6].

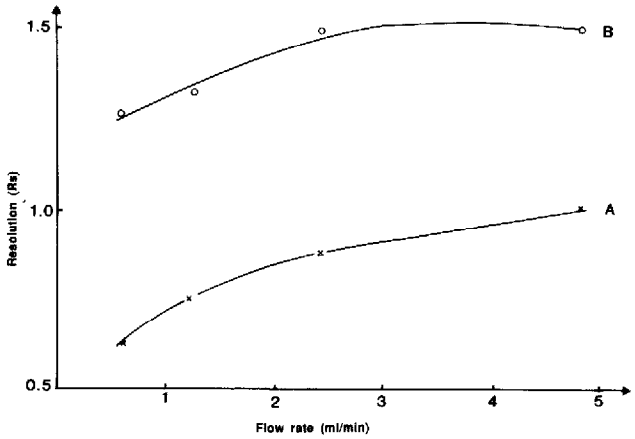

Fig. 2. Resolution as a function of flow-rate and dilution of Polybuffer. After application of 0.25 mg of human transferrin in 50 μl of starting buffer the column (3.0 cm × 0.6 cm I.D.) was eluted at the flow-rates 4.8, 2.4, 1.2 and 0.6 ml/min. (A) Polybuffer diluted 1:40; (B) Polybuffer diluted 1:80. The resolution was determined for the two transferrin peaks obtained.

The results are presented in Fig. 2, curve A. The experiment was then repeated, with the difference that the Polybuffer was diluted 1:80 (see Fig. 2, curve B).

Resolution as a function of sample load

The sample consisted of 0.25 mg of human transferrin dissolved in 100 μl of the buffer, 0.025 *M* Bis-Tris–HCl, pH 6.5. Following equilibration of the column bed (2.5 cm × 0.6 cm I.D.) with this buffer, the sample was applied and then eluted at a flow-rate of 2.0 ml/min with Polybuffer 74 (diluted 1:80)–HCl, pH 5.0. The resolution was calculated as in the previous section. The column was then washed with 2 *M* sodium chloride. Following equilibration of the column, a similar experiment was carried out with 0.5 mg of transferrin. In the same way, the resolution was determined for 1.0 and 2.0 mg of the same protein. A plot of the resolution against the sample load is presented in Fig. 3.

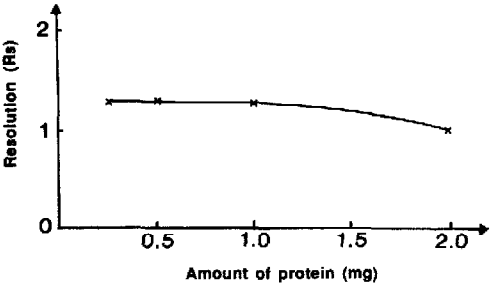

Fig. 3. Resolution as a function of sample load. Samples of human transferrin (0.25, 0.5, 1.0 and 2.0 mg in 100 μl of starting buffer) were applied on the column (2.5 cm × 0.6 cm I.D.) and eluted at a flow-rate of 2.0 ml/min. The resolution was calculated for the two transferrin peaks obtained.

Demonstration of the effect of elution buffer concentration on separation of human growth hormone components

The experiments were performed under the same conditions as given in *Resolution as a function of flow-rate and Polybuffer concentration*, with the difference that the flow-rate was constant at 2.0 ml/min. The column was equilibrated with starting buffer, 0.025 *M* Bis-Tris–HCl, pH 5.5. About 0.5 mg of human growth hormone (HGH) dissolved in the starting buffer were applied to the column and eluted with Polybuffer 74 (1:40)–HCl. After washing with 2 *M* NaCl and reequilibration with the starting buffer, the same amount of HGH was applied and eluted with 80-fold diluted Polybuffer 74–HCl, pH 4.0; the experiment was then repeated with 160-fold diluted Polybuffer 74–HCl, pH 4.0. The chromatograms are shown in Fig. 4.

Chromatofocusing of conalbumin

Since this protein has been used previously as a model substance in methodological studies of chromatofocusing[14], it was of interest to see how it behaved in our chromatographic system. The column (3.0 cm × 0.6 cm I.D.) was equilibrated with 0.025 *M* Bis-Tris–HCl, pH 6.0. Conalbumin (200 μg) was dissolved in 100 μl of this buffer and injected onto the column. Elution was accomplished with Polybuffer 74 (diluted 1:40)–HCl, pH 5.2, at a flow-rate of 1 ml/min (Fig. 5). The continuous

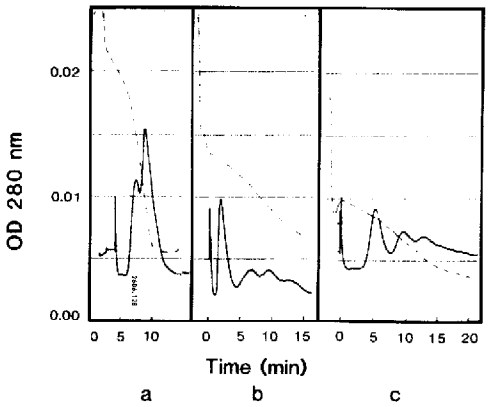

Fig. 4. Chromatofocusing of human growth hormone at different dilutions of the eluting buffer. Sample amount: 0.5 mg. (a) Polybuffer diluted 1:40; (b) Polybuffer diluted 1:80; (c) Polybuffer diluted 1:160. Column: 3.0 cm × 0.6 cm I.D. Flow-rate: 2.0 ml/min. The pH was measured continuously (the dashed curves).

increase in background absorption was probably caused by contaminating substances in the Polybuffer.

Purification of alcohol dehydrogenase from baker's yeast

Following equilibration of the column (4.0 cm × 0.6 cm I.D.) with 0.005 *M* Bis-Tris–HCl, pH 6.5, 400 μg of a yeast extract dissolved in 100 μl of this buffer was applied. For desorption we employed Polybuffer 74 (diluted 1:40)–HCl, pH 4.0. Fractions of 1 ml were collected and their alcohol dehydrogenase (ADH) activity was determined (Fig. 6). The recovery of enzyme activity was 100%, half (52%) being eluted in a peak close to the void peak and half (48%) at a pH around 4.8.

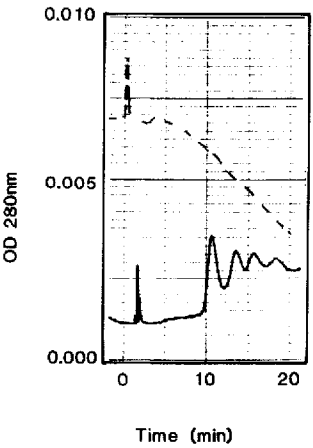

Fig. 5. Chromatofocusing of conalbumin. The column (3.0 cm × 0.6 cm I.D.) was equilibrated at pH 6.0. Conalbumin (200 μg) was eluted with Polybuffer 74 (1:40)–HCl, pH 5.2. Flow-rate: 1 ml/min.

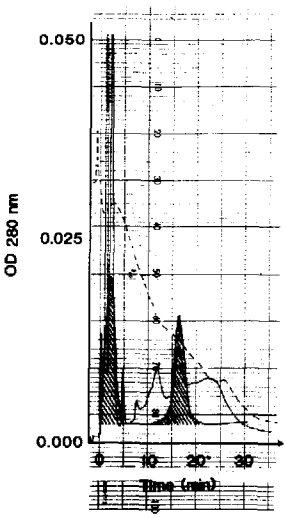

Fig. 6. Purification of alcohol dehydrogenase by chromatofocusing. After equilibration of the column at pH 6.5, 400 μg of the sample (yeast extract) was eluted with Polybuffer 74 (1:40)–HCl, pH 4.0. Enzyme activity was traced to the shaded peaks. Flow-rate: 1 ml/min.

DISCUSSION

Titration capacity

Fig. 1 shows that the titration capacity in different pH intervals of an ion exchanger synthesized from non-porous agarose beads has the same profile as that based on macroporous beads (Fig. 1 in Part I[6]), *i.e.*, the capacity is higher at basic than at acidic pH in contrast to the result reported by Sluyterman and Wijdenes[12]. The titration capacity was higher for the macroporous beads (compare Fig. 1 with Fig. 1c in Part I).

Protein capacity

The non-porous agarose beads were prepared from the macroporous beads used in the experiments described in Part I[6]. After shrinkage and cross-linking, the volume of the beads was about one third that of the original non-shrunken beads[2]. A bed of shrunken beads thus contains many more beads per unit than does a bed of non-shrunken beads. Furthermore, the surface area of a shrunken bead might be about the same as that of a non-shrunken bead, although the surface of the shrunken beads is more creased to judge from scanning electron microscopy pictures. These considerations indicate that the capacity of a bed of non-porous shrunken agarose beads, expressed in terms of the amount of protein adsorbed per ml bed, need not necessarily be much lower than that of a bed of macroporous beads (compare Fig. 3 with Fig. 2 in Part I[6]). The lower capacity is compensated by a shorter regeneration time (see below) and the possibility to operate a column packed with deformed non-porous beads at higher flow-rates without loss in resolution (Fig. 2). The throughput (amount of protein purified per unit time) may therefore be similar. An advantage of the non-porous beads from the viewpoint of preparation is that they can be relatively

large (15–50 μm in diameter) and have a wide size distribution and still give high resolution[1–3].

Resolution as a function of flow-rate

In chromatofocusing[15] as well as in other chromatographic methods based on the use of conventional beds the resolution decreases with an increase in flow-rate. We have shown in several papers that compressed beds of non-porous agarose beads are unique in the sense that they do not follow this rule. These beads have the same attractive feature when utilized for chromatofocusing, as shown in Fig. 2. Some possible explanations for this anomalous behaviour have been treated only briefly[1]. A more thorough discussion will be given elsewhere. Since the resolution increases with the flow-rate upon chromatofocusing on deformed non-porous agarose beads the experiments can be conducted very rapidly without any drawbacks (Fig. 2).

Resolution as a function of the concentration of the eluting buffer

In chromatofocusing on deformed non-porous agarose beads (as for macroporous beads) (see Part I[6]) the resolution increases (Fig. 2) upon dilution of the Polybuffer (although the peaks became broader) and even at dilutions as high as 1:40–1:160 where the buffer capacity is low there was no shoulder in the region of the pH gradient where the proteins were eluted (Figs. 4 and 5). This finding is of practical importance, since Polybuffers are expensive.

Time for regeneration

The macroporous and non-porous agarose beads used for chromatofocusing require 10–15 and 5 bed volumes of the equilibration buffer, respectively, for regeneration. A 1-ml column packed with the latter beads can therefore be regenerated in somewhat more than 1 min at a flow-rate of 4 ml/min.

pI Values

Isoelectric focusing of human growth hormone gives four main peaks with the p*I* values of 4.9, 4.8, 4.7 and 4.6[16]. The chromatofocusing experiments shown in Fig. 4 gave similar p*I* values.

A short comparison between high-performance isoelectric focusing in capillary tubes and high-performance chromatofocusing on deformed non-porous agarose beads

If the flow-rates in the experiments shown in Figs. 5 and 6 are increased to 4 ml/min the analysis times will be about 5–10 min, which are normal in free high-performance isoelectric focusing in capillary tubes with an inner diameter in the range 0.025–0.1 mm[17,18]. This method (which is the electrophoretic counterpart of chromatofocusing) has, however, an higher resolution than chromatofocusing because the separation medium in a carrier-free electrophoresis method (an aqueous solution) is more homogeneous than that in any chromatographic method (a non-uniformly packed bed). In this comparison between isoelectric focusing and chromatofocusing we have considered free high-performance isoelectric focusing (HPIF) in capillary tubes and high-performance chromatofocusing (HPCF) on deformed non-porous agarose beads. This might be justified, since HPIF and HPCF are among the most rapid and most highly resolving methods based on electrophoretic and chromato-

graphic focusing, respectively. The former method has the disadvantage of permitting separation only on a micro-scale.

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